STRESS, BLOOD-BRAIN BARRIER AND PYRIDOSTIGMINE TOXICITY

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PYRIDOSTIGMINE TOXICITY

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LIST OF ABBREVIATIONS

ACh	acetylcholine
AChE	acetylcholinesterase
ANOVA	analysis of variance
BBB	blood-brain barrier
BuChE	butyrylcholinesterase
ChE	cholinestase
CMV	cytomegalovirus
CNS	central nervous system
DXM	dexamethasone
ED_{50}	dosage causing 50% effect
FDA	Food and Drug Administration
GW	Gulf War
GWI	Gulf War Illnesses
HRP	horseradish peroxidase
IC_{50}	concentration causing 50% inhibition
IM	involuntary movements
i.m.	intramuscular
i.p.	intraperitoneal
IQR	inter-quartile range
kg	kilogram
LD_{50}	dosage causing 50% lethality
mg	milligram
ml	milliliter
MTD	maximum tolerated dosage
OP	organophosphorus
PO	pararoxon
p.o.	perioral
PHY	physostigmine
PYR	pyridostigmine
SLUD	salivation, lacrimation, urination, defecation

Chapter 1

INTRODUCTION

In response to Iraq's invasion of Kuwait in 1990, the United States deployed approximately 697,000 military personnel in Operations Desert Shield and Desert Storm from August 1990 to June 1991. After returning from the Persian Gulf War (GW), thousands of US military personnel have complained of a variety of symptoms including persistent fatigue, headaches, muscle and joint pain, sleep disturbances, cognitive difficulties and others (Joseph, 1997; Haley and Kurt, 1997). Epidemiological studies showed that these symptoms were reported more frequently in the GW veterans than in those who were not deployed (Joseph, 1997). As Table 1 shows, the wide array of the symptoms ranges from minor conditions such as fatigue or headache to some more serious disorders e.g. memory, sleep or concentration problems. One characteristic of the reported illnesses is that complaints from veterans can non-specifically be associated with multiple conditions, e.g. 1) headache might be caused by a lack of oxygen supply to the brain, intoxication from biological or chemical agents, or common migraine, 2) joint and muscle pain could arise from the common cold, bacteremia, rheumatoid arthritis or a number of other medical conditions. Another characteristic is that these illnesses have a poor organic or systemic explanation and appear to be disturbances of multiple systems.

Symptoms	As chief complaint %	As any complaint %
Fatigue	20	59
Headache	9	44
Memory problems	6	40
Sleep disturbances	5	40
Rash	4	30
Joint pain	4	47
Shortness of breath	2	19
Abdominal pain	1	16
Muscle pain	1	22
Difficulty concentrating	1	31
Diarrhea	0.5	18
Depression	0.3	22
Cough	1	1

Table 1: The most frequent symptoms among 3558 Comprehensive Clinical Evaluation Program participants with a Primary Diagnosis of "Symptoms, Signs, and Ill-Defined Conditions" (Adopted from Joseph, 1997).

Based on a study involving 249 soldiers from the Gulf War, Haley and coworkers (1997 a, b) identified and characterized the "syndromes" into 6 categories: 1) impaired cognition, 2) confusion-ataxia, 3) arthro-myo-neuropathy, 4) phobia-apraxia, 5) fever-adenopathy, and 6) weakness-incontinence. The first 3 syndromes are considered the major, involving the central nervous system (CNS) functions.

The exact mechanisms for the Gulf War Illnesses (GWI) are still not clear. Several risk factors have been postulated to contribute to the development of GWI including infectious diseases, pyridostigmine (PYR) toxicity, immunizations, stress, chemical and biological warfare agents, oil well fires, depleted uranium and pesticides. Among these possible etiological factors, we were particularly interested in the hypothesis that stressors increase BBB permeability and allow PYR penetration into the CNS to cause central cholinergic dysfunction.

PYR is a carbamate cholinesterase (ChE) inhibitor. It was approved by the United States Food and Drug Administration (FDA) in 1955 as a therapeutic agent for myasthenia gravis, an autoimmune disease characterized by gradually developing muscle weakness and fatigability (Breyer-Pfaff et al., 1985; Drachman, 1994; Antonini et al., 1996). As an effective therapeutic for myasthenia gravis, PYR is given initially at a dosage of 60 mg 3 times a day. The dosage is increased until the maximum clinical benefit is reached. The average dose of PYR given orally is around 600 mg/day with a dose range from 200 to 1400 mg/day.

During the GW from 1990 to 1991, PYR bromide tablets, under the status of "investigational new drug" conferred by FDA, were given to United States and allied military personnel as a pretreatment to protect against possible organophosphorus (OP)

nerve agent (e.g., soman) attack from Iraq (Keeler et al., 1991; Gunderson et al., 1992). The military doctrine for PYR use as prophylactic agent against OP nerve gas is administration as oral tablet, at a dosage of 30 mg every 8 hours. PYR could be given for up to 2 weeks as long as the soldiers were under high risk of nerve agent attack. Few adverse effects were reported while PYR used as drug for either Myasthenia Gravis at lower end of dose range or for military purpose before GW.

PYR is a charged drug and has been reported to have relatively poor and erratic absorption into the circulation from the gastrointestinal tract. First-pass metabolism also contributes to the low bioavailability of PYR. A previous study (Aquilonius et al., 1980) of PYR pharmacokinetics in human volunteers suggested the kinetic constants (e.g. elimination half-life, volume of distribution, and plasma clearance) were very similar between 2.5 mg intravenous injection and 120 mg oral administration, giving the bioavailability of about 7.6%. Most of the PYR given orally is excreted in feces unchanged while most of the absorbed PYR is excreted in the urine in the original form (Keeler, 1990). A small portion of the PYR is mainly metabolized into 3-hydroxy-N-methyl pyridium, which is both a ChE inhibitor and acetylcholine (ACh) receptor antagonist (Lee et al., 1992).

The rationale for PYR use as pre-exposure antidote to OP nerve agent relies on the short duration of action of PYR on acetylcholinesterase (AChE). As shown in Figure 1, the interaction between AChE and its natural substrate (ACh) or inhibitors (carbamate or OP) can be illustrated in three steps, with the difference between substrate and inhibitors primarily in the reaction rate of the final step. With PYR, this reaction rate is slower than that for ACh but much faster than for OPs. Therefore, PYR is

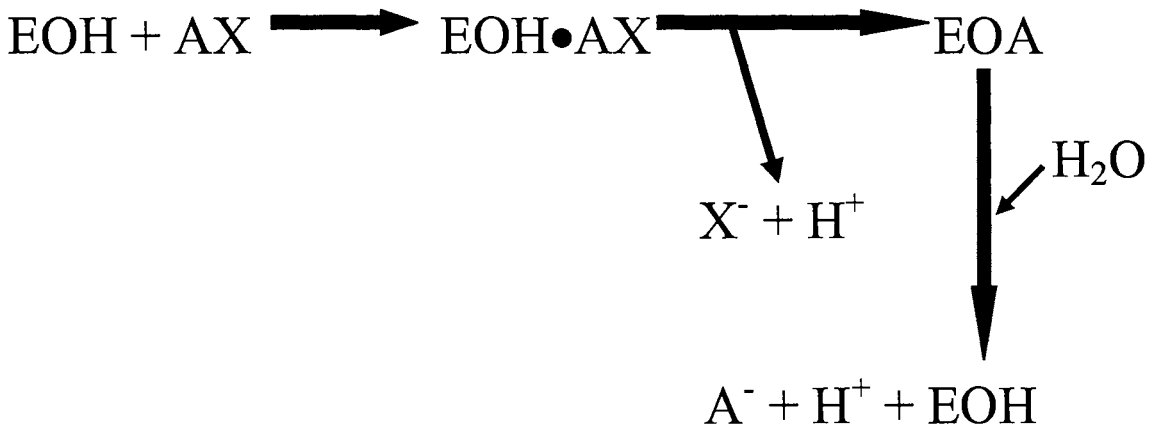

Figure 1: Reaction of AChE with either the natural substrate (acetylcholine) or inhibitors (carbamate or OP). EOH represents AChE. AX represents acetylcholine, carbamate or OP. In structure of AX (substrate or inhibitor), A is binding group while X is the leaving group.

referred to as a "reversible" ChE inhibitor while OPs are referred to as "irreversible" inhibitors. Reversible inhibition of ChE by PYR prevents the irreversible inactivation by an OP. This forms the basis for the rationale for PYR prophylaxis against OP chemical warfare agent exposure. Soman, one of the OP nerve agents, leads to lethality by irreversibly inactivating AChE. Pretreatment with PYR complexes a certain proportion of the AChE molecules. Occupancy of PYR on AChE prevents the further binding on the same active site by an OP. After withdrawal from OP exposure, PYR-AChE complex undergoes hydrolysis and decarbamylation since PYR has a short duration of action, regenerating the AChE activity. PYR given to soldiers as during GW caused about 20 to 40% ChE inhibition, which assures this portion of AChE molecules are unavailable for inhibition by an OP. On the other hand, the remaining AChE molecules (60 to 80%) would be generally sufficient for normal function without compromising performance or eliciting side effects associated with AChE inhibition. Compared to other carbamates such as physostigmine, PYR is preferred as a protective agent because it is not expected to enter the CNS and influence brain function. PYR therefore does not significantly impair higher order mental performance.

Atropine and pralidoxime (2-PAM), used as post-exposure antidotes, are the traditional therapeutic agents against nerve agent poisoning. Pralidoxime, however, is not effective in treating OP poisoning once "aging", a process in which AChE is permanently inactivated by the bound OP inhibitor, occurs. Therefore, pretreatment with PYR can protect the AChE molecules against an OP such as soman that ages rapidly. Since only minutes are needed for soman to irreversibly inhibit 50% of ChE, aging by the bound soman could occur before an intoxicated soldier could administer post-exposure

treatments. PYR administered prophylactically is therefore an effective and complementary adjunct to atropine and pralidoxime as treatment for OP intoxication with very rapid inhibitory effects on AChE activity.

An overdose of either PYR or OP elicits toxicity by AChE inhibition, which leads to disturbances of cholinergic signaling. Figure 2 demonstrates the normal cholinergic signal transduction at the neuronal synapse. ACh is formed from the 2 immediate precursors, choline and acetyl coenzyme A by the catalytic enzyme choline-acetyl transferase. The reaction rate is determined by the availability of choline, which is supplied from the plasma and through high affinity choline uptake following degradation of ACh. The synthesized ACh is concentrated in neurotransmitter vesicles within the presynaptic terminal. A slow release of ACh is considered as baseline release for "house-keeping" purposes and occurs at all cholinergic synapses at rest condition. When a neuron fires, an action potential propagates to the presynaptic terminal. Depolarization initiates opening of the voltage-sensitive calcium ion channels. The resulting elevation of Ca++ ions triggers the fusion of neurotransmitter vesicles with the presynaptic membrane and subsequent release of ACh into the synaptic cleft. ACh binds with and activates cholinergic receptors on the postsynaptic membrane inducing the physiological events, e.g., gland secretion, muscle contraction or further downstream signaling through second messenger formation. AChE hydrolyzes ACh thereby terminating the action of the neurotransmitter.

There are two major subtypes of cholinergic receptors located on the post-synaptic membrane, nicotinic and muscarinic classified originally by the high affinity of

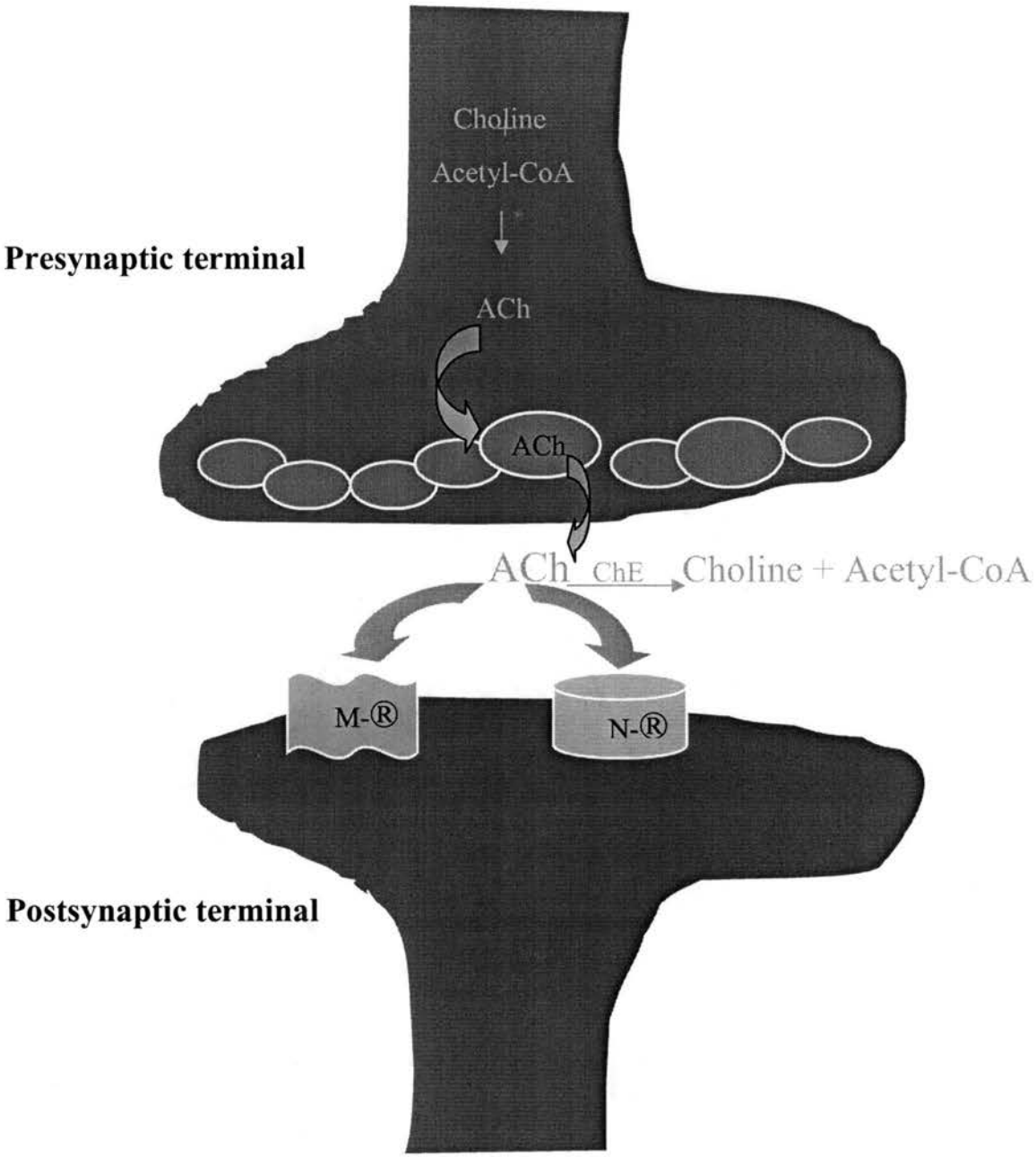

Figure 2: Cholinergic signal transduction at neuronal synapse. M-® and N-® represent muscrarinic and nicotinic receptor, respectively.

2 natural compounds (nicotine and muscarine) that bind to these receptors. Nicotinic receptors are essentially found in the CNS, autonomic ganglia, skeletal muscles and the adrenal glands. Most nicotinic ACh receptors consist of 5 peptide subunits, 2 copies of α subunits and a single copy of β, γ or δ subunit. The 5 protein subunits are arranged around a central cavity, which is the channel for the passage of ions across the membrane. Once the nicotinic receptor is activated by ACh, the ion channel is opened for the flow of selective ions. Therefore, the nicotinic ACh receptor is referred to as a "ligand-gated ion channel". Muscarinic ACh receptors are mainly located in the CNS (some presynaptic sites), myocardium, smooth muscles, and exocrine glands. In contrast to nicotinic receptor, the muscarinic receptor is a single polypeptide with 7 transmembrane domains, by which the signal is transduced by interacting with associated G-proteins. Upon binding with agonists, activation of muscarinic receptors generally causes inhibition of adenylyl cyclase or stimulation of phospholipase C, followed by the biochemical events including decreased production of cyclic AMP, and increased formation of inositol 1,4,5-trisphosphate and diacylglycerol, respectively. The change of the amount of these second messengers will affect other metabolic sequelae and trigger diverse cellular responses.

In the presence of anti-ChE agents, AChE is inactivated and therefore unable to hydrolyze ACh. Consequently, ACh accumulates in the synapse and over-stimulates the cholinergic receptors on the postsynaptic membrane leading to cholinergic toxicity. The cholinergic toxicity could be either central or peripheral in nature. Central nervous system disturbances include emotional lability, anxiety, headache, excessive dreaming, convulsion, ataxia, memory difficulty, respiratory paralysis and coma (Gutmann and

Besser, 1990). The peripheral cholinergic toxicity caused by overstimulating nicotinic receptors includes muscle involuntary movements such as twitches, fasciculation, cramps, and tremor followed by muscle weakness and paralysis leading to breathing difficulties and respiratory failure. Overstimulation of the peripheral muscarinic receptors leads to 1) smooth muscle spasms and increased gastrointestinal motility, nausea, vomiting abdominal cramping, bronchospasm and ureteral spasm etc, 2) extensive glandular secretion such as salivation, lacrimation, urination, defecation, rhinorrhea and bronchorrhea.

PYR has a quaternary ammonium group in the molecule as shown in Figure 3. This drug is therefore positively charged at physiological pH. In contrast to another carbamate ChE inhibitor physostigmine (PHY) with central ChE inhibitory capability, PYR is not expected to enter the brain due to the restriction by the blood-brain barrier (BBB) from entrance of polar or large molecules into the CNS.

The BBB consists of cerebral endothelial cells and the associated accessory structures including the tight junctions between endothelial cells, pericytes, astrocytes and the basal membrane. Figures 4 and 5 demonstrate the longitudinal and cross-sectional views of the blood vessels in the brain. The endothelial cells and the tight junctions between them completely enclose the blood lumen forming a barrier that distributes along the length of the blood circulation in the brain. The basal membrane covers the abluminal surface of the endothelial cells, with the pericytes providing mechanical support to the endothelium. Pericytes associated with the blood vessels have been suggested to regulate endothelial cell proliferation, survival, migration, differentiation, vascular branching, thus essentially to maintain the normal morphology

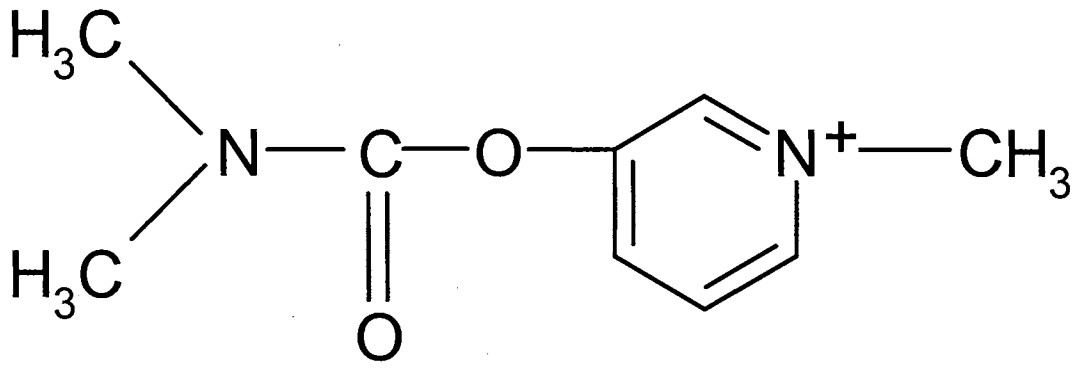

Figure 3: Molecular structure of pyridostigmine.

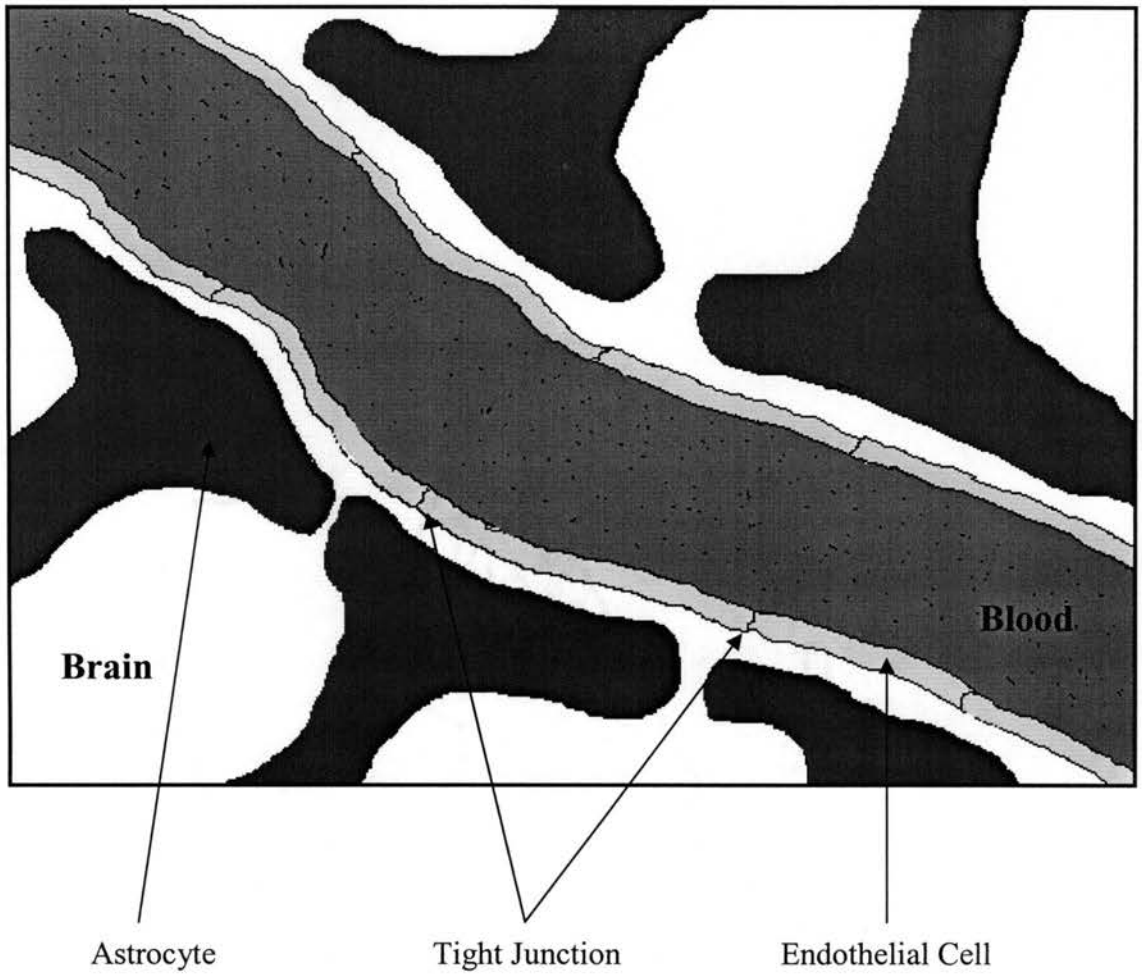

Figure 4: Longitudinal view of blood capillaries in the brain.

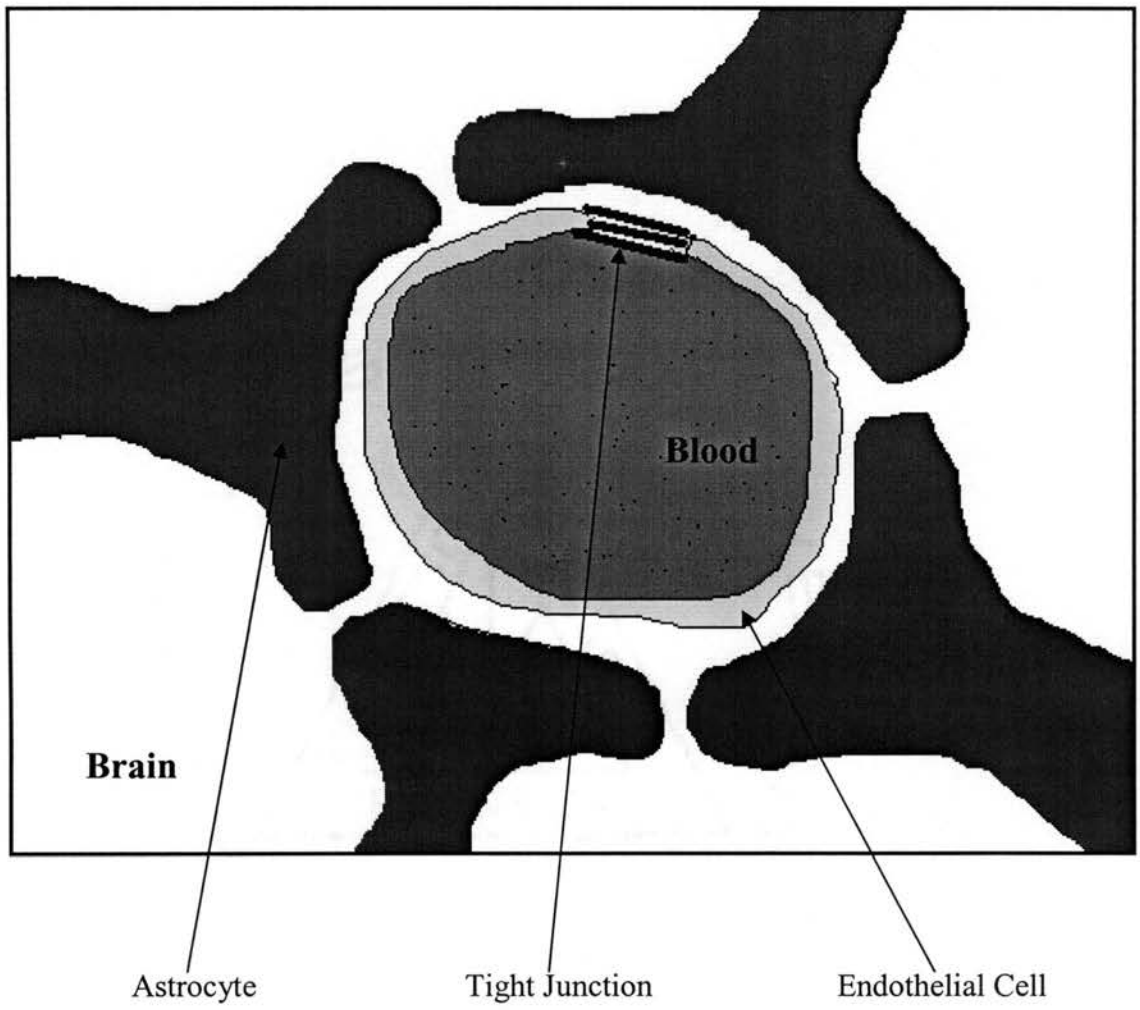

Figure 5: Cross-sectional view of the blood capillaries in the brain.

and function of the brain microvessels (Lindahl et al., 1997; Gerhardt et al., 2000; Hellstrom et al., 2001). Astrocytes are glial cells. With the end foot sharing the basal membrane, astrocytes cover most of the outer surface of the BBB endothelium and form another layer. Association of astrocytes with the endothelium is also important to the development and function of the BBB (Arthur et al., 1987; Wolburg et al., 1994).

The tight junction is the key structure for maintenance of BBB integrity. It seals the clefts between endothelial cells at the apical region forming continuous physical strands that course through the brain vasculature. The tight junctions and the neighboring endothelial cells form a remarkably high transmembrane electrical resistance in the range of 1000 to 2000 ohms-cm^2, as compared to less than 50 ohms-cm^2 of transmembrane electrical resistance of the capillaries in other tissues (Butt et al., 1990; Cereijido et al., 2000). The high electrical resistance at the blood-brain interface significantly limits molecule transport across the endothelial cells in the brain. Anatomy of cerebral endothelial cells shows 1) the cytoplasm is relatively similar in thickness and has very few pinocytotic vesicles that allowing formation of vacuole for molecule transport, 2) lack of fenestrations as noted in the capillaries in peripheral tissues and thus less openings for chemical penetration, 3) more and larger mitochondria (Oldendorf and Brown, 1975; Oledndorf et al., 1977) resulting in enhanced energy production capability, which is believed to be required for active transport of "favorable" substances into the brain. These anatomical characteristics suggest that blood to brain molecule transport is more favored of energy-dependent active transport over free diffusion, which allows the brain to gain necessary while rejecting unwanted substances.

The BBB strictly regulates and limits the exchange between the circulation and the cerebral extracellular space. To pass from the blood stream to the brain parenchyma, a molecule needs to penetrate in order 1) luminal membrane of endothelial cell, 2) the cytoplasma of the endothelial cell, 3) the abluminal membrane of the endothelial cells, 4) pericytes, basal membrane or both.

While relatively small or lipophilic molecules could pass the barrier through the clefts between endothelial cells or directly through the endothelial cells, other types of molecules have little access into the CNS. This mechanism protects the brain from composition changes that may result in pathophysiological events such as edema or swelling in brain tissues. The BBB integrity is maintained by the continuous tight junction expressed by the endothelial cells (Brightman and Reese, 1969). The compound of interest for our studies, PYR, has little access into the CNS under normal conditions. Some recent studies, however, suggested that stressful conditions may increase BBB permeability and allow PYR to enter the brain causing cholinergic toxicity.

Stress is stimulation or pressure from the ever-changing environment and the associated imbalance between exogenous environment demands and the living system's internal adaptation capacity. Existence of stress has allowed the organism to evolve strategies to deal with the critical changes of the surrounding environment. Stress of limited degree and duration is believed to have generally positive effects on living system. For instance, stressors may increase mental and physical performance, prepare the organism for high energy needs etc. A number of studies suggested, however, extreme and persistent stress caused adverse effects including injury or disturbance of brain structures or functions (Magarinos et al., 1996; McEwen and Magarinos, 1997;

Fuchs and Flugge, 1998; McEwen, 1999; McEwen, 2000). Specifically, stress was found to compromise the BBB integrity and facilitate chemical toxicity in the CNS.

Friedman and coworkers (1996) reported a critical finding that initiated significant interest and extensive studies in effects of stress on BBB permeability and PYR penetration into the CNS. In Friedman's work, adult FVB/N mice were subjected to 2 periods of 4-min forced swim with a 4 min rest between the 2 stress sessions. Ten minutes after the last swim, mice were injected with either saline or PYR and brain ChE activity was measured 10 min after PYR exposure. Results showed the dose of PYR needed to cause 50% brain ChE inhibition under normal condition was 1.50 mg/kg whereas the equi-effective dose was reduced to 0.01 mg/kg following the forced swim stress. The greater than 100 fold change in PYR ED_{50} under normal and stressful conditions suggested a robust penetration of PYR into the CNS, presumably due to BBB disruption by the brief physical stress. Friedman and colleagues (1996) also evaluated BBB permeability after the acute sessions of swim stress by doing the following experiments. In one study, Evans blue dye was injected into the heart of the mice and the concentration of albumin-bound Evans blue in the brain was determined spectrophotometrically. Data suggested the leakage of Evans blue dye into the brain of the stressed mice increased 10 times compared to that of the non-stressed mice. In another study, cytomegalovirus (CMV) AChE plasmid was injected (i.p.) into stressed or non-stressed mice. PCR amplification showed the CMV AChE DNA was detected from cycle reaction 21 in stressed mice while the PCR products were weak from cycle reaction 24 in non-stressed mice. Results suggested the penetration of plasmid DNA into the brains of the stressed mice was at least 8 times higher than that of the non-stressed mice.

Furthermore, central disruptions were noted. The level of PYR-induced c-fos oncogene was found to increase over 100 fold in the stressed mice compared to controls. In vitro incubation of brain slices with PYR caused similar enhanced c-fos mRNA level and neuronal excitability. Results from this study suggested forced swim stress facilitated PYR entry into the brain across a "leaky" BBB.

Other studies also suggested a variety of physical stressors may compromise BBB integrity and increase barrier permeability. Physical immobilization caused the vital dye trypan blue to enter into brain areas such as hypothalamus and the reticular formation (Belova and Jonsson, 1982). Acute heat at 38°C for 4 hrs, chronic heat at 36°C, and continuous 30 min forced swim were found to render the BBB leaky to tracers including Evans blue albumin complex and radiolabeled iodide sodium in selected brain areas (Sharma and Dey, 1986; Sharma et al., 1991, 1992, 1995). Physical immobilization stress for 30 min was reported to increase BBB permeability to intravenously administered ^{99}Technetium gluceptate in the diencephalon and cerebellum (Esposito et al., 2001). In the same study, acute restraint stress was found to cause activation of brain mast cells. Also, enhanced BBB permeability in response to restraint was blocked by pretreatment with disodium cromoglycate, a mast cell stabilizer. The combined findings suggested immobilization might increase BBB penetrability through activation of brain mast cells.

Stress activates the hypothalamic-pituitary-adrenal axis causing secretion of corticotropin-releasing hormone that in turn leads to release of glucocorticoids and catecholamines (White-Welkley et al., 1996; Dishman et al., 2000; Wong et al., 2000; Chennaoui, et al., 2002). Numerous studies suggested the enhanced release of stress hormones could elicit hippocampus injury or other neurotoxicity (Chan et al., 1996;

McIntosh and Sapolsky, 1996; Sapolsky, 1996; Bremner, 1999, 2001; Villarreal et al, 2002). Deployment and operation during any war are obviously stressful events. The experience in the GW was not an exception. First, unfavorable living conditions (e.g. change in diet, separation from family, isolation from the civilian society, etc) potentially enhanced psychological stress associated with war. Those conditions also included but were not limited to extreme weather in the battlefield in the Middle East, confinement in small camps and repeated military practice with little entertainment or recreation. Another severe stressor during the GW was apprehension about Iraqi military capabilities, such as the large fighting force, weapons of mass destruction, possible SCUD missile attacks on random targets and storage of highly lethal chemical and biological warfare agents with recognition of Iraq's past experience of using these agents in war time settings. In general, experience during the GW was a substantially stressful event to those military personnel, which may have led to deleterious effects in the nervous system as previously suggested, e.g., BBB disruption (Friedman et al., 1996).

Chemical stressors such as some irreversible OP ChE inhibitors have been reported to increase BBB permeability to systemically administered tracers (Ashani and Catravas 1981; Carpentier et al., 1990; Grange-Messent et al., 1999). The exact mechanism of BBB breakdown by relatively high dose OP exposure is not clear, but is associated with induction of seizures with no remarkable changes in the structure of the tight junctions (Grange-Messent et al., 1999; Nitsch and Klatzo 1983). OP exposure was also previously reported to facilitate entry of peripherally-acting ChE inhibitor into the brain. Co-exposure of chlorpyrifos, a centrally-acting ChE inhibitor, with PYR led to greater inhibition of brain ChE and neurotoxic esterase activity than chlorpyrifos

exposure alone (Abou-Donia et al., 1996). The mechanisms for such interactive effects between chlorpyrifos and PYR are unclear. The results suggested, however, that OPs might increase BBB permeability to a peripherally-acting ChE inhibitor, therefore contributing to the synergistic ChE inhibition after carbamate and OP co-exposure. Ashani and Catravas (1981) reported that paraoxon, at dosages causing seizures, compromised BBB integrity and allowed the peripheral ChE inhibitor phospholine iodide to enter the brain. In general, according to these previous findings, relatively high dosage OP may alter the BBB and render the cerebral endothelial cell layer permeable to primarily peripherally-acting agents that have little access to the brain under normal conditions. Preliminary results suggested, however, that low dose paraoxon exposure associated with no seizures may also increase BBB permeability to the marker horseradish peroxidase (HRP).

It has been widely accepted that PYR is a peripherally acting agent with little central effects. PYR, however, is not absolutely excluded from entering into the brain. Several studies suggested PYR with relatively high levels in the plasma could enter the brain causing observed CNS symptoms or functional changes. Loewenstein-Lichtenstein and co-workers (1995) demonstrated that high "effective" PYR levels resulting from the decreased detoxification capability of atypical BuChE caused severe CNS-associated symptoms. Results from Xia and colleagues (1981) suggested high dosage of PYR exposure (0.5 X LD_{50}) associated with extensive plasma ChE inhibition caused slight ChE inhibition (about 17%) in the brain. In our pilot study, the maximum tolerated dosage (MTD) of PYR (which caused more than 80% ChE inhibition in whole blood and diaphragm) inhibited about 20% ChE activity in the frontal cortex. Recently, repeated

treatment of PYR given at dosage of 1.85 mg/kg (i.p. injection) was also found to induce apoptotic cell death in cortex, hippocampus and striatum of rats (Li et al., 2000). PYR-induced apoptosis was blocked by pretreatment with atropine, suggesting the apoptotic cell death was a muscarinic receptor mediated response. These results suggest that PYR, a ChE inhibitor limited to primarily peripheral actions, may penetrate into the brain and interact with other macromolecular targets but have little effect on the "primary" target – AChE.

Several other studies reported interactions between PYR and muscarinic/nicotinic receptors. Using the patch clamp technique, PYR was found to interact with the ACh receptor-ionic channel complex, binding with the receptors as a weak agonist and inducing desensitization (Akaike et al., 1984; Albuquerque et al., 1984; Pascuzzo et al., 1984). Sherby and co-workers (1985) observed displaced binding of ^{3}H-ACh and ^{125}I-alpha-bungarotoxin to the ACh receptor sites by PYR pre-treatment. The bound PYR could act as a partial agonist and potentiate receptor desensitization (Santos et al., 2003). Therefore, it would be interesting to investigate the interaction of PYR with such additional targets (e.g. muscarinic and nicotinic receptors) in the brain, the subsequent pathological changes (e.g. cell death, etc), and the effects of stress on those changes.

Based on previous studies and our pilot findings, we hypothesized that physical or chemical stressors compromise BBB integrity and enhance PYR penetration into the brain. Enhanced CNS entry of PYR by stress may lead to increased neurotoxicity and be a contributing factor in unexplained GWI.

To test our hypothesis, we proposed the following research objectives:

1) To determine the acute toxicity of PYR in immature male rats following oral dosing
2) To evaluate the effects of either acute or repeated restraint stress on acute and subacute PYR toxicity
3) To study the effects of acute restraint stress on BBB permeability
4) To evaluate biochemical markers of stress following immobilization
5) To study the effects of chemical stressor on PYR toxicity
6) To investigate the effects of chemical stressor on BBB permeability

Chapter 2

MATERIALS AND METHODS

Animals

Six-week old Sprague Dawley male rats were used for study of the effects of acute restraint stress on pyridostigmine toxicity while Sprague Dawley rats used for the study of repeated restraint stress were 5 weeks old at the start and 7 weeks old at the end of the experiment. For study of chemical stressors, 1) male Long Evans rats, 25-30 days of age, 2) male Sprague Dawley rats, 25-30 days of age, and 3) male Sprague Dawley rats, 6 weeks of age were used. Rats were maintained and handled according to NIH/NRC Guide for the Care and Use of Laboratory Animals and reviewed by the Institutional Animal Care and Use Committee at Oklahoma State University. Animals were acclimated for 7 days before experimentation and housed in a temperature and light controlled room (23±1C°; 12 h : 12 h, light : dark cycle). Rats were given free access to food and water and isolated from environmental stress throughout the acclimation and experimentation periods.

Chemicals

Peroxidase (type II, from horseradish, activity 158 units/mg), pyridostigmine bromide (PYR, 3-dimethylaminocarbonyloxy-N-methylpyridinium bromide), glucose

oxidase, 3,3'-diaminobenzidine (DAB), and acetylcholinesterase (Type V-S) purified from electric eel were purchased from Sigma Chemical Company (St. Louis, MO). Paraoxon (O, O'-diethyl-O-(p-nitrophenyl) phosphate) was purchased from Chem Service (West Chester, PA). ^{3}H-Acetylcholine iodide (specific activity = 82.0 mCi/mmol) was obtained from New England Nuclear Company (Boston, MA). PYR and paraoxon were prepared in 0.9% saline fresh on the day of experiment and given by oral gavage and intra-muscular injection, respectively. DAB was prepared in a safety hood immediately before the start of the reaction for peroxidase histochemistry. All chemicals were reagent grade.

Determination of the Maximum Tolerated Dosage of PYR and PHY

Rats (n=6/treatment) were treated with one of four dosage levels of PYR (23, 30, 39 and 50 mg/kg) and observed for functional signs of toxicity and lethality for 24 hours. The highest dosage of PYR associated with no lethality in treated rats was determined as the acute, oral maximum tolerated dosage (MTD) for the 6-week old male Sprague Dawley rats. The MTD of PHY was determined the same way except that rats were given PHY at the dosage of 11, 14.5, 19 and 25 mg/kg.

Restraint Stress Protocols

Rats were immobilized by placement in plexiglass cylindrical restrainers (Model #51336, Stoelting Research Instruments, Wood Dale, IL). Three protocols for the acute restraint stress model were designed as shown in Figure 6. Rats were either 1) placed in the restraint tubes for 90 min and immediately challenged with PYR, 2) treated with PYR

Acute Restraint Stress Protocols

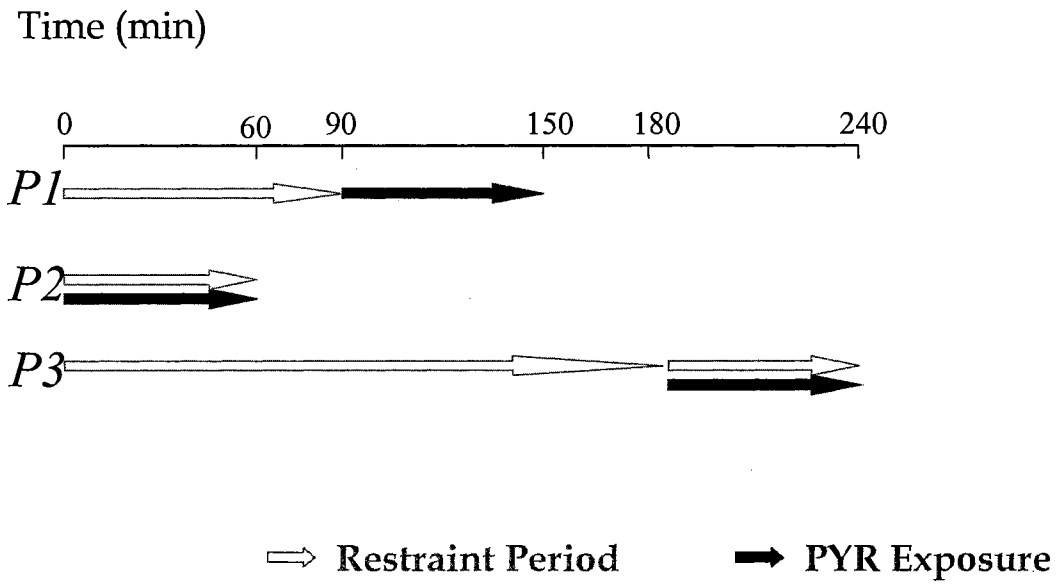

Figure 6: Acute restraint stress protocols. Protocol 1 (P1): Rats were placed in the restraint tubes for 90 min followed by administration of PYR and placed back in the home cage for 60 min. Protocol 2 (P2): Rats were challenged with PYR and immediately placed into the restraint tubes for 60 min. Protocol 3 (P3): Rats were placed in the restraint tubes for 3 hrs, briefly removed, treated with PYR and replaced into the restraint tubes for an additional 60 min.

immediately before placing them into the restraint tubes for 60 min or 3) placed in the restraint tubes for 3 hrs, briefly removed, treated with PYR and replaced into the restraint tubes for an additional 60 min. In all three acute stress protocols, rats were given the MTD of PYR (30 mg/kg, p.o.) and observed for functional signs, i.e., SLUD (acronym for salivation, lacrimation, urination and defecation) and involuntary movements. Rats were sacrificed for collection of tissues at 60 min after PYR dosing. For repeated restraint studies, protocol 2 of acute stress was utilized on a daily basis for 14 consecutive days, i.e., rats were given PYR (0, 3 or 10 mg/kg/day, p.o.) and immediately placed in the restraint tubes for 60 min each day. SLUD signs and involuntary movements were scored daily at the termination of the restraint stress. In all acute and repeated restraint studies, rats were divided into four experimental groups with 6 rats per treatment group: 1) saline only (rats were kept in their home cage and treated with saline, 1 ml/kg, p.o.), 2) PYR only (rats were kept in their home cage and treated with PYR (in saline, 1 ml/kg, p.o.), 3) restraint only (rats were immobilized in one of the above stress protocols and given saline, p.o.), and 4) restraint + PYR (rats were immobilized and given PYR, p.o.).

Chemical Stress Protocols

Three chemical stress protocols were designed as shown in Figure 7. In protocol 1, male 25 to 30 day-old Long Evans rats were given either PYR (30 mg/kg) or saline by oral gavage. Paraoxon (100 μg/kg) or vehicle was injected in the thigh muscle 50 min after PYR exposure. Rats were sacrificed 10 min after paraoxon treatment for tissue collection. In protocol 2, male 6-week old Sprague Dawley rats were treated the same as the Long Evans rats except the dosage of paraoxon was 220 μg/kg. In protocol 3, male 25

Chemical Stress Protocols

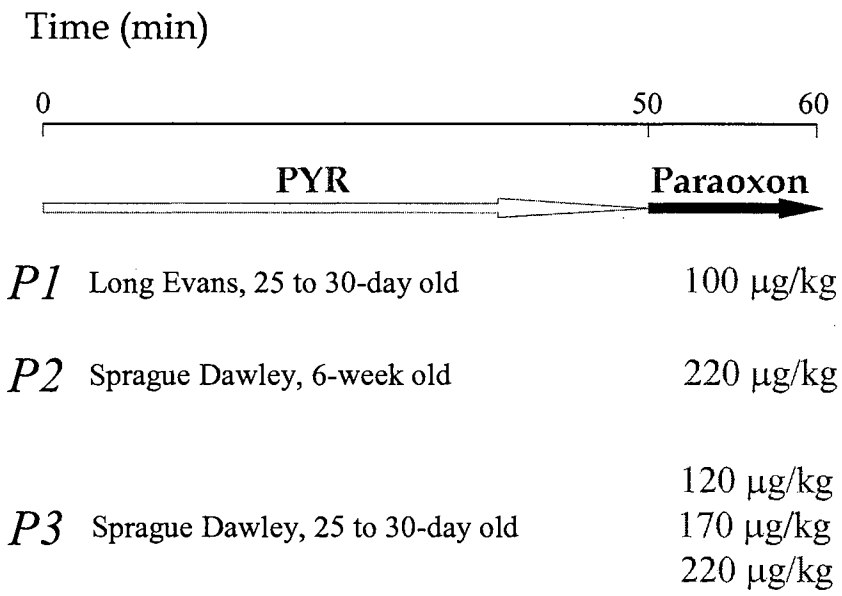

Figure 7: Chemical stress protocols. In all protocols, male rats were given PYR (30 mg/kg, p.o.) for 50 min followed by paraoxon treatment (i.m. injection) for 10 min. In protocol 1 (P1), 25 to 30 days old rats were used and the paraoxon dosage was 100 μg/kg. In protocol 2 (P2), 6 weeks old Sprague Dawley rats were used and paraoxon dosage was 220 μg/kg. In protocol 3 (P3), 25 to 30 days old Sprague Dawley rats were given one of three levels of paraoxon (120, 170 and 220 μg/kg).

to 30 day-old Sprague Dawley rats were given either PYR (30 mg/kg, p.o.) or saline. Three levels of paraoxon (120, 170, or 220 μg/kg) were given 50 min after PYR. Tissues were collected 10 min after paraoxon.

Functional and Behavioral Measurements

Animals were observed by two investigators before sacrifice (i.e., 60 min following PYR exposure) for involuntary movements and SLUD signs by the methods of Moser and coworkers (1988) as described before (Liu and Pope, 1996). Involuntary movements were scored as 2 = normal quivering of vibrissae, head and limbs; 3=mild, fine tremor typically seen in the forelimbs and head; 4=whole body tremor; 5=myoclonic jerks; 6=clonic convulsions. Autonomic dysfunction was scored as 1=normal, no excessive secretion; 2=slight, one SLUD sign or very mild multiple signs; 3=moderate, multiple, over SLUD signs; 4=severe, multiple, extensive SLUD signs.

AChE Activity Evaluation

Regional brain (frontal cortex, cerebellum and hippocampus) and diaphragm samples were collected, dissected on ice and washed with saline to remove contaminating blood. Tissues were homogenized in 50 mM potassium phosphate (KPi) buffer (pH = 7) using a Polytron PT-3000 homogenizer (Brinkman Instruments, Westbury, NY) at 28,000 rpm for 20 sec. Blood samples were collected in Eppendorf tubes (1.5 ml) containing heparin (20 μl, 10,000 units/ml) immediately after decapitation. All tissues were stored at -70°C, thawed and homogenized/diluted on the day of assay. AChE activity was measured radiometrically by the method of Johnson and Russell (1975) as previously

described (Pope et al., 1991) using 1 mM [^{3}H]acetylcholine iodide as the substrate. Incubation times and tissue concentrations required for linear rates of substrate hydrolysis were confirmed in preliminary assays. The radiometric assay was conducted in 7 ml scintillation vials at room temperature. Following 60 µl of triton X-100 (1% in 50 mM potassium phosphate buffer, pH=7.0), 20 µl of tissue homogenate were added, 20 µl of the hot substrate working solution was then added into the reaction mixture for a defined incubation time determined by preliminary study. "Stop" solution was then added to terminate the reaction followed by addition of 5 ml of scintillation solution. Reaction vials were vortexed thoroughly each time after solution was added. Capped vials were placed into a Wallac Liquid Scintillation Counter (Model 1409 DSA, PerkinElmer Inc., Boston, MA) for 2 min counting. Blank vials containing reaction solution but no tissue homogenate were also counted for the purpose of correcting for non-enzymatic hydrolysis of hot substrate. Reactions containing 20 µl of electric eel AChE (50 units/ml in phosphate buffer, pH 7) were also included for determination of the maximum hydrolysis of substrate. To minimize possible spontaneous reactivation of carbamylated ChE, conditions of limited tissue dilution and rapid assay following thawing and homogenization were adopted (Padilla and Hooper, 1992) as reported previously (Tian et al., 2002). Protein content was evaluated (Lowry et al., 1951) using 1 mg/ml bovine serum albumin as standard. Regional brain and plasma ChE activity was expressed as nmol/min/mg protein whereas blood AChE activity was expressed as nmol/min/µl blood.

Preparation of hot substrate

A stock solution of radiolabeled acetylcholine iodide was made by suspension of 1 mCi of [^{3}H]acetylcholine iodide (specific activity ≈ 75 mCi/mmol) into 2 ml 50 mM potassium phosphate buffer (pH=7.0). The stock solution was kept at –70°C in the ultra low freezer. To make working solution of radiolabeled acetylcholine iodide, 95 µl of stock solution was thawed and transferred into clean vial followed by addition of 475 µl of 24 mM non-radiolabeled acetylcholine iodide and 18.525 ml of 50 mM potassium phosphate buffer (pH=7.0). The final solution was 0.126 mM acetylcholine (specific activity of 3.944 µCi/µmol) and separated into 1 ml aliquots stored at –70°C until the time of ChE assay.

Preparation of stop solution

Stop solution was prepared by adding 9.45 g chloroacetic acid, 2 g sodium hydroxide and 11.6 g sodium chloride into distilled water with a final volume of 100 ml.

Preparation of scintillation cocktail

To prepare a liter scintillation cocktail solution, 5.0 g of PPO (2,5-diphenyl oxazole) and 0.3g of POPOP (1,4-bis[2-(5-phenyloxazolyl)]benzene) were dissolved in 100 ml of isoamyl alcohol and diluted in toluene with a total volume of 1 liter. Solution was stirred with a magnetic bar in the hood overnight.

Lowry Protein Assay

A standard curve of protein (0, 10, 25, 50, 75 and 100 µl of 1 mg/ml BSA) was included with each assay. For standard curve reactions, potassium phosphate buffer (pH 7.0) was added to each mixture to a total volume equal to the tissue volume in the sample reaction tubes. The volume of diluted sample tissue added was determined such that the protein content would be less than 100 µg/ml which is the maximum concentration on the standard curve. The final volume for either standard reactions or sample reactions was adjusted to 200 µl with water. Two ml of working agent #1 was then added followed by vortexing. After 10 min at room temperature, 200 µl working reagent #2 was added. The tubes were then vortexed and allowed to stand 30 min at room temperature. Absorbance was measured at 720 nm in a Beckman UV-VIS Spectrophotometer (Beckman-Coutler, Fullerton, CA).

Preparation of working reagents

Working reagent #1 was prepared by mixing 1 part of 0.5% copper sulfate solution, 1 part of 1% sodium potassium tartrate solution, and 100 parts of 2% sodium carbonate solution. Working reagent #2 was prepared fresh by mixing 1 part of folin phenol solution with 1 part of deionized water.

Measurement of Stress Hormones

For the acute restraint study, blood samples (n=6/treatment) were collected immediately, 1 and 3 hours following 60 min stress. For the repeated restraint study, blood samples (n=4/treatment) were collected immediately after removal from the

restraint tubes on days 1, 7 and 14. All procedures for collecting blood were completed within one minute to minimize hormone secretion caused by handling stress. Blood in a heparinized Eppendorf tube was immediately centrifuged (10,000 rpm, 1 minute at 4°C) to separate plasma, which was then frozen at -70°C until assay. Plasma corticosterone level was quantified using a radioimmunoassay kit (Rat Corticosteroid Coat-a-Count Kit, Diagnostic Products Corp., Los Angles, CA).

PYR Treatment for Neuronal Cell Death Study

Rats were given PYR (1.85 mg/kg, i.p.) every 12 hrs for 4 consecutive days as described by Li and colleagues (2000). Three hrs after the last dose, rats were perfused and fixed. Brains were collected, histochemically processed and evaluated for cell deaths under light microscope.

Whole Body Perfusion for Neuronal Cell Death Study

Rats were given a single injection of pentobarbital (50 mg/kg, i.p.). After being anesthetized, rats were fixed in dorsal recumbency on a rack in the pan. The thoracic cavity was opened by cutting from the left side of the sternum up to the level of the first rib. The sternum was reflected forward utilizing a hemostat clamp to expose the heart. Flushing solution began flowing at the rate of 40 ml/min. A blunted needle was inserted into the heart through the left ventricular apex towards the aorta. After the right heart was noted to bulge, the right atrium was lacerated with scissors. Blood, flushing solution and perfusate exited from the right side of the heart. Immediately following 60 sec perfusion with the flushing solution, fixative perfusion was started at the same pressure for 10 min.

Muscle twitching leading to whole body spasms was noted 1 to 2 min after start of the flowing of fixative indicating the systemic fixation of tissues. At the termination of perfusion, the whole rat body including the hind limbs was rigid as the indication of the quality perfusion. Brain was collected, processed and sliced for observation of cell death.

Preparation of flushing solution

For a volume of 1 L flushing solution, 1 ml heparin solution at the concentration of 1000 units/ml, 1 ml sodium nitrite solution (1%), and 8.5 g sodium chloride were added into deionized water to make a total volume of 1 L.

Preparation of fixative

Fixative was prepared fresh on the day of experiment. Paraformaldehyde (30 g) and 2 to 3 drops of 5 N sodium hydroxide solution were added into 380 ml deionized water. Mixture was stirred and heated (but not to boil) until the paraformaldehyde dissolved. The clear solution was allowed to cool and then added to 120 ml glutaraldehyde (25%), 500 ml phosphate buffer, and 1 g picric acid. Solution was well mixed and filtered through a syringe.

HRP Injection for BBB Permeability Evaluation after Restraint

Immediately after being restrained for 60 min, rats were anesthetized with pentobarbital (50 mg/kg, i.p.). HRP (50 mg/ml) in saline containing 2% Evans blue was injected directly into the heart (2 ml/kg) by cardiac puncture over a 30-second period. Blue discoloration of the skin, eyes and tail within about 1 minute after the injection was

regarded as a positive indicator of systemic injection and only those rats were used for subsequent analyses. Rats were decapitated 10 minutes after HRP injection. Whole brain was removed and rinsed with saline. Frontal cortex, cerebellum, and hippocampus were dissected and stored at -70°C until assay.

HRP Injection for BBB Permeability Evaluation after Paraoxon

Chemical stress protocol 1 was chosen to study the effects of paraoxon on BBB permeability. Rats were given PYR (30 mg/kg, p.o.). Fifty min later, paraoxon (100 μg/kg, i.m., 1 ml/kg) was injected in the thigh. At exactly 7.5 min after paraoxon treatment, rats were anesthetized by injection of xylaket (14.2 ml of ethanol, 2.5 ml of xylazine at 100 mg/ml, and 25 ml of ketamine HCl at 100 mg/ml were diluted into 0.9% saline in a final volume of 100 ml). The thoracic cavity of the rats was then opened to expose the whole heart. Exactly ten min after paraoxon treatment, 200 μl HRP in 2% Evans blue solution (40 mg/1 ml, HRP : Evans blue) was injected into the left ventricle of each rat over a 10-sec period. The eyes, skin, feet and tail of the rats turned blue as the consequence of Evans blue circulating throughout the whole body. Rats were sacrificed 1 min after the end of HRP injection. For ChE evaluation, rats were treated identically as above except that rats were not injected with HRP but sacrificed 10 min after paraoxon treatment. Whole brains were removed and cleaned of surface blood. Frontal and temporal cortex was dissected for ChE activity measurement.

HRP Biochemical Assay

HRP activity was measured essentially by the method of Stewart and coworkers (1992) as reported previously (Tian et al., 2002). Regional brain tissues were homogenized in 50 mM sodium acetate buffer (pH = 5.0) using a Polytron PT-3000 homogenizer for 20 seconds at 28,000 rpm. Samples (0.5 ml) were transferred to Eppendorf tubes (1.5 ml) and incubated with 0.1 ml 1% Triton X-100 in sodium acetate buffer for 20 minutes. Samples were vortexed twice during the 20 min incubation and centrifuged (4 min, 12,500 rpm, 4°C) following incubation. Reaction mixtures contained 1 ml substrate (10 mg o-dianisidine HCl + 0.5 ml deionized water + 49 ml 50mM acetate buffer + 0.5ml 100mM EDTA), 16.6µl tissue sample and 20 µl 0.3% H_2O_2. Absorbance at 460 nm was determined every 30 seconds after the start of reaction for 2 minutes. HRP activity was expressed as ng HRP/mg tissue wet weight.

HRP Histochemical Visualization

The method of HRP visualization in the brain was essentially as described by Stewart and co-workers (1992). Brains were removed, surface blood was cleaned, and they were then placed overnight in glutaraldehyde (2.5% in 0.1 M phosphate buffer, pH 7.4) followed by paraformaldehyde (4%) for at least 8 hours. Brains were then crude-dissected and temporal and frontal cortex were collected separately and replaced in paraformaldehyde for histochemical evaluation. Fixed brains were sliced at 70 µm on a vibratome and kept in ice cold 10 µM PBS buffer. Sections were sequentially washed with 1) 10 µM PBS buffer, 2) paraformaldehyde (4%), 3) 10 µM PBS buffer, 4) twice with 0.1 M Tris buffer (pH 7.4) and then treated with 0.5% cobalt chloride in Tris buffer

for 10 min at room temperature. Sections were washed three times with 0.1 M Tris buffer (pH=7.4) and twice with 0.1 M phosphate buffer (pH=7.4). Substrate (100 ml phosphate buffer containing 50 mg diaminobenzidine (DAB), 40 mg ammonium chloride, 200 mg β-D-Glucose and 3 mg glucose oxidase) was added. Slices were incubated in the dark at 37°C for 1 to 2 hrs untill a dark brown/black color was noted on the sections. Slices were mounted on glass slides and cover slips were positioned with 90% glycerol. Slides were dried for 10 min prior to observation under the light microscope (see below).

Preparation of fixative for HRP histochemistry

Solution of 0.4 M phosphate buffer was prepared first by adding 10.49 g sodium phosphate monobasic and 45.98 g sodium phosphate dibasic into 1 liter of water. Buffer was then well mixed and filtered to get the working solution. To make 1 liter 4% paraformaldehyde solution, 40 g paraformaldehyde was added into 400 ml deionized water and heated to 90°C for up to 45 min without boiling. A few drops of 10 M sodium hydroxide was added and the solution was then filtered using a filter syringe into 375 ml of 0.4 M phosphate buffer. Deionized water was then added to a final volume of 1 liter.

BBB leakage Quantitation

BBB disruption was visualized by HRP extravasation into the brain using light microscope. Eight sections from frontal and temporal cortex were selected for BBB leakage evaluation. Two investigators separately counted the HRP leaks under light microscope (10X magnification). Number of leaks observed on sections were

summarized and mean leaks/section was calculated for each of the frontal and temporal cortex.

Dexamethasone Injection

Male Long Evans rats (25 to 30 days old) were given 2 injections of dexamethasone (2 mg/kg, i.p.) with 24 hrs between the 2 treatments. Three hrs after the last dexamethasone injection, rats were given paraoxon (100 μg/kg, i.m.) followed by intra-cardiac injection of HRP 10 min later. Brains were collected 1 min after HRP perfusion and evaluated for HRP leakage in the frontal and temporal cortex.

Corticosteroid Secretion after PYR Exposure

Male Sprague Dawley rats (6-week old) were given MTD of PYR (30 mg/kg, p.o.). Whole blood was collected at 1, 2, 3, 6, and 24 hrs after PYR exposure. Plasma was separated and corticosteroid was measured by radioimmunoassay.

Statistical Analysis

Functional signs of toxicity were expressed as median ± interquartile range (IQR) and analyzed for significance by the Pearson Chi-square test. AChE activity was tested for significance by one-way or two-way analysis of variance (ANOVA). HRP activity was tested by one-way ANOVA followed by linear contrasts. HRP leakage (calculated as leaks/section) was analyzed by two-way ANOVA followed by linear contrasts. Plasma corticosteroids levels were also tested by two-way ANOVA. All statistical analyses were

done with the JMP statistical computer software (SAS, 1995). In all cases, P value < 0.05 was considered statistically significant.

Chapter 3

RESULTS

Part I: Effects of Physical Stress on PYR-induced Cholinergic Toxicity

Maximum Tolerated Dosage of PYR and Physostigmine

Table 2 demonstrates lethality in the 6-week old rats following PYR or physostigmine (PHY) exposure. After treatment with PYR, no death was noted in rats given 23 or 30 mg/kg PYR while one death occurred within 24 hrs in the rats given 38.5 or 50 mg/kg. After PHY treatment, all rats given 11 mg/kg survived while 1 death was noted within 24 hrs in rats given 14.5 and 19 mg/kg and 2 deaths in 25 mg/kg. The acute oral MTDs of PYR and PHY in 6-week old male Sprague Dawley rats were therefore estimated at 30 and 11 mg/kg (in saline, 1 ml/kg), respectively.

Timecourse and Dose-response of ChE Inhibition Following PYR or PHY

Figure 8 illustrates inhibition of whole blood ChE activity from 0.5 to 24 hrs following PYR exposure (30 mg/kg, p.o.). Peak inhibition occurred from 0.5 to 4 hours after dosing, and less than 50% of control activity was still noted at 24 hrs after treatment. There was a slight (<20%) but significant reduction of frontal cortex ChE activity at both 1 and 2 hr following PYR exposure. Figure 9 shows dose-related inhibition of blood ChE

activity 1 hr after PYR dosing, with an estimated ED_{50} of 3 mg/kg. As expected, lower dosages of PYR had little effect on cortical ChE activity. Figure 10 shows the *in vitro* inhibition of brain ChE activity by PYR. ChE inhibition increased in a concentration dependent manner with an IC_{50} of 0.66 μM (95% CI =0.4 to 1.1 μM).

Figures 11 and 12 show the timecourse of ChE inhibition following MTD dosing and dose-response evaluation following PHY exposure. Both blood and brain ChE was significantly inhibited by PHY as shown in Figure 11. Inhibition occurred rapidly after PHY dosing, with 50% inhibition noted at 15 min following treatment. ChE activity started to recover from 2 hrs after exposure and nearly all activity recovered by 24 hrs after treatment. More extensive dose-related inhibition of brain ChE occurred after PHY than after PYR as noted in Figure 12, presumably due to the ability of PHY to penetrate into the brain through the BBB.

These results confirm that PYR is essentially a peripherally-acting anti-ChE agent with limited effects on ChE activity in the frontal cortex, even at the highest dosage level (30 mg/kg, p.o.) that rats could tolerate (i.e., the MTD). However, brain ChE was sensitive to PYR *in vitro*.

Functional Toxicity Measurements

Figures 13 to 15 illustrate the effects of acute restraint stress on functional signs of cholinergic toxicity, i.e., SLUD and involuntary movements (IM), following the MTD of PYR. Rats (either stressed or non-stressed) treated with PYR showed only moderate signs of cholinergic toxicity (SLUD ranged from 2 to 2.5, IM ranged from 3 to 3.25). None of the acute stress protocols, however, influenced PYR-induced toxicity since the

PYR-treated rats under restraint stress showed similar levels of toxicity as those without stress. Figures 16 to 19 illustrate the effects of daily restraint stress (60 min) on functional signs of toxicity in rats dosed daily with PYR (0, 3 or 10 mg/kg PYR) for 14 days. Rats (either stressed or non-stressed) given PYR (either 3 or 10 mg/kg, p.o.) initially exhibited SLUD signs and IM in the first 3 to 5 days of dosing, after which no functional signs were noted suggesting the development of tolerance. The degrees of toxicity signs for stressed PYR-treated rats and unstressed PYR-treated rats were similar, however suggesting repeated restraint stress had no apparent effects on PYR toxicity.

ChE Activity

Figures 20 to 41 show the effects of acute or repeated PYR exposures on ChE activity in central (frontal cortex, cerebellum and hippocampus) and peripheral (blood and diaphragm) tissues in the acute and repeated stress studies. All the ChE activities were expressed as % of control and summarized in Table 3. In general, PYR caused substantial inhibition of ChE activity in peripheral tissues, while having minimal and inconsistent effects on brain regional ChE activity. Acute PYR exposure in the acute restraint study caused marked inhibition of whole blood ChE activity (86-95%). Daily PYR exposure (3 or 10 mg/kg) for 14 days caused marked inhibition (52-85%) in peripheral tissues (whole blood and diaphragm) but little change in brain regional ChE activity. None of the acute or repeated restraint procedures increased ChE inhibition by PYR in either peripheral or central tissues, however.

BBB Permeability Following Acute Restraint

HRP accumulation in brain regions was used to evaluate the change of BBB permeability following acute stress (60 min restraint). Figure 42 shows that HRP activity was not increased in frontal cortex, cerebellum or hippocampus by prior restraint stress.

Corticosteroid Levels

Figure 43 shows the plasma corticosteroid levels in rats following 60 min restraint stress. Plasma corticosteroid level was increased significantly (greater than 6-fold) immediately after termination of restraint and returned to normal 1 hour later. Figure 44 shows plasma corticosteroid levels in rats following repeated daily restraint stress of 1 hr. Corticosteroid level was significantly elevated immediately after termination of restraint on days 1 (about 7-fold) and 7 (about 4-fold), but not increased on day 14 of the study. These results suggest that both acute and repeated restraint models induced a significant stress response, and that tolerance to immobilization may eventually develop with daily restraint stress.

Neuronal Cell Death after PYR Treatment

Figures 45 to 47 show neurons in the hippocampus and cortex following repeated PYR (1.85 mg/kg, i.p., every 12 hrs for 4 days) exposure. Micrographs of brain slices with Hematoxylin-Eosin (H-E) staining demonstrated the viable neurons with intact cell membranes and normal nucleus. No cell deaths were noted in the slices of brain from rats treated repeatedly with this relatively high dosage PYR exposure.

Part II: Effects of Chemical Stressor on PYR Penetration into the Brain

BBB Leakage Visualization after PYR/Paraoxon

Figure 48 is the result of pilot study from our collaborator (Dr. J. Bressler, Kennedy Krieger Institute, Baltimore, MD) showing BBB leakage visualized as the HRP extravasation from the cerebral capillaries into rat brain 10 min after paraoxon (100 μg/kg, i.m.). Figures 49 to 52 illustrate HRP leaks (as indicated with black solid arrows) in the cortex after paraoxon and/or PYR exposure (treatments described in methods). Sporadic HRP leaks were noted in brain slices from control rats, which was considered as the baseline of HRP leakage in normal rats. Single high dose PYR treatment caused no apparent increase of HRP leaks. Diffuse leaks were observed, however in frontal and temporal cortexes of the rats given a single paraoxon dose (100 μg/kg, i.m.). Interestingly, only a few HRP leaks were noted in the brain slices of rats following PYR and paraoxon co-exposure, similar to that seen in the control or PYR-only groups. These results suggest paraoxon-associated HRP leaks were blocked by PYR pre-treatment.

Quantitation of BBB Leakage after PYR/Paraoxon

Figures 53 and 54 show the BBB disruptions quantified by the amount of HRP leaks in frontal and temporal cortex following PYR, paraoxon or both. Baseline BBB leakage (about 6 leaks/section) was noticed in the brain of control rats. Sporadic BBB leaks (about 10 leaks/section) with relatively similar changes as in the control group were found in brain of the rats given a single dose of PYR. Relatively low dosage paraoxon (100 μg/kg, i.m.) elicited significantly higher number of HRP leaks into the brain (20, 24

leaks/section in frontal and temporal cortex, respectively). BBB leakage (5, 11 leaks/section in frontal and temporal cortex, respectively) of the rats following PYR and paraoxon co-exposure, however, was significantly lower than that in the rats treated with paraoxon only. These results suggest that paraoxon exposure induces significant BBB leakage in both frontal and temporal cortex. The paraoxon-induced HRP leaks into the brain were, however, at least partially blocked by the PYR pre-exposure.

Functional and Behavioral Measurements

Figure 55 shows functional signs of toxicity in rats given PYR, paraoxon or both. Rats given high dose of PYR (30 mg/kg, p.o.) showed moderate signs of cholinergic toxicity (SLUD signs median score=2; involuntary movement median score=3). Rats showed no overt signs of toxicity following paraoxon exposure (100 μg/kg), however. Rats also showed minimum but significant signs of toxicity following PYR and paraoxon co-exposure (SLUD signs median score=2; involuntary movement median score=3) with the toxicity scores similar to those noted in rats treated with PYR only. These results suggest that low dosage of paraoxon caused no cholinergic toxicity while a high dosage of PYR elicited moderate signs of toxicity. Paraoxon did not, however, influence PYR-induced SLUD signs or involuntary movements.

ChE Activity

Figures 56 to 59 demonstrate ChE activity in the frontal cortex, temporal cortex, blood, and diaphragm following PYR, paraoxon or both in chemical stress protocol 1. For peripheral tissues, PYR caused remarkable ChE inhibition (>80%) while paraoxon

inhibited 40 to 65% ChE activity in whole blood and diaphragm. PYR and paraoxon combined exposure also caused more than 80% ChE inhibition in blood and diaphragm, which was similar as the inhibition levels following PYR only. For central tissues, PYR had little inhibitory effects on either frontal or temporal ChE activity. Paraoxon exposure caused 54-58% ChE inhibition in frontal and temporal cortex. PYR and paraoxon co-exposure caused 40 to 42% ChE inhibition in frontal and temporal cortex. These results suggest relatively low dose of paraoxon (100 μg/kg, i.m.) inhibited about 50% of total ChE activity in the brain, however, paraoxon and PYR co-exposure did not elicit more extensive brain ChE inhibition. In fact, PYR pre-exposure appeared to block some degree of ChE inhibition caused by paraoxon (see Figure 57).

Figures 60 to 64 illustrate ChE activity in frontal cortex, cerebellum, hippocampus, whole blood and diaphragm after PYR, paraoxon or both in chemical stress protocol 2. PYR had little effect on central ChE activity while paraoxon (220 μg/kg) caused about 50% ChE inhibition in frontal cortex and cerebellum. Rats treated with both PYR and paraoxon had similar brain ChE inhibition (45-49%) as noted in rats treated with paraoxon alone. PYR caused 87-88% inhibition while PYR and paraoxon co-exposure caused 90-93% ChE inhibition in the whole blood and diaphragm. The two inhibition levels were statistically similar. Results suggested paraoxon did not facilitate PYR-induced ChE inhibition in the brain or peripheral tissues.

Figures 65 to 68 demonstrate ChE activity in frontal cortex, cerebellum, hippocampus and whole blood after PYR, paraoxon or both in chemical stress protocol 3. PYR treatment did not lead to ChE inhibition in the central tissues. Paraoxon at 3 different dose levels caused 48 to 75% ChE inhibition in cortex and cerebellum. Brain

ChE inhibition (44-72%) after co-exposure to PYR and each of the 3 levels of paraoxon was similar as that noted after the paraoxon exposure alone, which suggests paraoxon did not increase the PYR-induced brain ChE inhibition. Blood ChE inhibition (87%) after PYR dosing was relatively similar to that after PYR and paraoxon co-exposure (95%). Therefore, paraoxon and PYR did not appear to interact to yield different levels of ChE inhibition compared to individual chemical treatments.

In summary, in all chemical stress protocols, paraoxon did not apparently enhance PYR penetration into the brain or influence the degree of PYR-induced ChE inhibition in any tissues.

Effects of Dexamethasone on BBB Leakage

Figure 69 shows the effect of dexamethasone on BBB leakage caused by paraoxon exposure. Dexamethasone alone (2 mg/kg, 2 injections) induced baseline HRP leaks (6 leaks/section) in the brain similar to that noted in control rats (7 leaks/section). Paraoxon (100 μg/kg) consistently elicited increased HRP leakage (16 leaks/section). Rats pre-treated with dexamethasone before paraoxon exposure showed baseline HRP leaks. The results suggested that dexamethasone pre-exposure could block the paraoxon-induced BBB disruption.

Corticosteroid Secretion after PYR Exposure

Figure 70 shows time-dependent effects of PYR on corticosteroid secretion. PYR (30 mg/kg, p.o.) significantly increased plasma corticosteroid levels at 1, 2 and 3 hrs

(greater than 10 times control levels). Corticosteroid secretion returned back to normal levels 6 hrs after PYR dosing.

Table 2: Determination of the maximum tolerated dosage of PYR and PHY in 6-week old male Sprague Dawley rats.

A. Lethality after PYR exposure

Dosage (mg/kg)	23	30	38.5	50
Death	0/6	0/6	1/6	1/6

B. Lethality after PHY exposure

Dosage (mg/kg)	11	14.5	19	25
Death	0/6	1/6	1/6	2/6

Table 3: Influence of restraint stress on ChE inhibition following PYR exposure in rats.

Conditions	Treatment[1]	ChE Activity[2] (% control ± SEM)				
		Cortex	Cerebellum	Hippo	Blood	Diaphragm
Restrain-Trt[3]	Con-sal	100.0±6.5	100.0±4.7	100.0±2.5	100.0±3.3	
	Con-PYR	90.6±10.8	99.6±9.1	93.6±5.0	9.5±1.2*	
	Str-sal	93.6±9.4	99.9±4.4	91.4±4.4	84.1±7.9	
	Str-PYR	103.2±5.1	83.0±4.5	101.6±5.5	7.7±0.7*	
Restraint/Trt[4]	Con-sal	100±3.3	100.0±6.4	100±2.3	100.0±5.8	
	Con-PYR	79.8±4.7*	97.8±7.6	86.2±5.7	13.8±1.1*	
	Str-sal	106.9±4.3	106.4±6.4	93.8±5.1	102.9±5.6	
	Str-PYR	78.6±8.1*	88.3±2.5	93.0±6.8	12.8±0.6*	
Restrain-Trt/Restraint[5]	Con-sal	100.0±4.4	100.0±3.0	100.0±9.9	100.0±13.0	
	Con-PYR	76.5±7.4	88.8±5.0*	108.7±3.0	4.9±0.7*	
	Str-sal	96.7±5.1	111.4±5.5	110.1±7.4	96.5±8.6	
	Str-PYR	81.0±11.1	78.3±3.4*	101.4±4.0	8.3±0.5*	
Repeated Restraint(3mg/kg/day)[6]	Con-sal	100.0±6.4	100.0±2.7	100.0±3.2	100.0±9.6	100.0±4.7
	Con-PYR	93.1±9.8	101.4±2.6	106.5±12.1	46.6±3.3*	23.3±1.6*
	Str-sal	110.3±4.7	100.3±2.1	99.3±9.2	110.9±6.6	96.9±6.1
	Str-PYR	111.0±7.0	106.2±4.6	98.4±2.8	46.5±3.2*	17.7±4.6*
Repeated Restraint(10mg/kg/day)[7]	Con-sal	100.0±3.5	100.0±4.2	100.0±8.6	100.0±8.8	100.0±6.4
	Con-PYR	96.3±2.7	82.2±1.3*	92.1±10.7	19.8±2.0*	30.0±2.0*
	Str-sal	105.0±3.6	98.5±3.1	89.0±10.4	98.3±11.1	91.9±7.7
	Str-PYR	99.3±3.8	84.5±3.8*	87.5±5.5	15.4±1.8*	21.9±2.1*

[1] For each experiment, 24 rats were divided into 4 groups with 6 rats per treatment: Rats were either not stressed (Con) or restraint-stressed (Str) and either given saline (sal) or pyridostigmine (PYR)

[2] ChE activity was measured by the radiometric method as described in Methods. Whole blood ChE activity was calculated as nmol/min/µl blood (combined control value: 0.48 ± 0.09) whereas regional brain and diaphragm ChE activity was calculated as nmol/min/mg protein (combined control value: frontal cortex, 39.2 ± 7.2; cerebellum, 20.6 ± 3.8; hippocampus, 24.2 ± 4.4; diaphragm, 4.3 ± 1.2). ChE activity was expressed as mean percentage of non-stressed, saline control group (± SEM).

[3]Rats were restrained for 90 min, removed from restraint tubes and given PYR (30 mg/kg), and then sacrificed 1 h later for cholinesterase measurements.

[4]Rats were given PYR (30 mg/kg), immediately placed in the restraint tubes and then sacrificed 1 hr later for cholinesterase measurements.

[5]Rats were placed in restraint tubes for 3 hrs, removed and given PYR (30 mg/kg), replaced back into the restraint tubes for an additional 1 hr and then sacrificed for cholinesterase measurements.

[6]Rats were given PYR (3 mg/kg/day) and placed in the restraint tubes for 1 hr each day for 14 consecutive days. On the last day, rats were sacrificed immediately upon removal from the restraint tubes for cholinesterase measurements.

[7]Rats were given PYR (10 mg/kg/day) and placed in the restraint tubes for 1 hr each day for 14 consecutive days. On the last day, rats were sacrificed immediately upon removal from the restraint tubes for cholinesterase measurements.

*Asterisk indicates significant difference from Con-sal group.

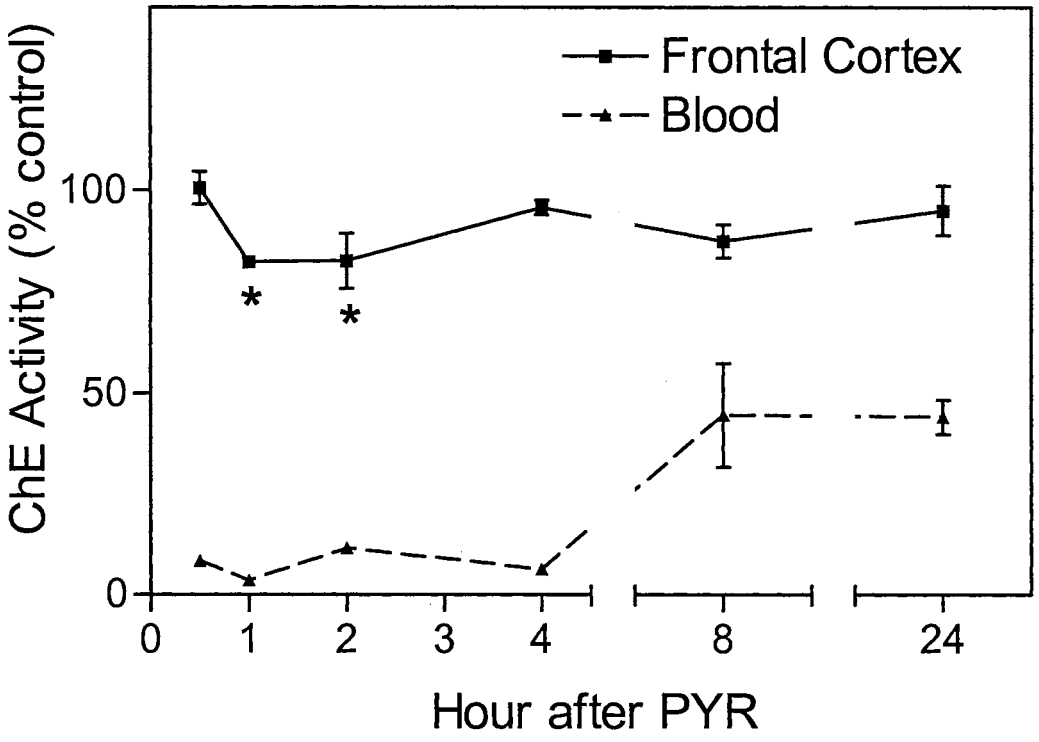

Figure 8: Inhibition of ChE activity in whole blood and frontal cortex following maximum tolerated dosage of pyridostigmine bromide administration. Rats (n=6/treatment group) were treated with PYR (30 mg/kg, p.o. in saline) and sacrificed 0.5, 1, 2, 4, 8 or 24 hrs following exposure. Frontal cortex and blood were collected for ChE activity measurement. ChE activity was measured by the radiometric method as described in Methods. Whole blood ChE activity was calculated as nmol/min/μl blood whereas frontal cortex ChE activity was calculated as nmol/min/mg protein. ChE activity was expressed as mean percentage of control group ± SEM.

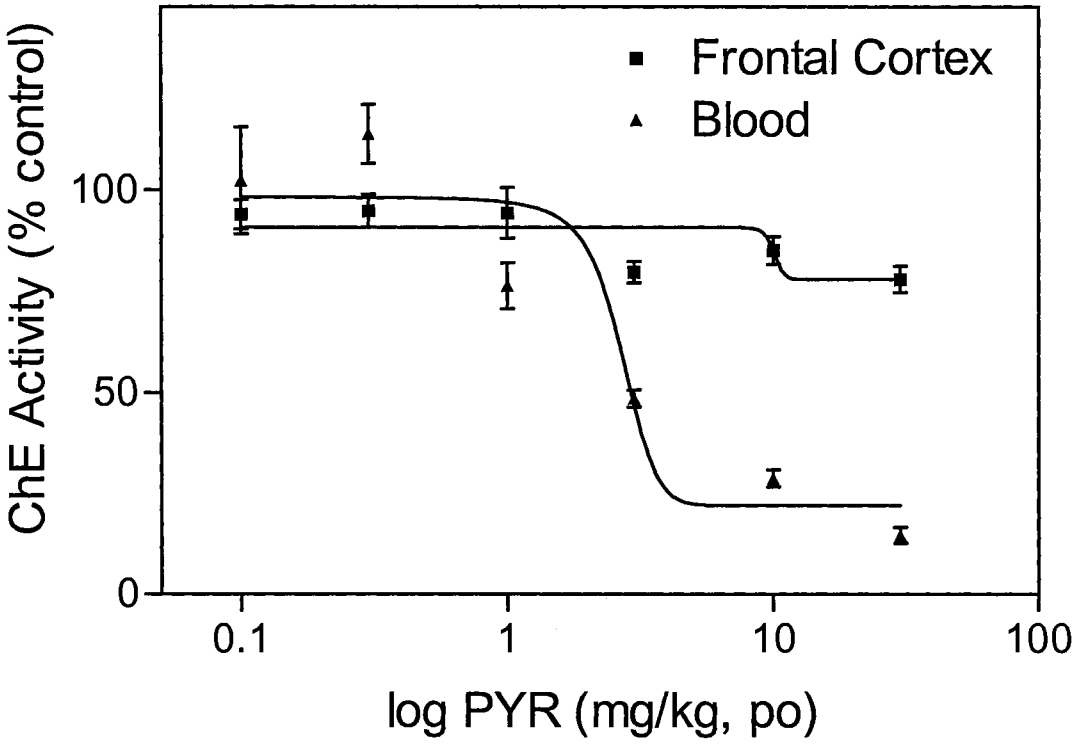

Figure 9: In vivo dose-related ChE activity in frontal cortex and whole blood following PYR exposure. Rats (n=6/treatment group) were treated with PYR (0.1, 0.3, 1, 3, 10 or 30 mg/kg, p.o.) and sacrificed 1 hr following exposure for measurement of ChE activity. Whole blood ChE activity was calculated as nmol/min/μl blood whereas frontal cortex ChE activity was calculated as nmol/min/mg protein. ChE activity was expressed as mean percentage of control group ± SEM.

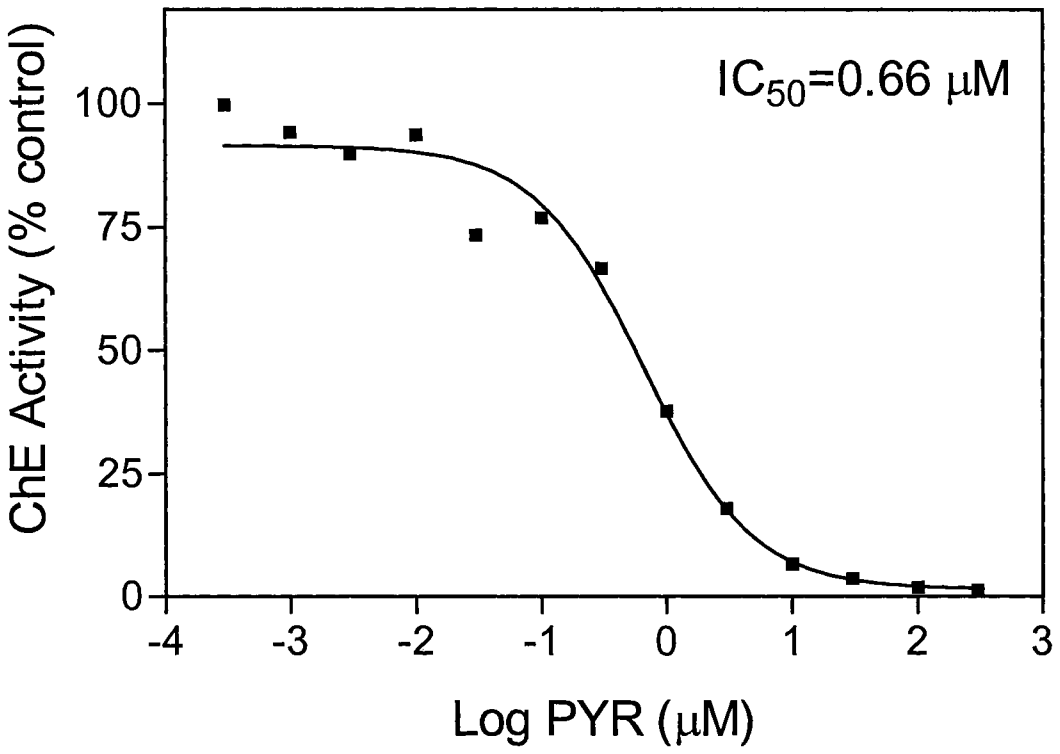

Figure 10: In vitro concentration dependent frontal cortex ChE activity following PYR exposure. Rats frontal cortex was incubated with PYR with a series of concentrations (0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, 300 μM) at 37°C for 30 min and then measured for ChE activity. Frontal cortex ChE activity was calculated as nmol/min/mg protein. ChE activity was expressed as mean percentage of control group.

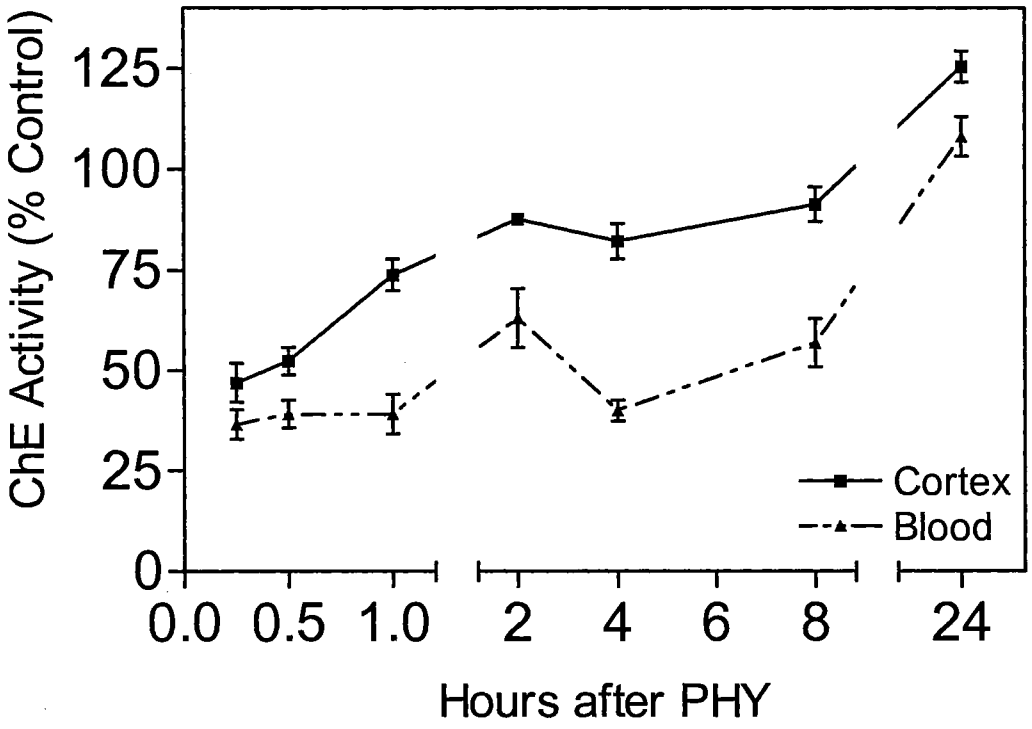

Figure 11: Inhibition of ChE activity in whole blood and frontal cortex following maximum tolerated dosage of physostigmine administration. Rats (n=6/treatment group) were treated with PHY (11 mg/kg, p.o. in saline) and sacrificed 15 min, 0.5, 1, 2, 4, 8 or 24 hrs following exposure. Frontal cortex and blood were collected for ChE activity measurement. ChE activity was measured by the radiometric method as described in Methods. Whole blood ChE activity was calculated as nmol/min/µl blood whereas frontal cortex ChE activity was calculated as nmol/min/mg protein. ChE activity was expressed as mean percentage of control group ± SEM.

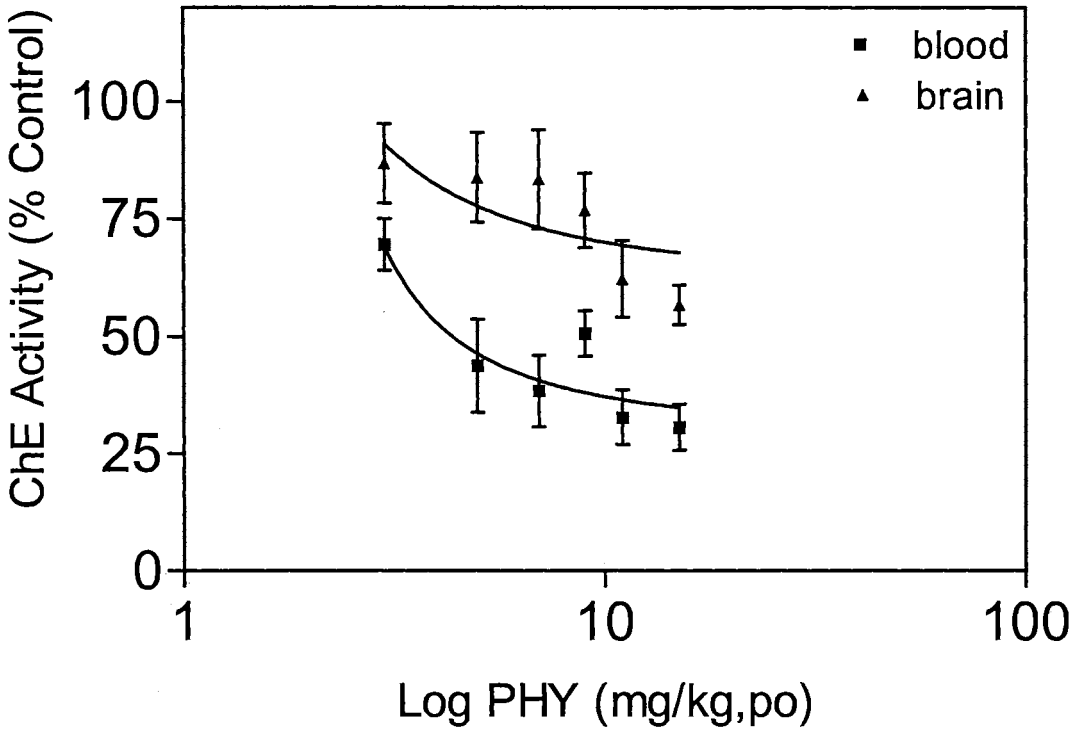

Figure 12: In vivo dose-related ChE activity in frontal cortex and whole blood following PHY exposure. Rats (n=6/treatment group) were treated with PYR (3, 5, 7, 9, 11 or 15 mg/kg, p.o.) and sacrificed 30 min following exposure for measurement of ChE activity. Whole blood ChE activity was calculated as nmol/min/μl blood whereas frontal cortex ChE activity was calculated as nmol/min/mg protein. ChE activity was expressed as mean percentage of control group ± SEM.

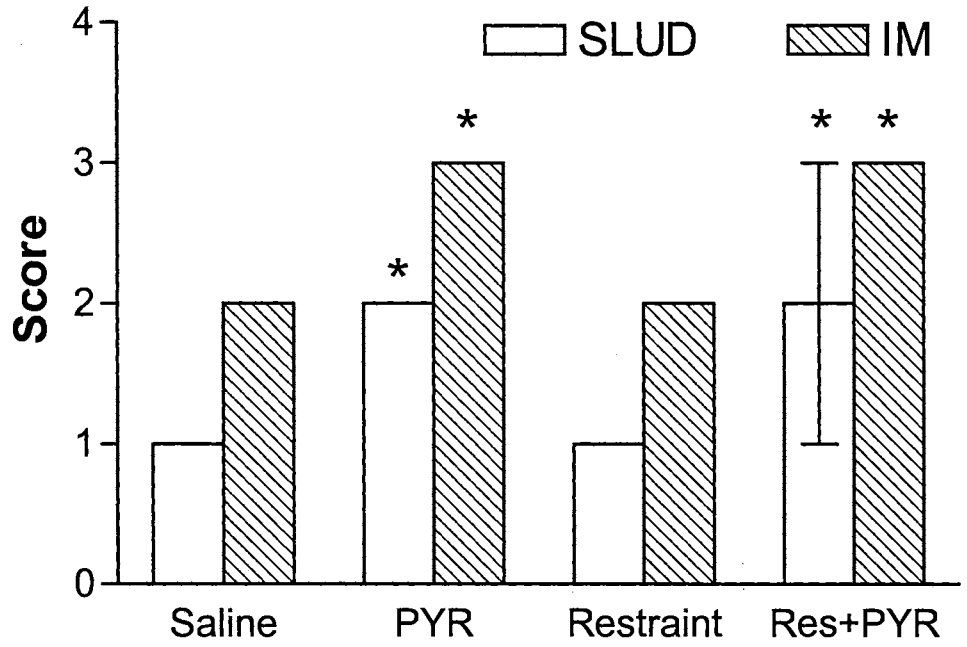

Figure 13: Effects of acute restraint stress protocol 1 on PYR-induced cholinergic toxicity. Rats (n=6/treatment) were immobilized (or not restrained) for 90 min and then given PYR (or saline) for 60 min. Rats were observed for functional signs of toxicity (SLUD signs and IM, reported as median ± IQR) before sacrifice. Asterisk indicates significant ($p<0.05$) difference from control group.

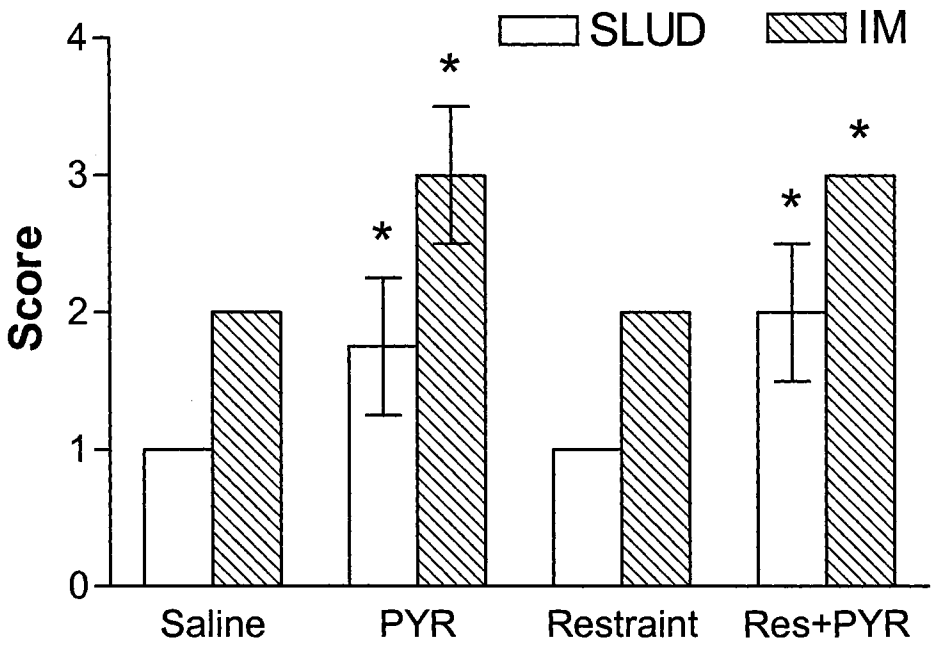

Figure 14: Effects of acute restraint stress protocol 2 on PYR-induced cholinergic toxicity. Rats (n=6/treatment) were given PYR (or saline) and immediately placed in restraint tubes (or acclimation cage) for 60 min. Rats were observed for functional signs of toxicity (SLUD signs and IM, reported as median ± IQR) before sacrifice. Asterisk indicates significant ($p<0.05$) difference from control group.

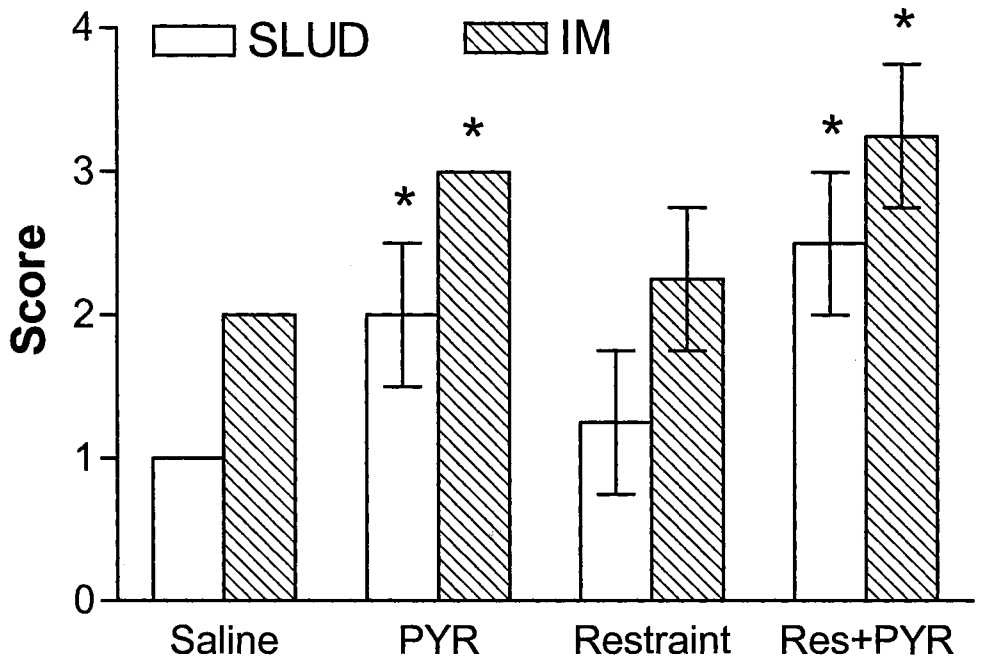

Figure 15: Effects of acute restraint stress protocol 3 on PYR-induced cholinergic toxicity. Rats (n=6/treatment) were immobilized (or not restrained) for 3 hrs, given PYR (or saline) and replaced into the restraint tubes (or acclimation cage) for an additional 60 min. Rats were observed for functional signs of toxicity (SLUD signs and IM, reported as median ± IQR) before sacrifice. Asterisk indicates significant ($p<0.05$) difference from control group.

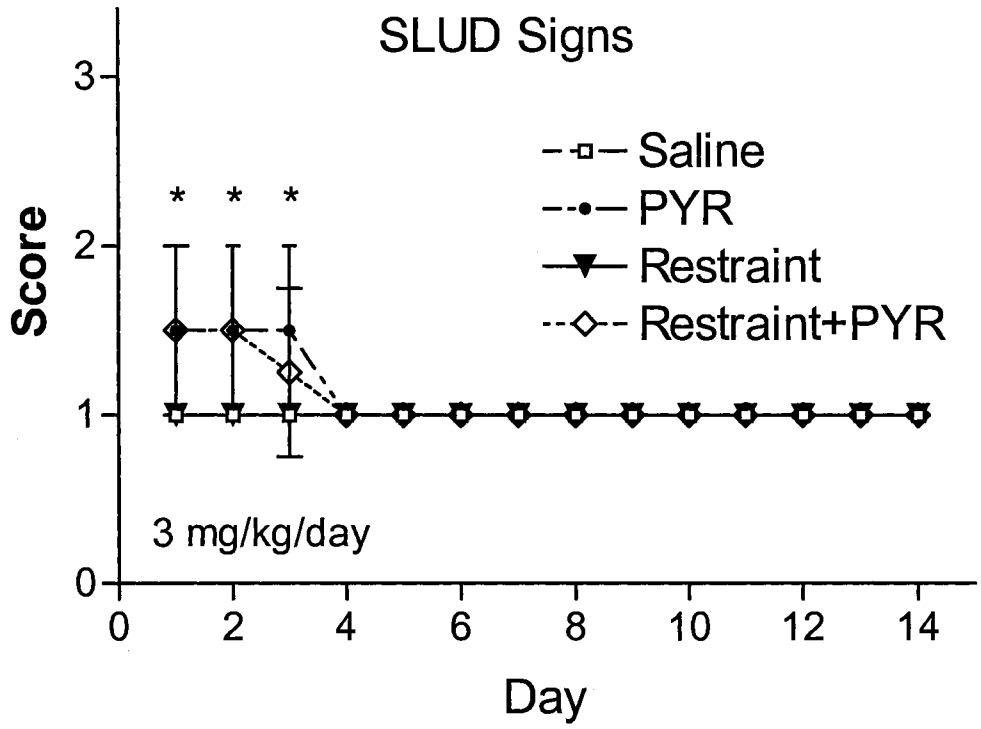

Figure 16: Effects of repeated restraint stress on PYR-induced SLUD signs of toxicity. Rats (n=6/treatment group) were given PYR (3 mg/kg/day, p.o.) or saline each day immediately prior to placement into restraint tubes or acclimation cage for 60 min over 14 consecutive days. SLUD signs were recorded at the end of the 60 min restraint session each day and reported as median ± IQR. Asterisk indicates significant ($p<0.05$) difference from control group.

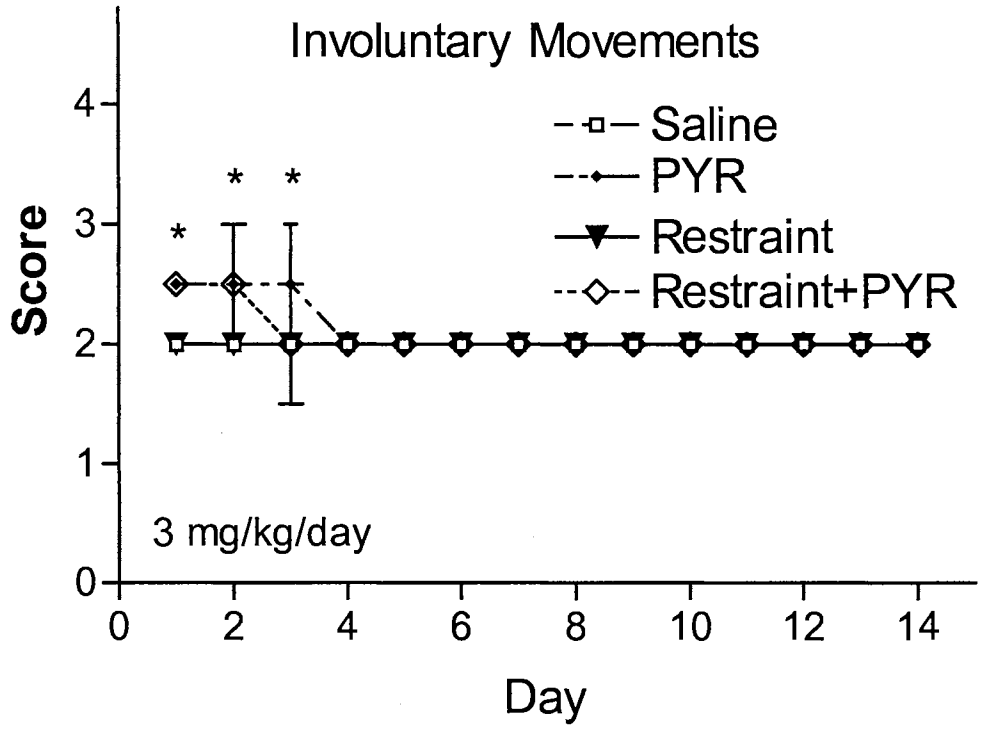

Figure 17: Effects of repeated restraint stress on PYR-induced IM signs of toxicity. Rats (n=6/treatment group) were given PYR (3 mg/kg/day, p.o.) or saline each day immediately prior to placement into restraint tubes or acclimation cage for 60 min over 14 consecutive days. IM signs were recorded at the end of the 60 min restraint session each day and reported as median ± IQR. Asterisk indicates significant ($p<0.05$) difference from control group.

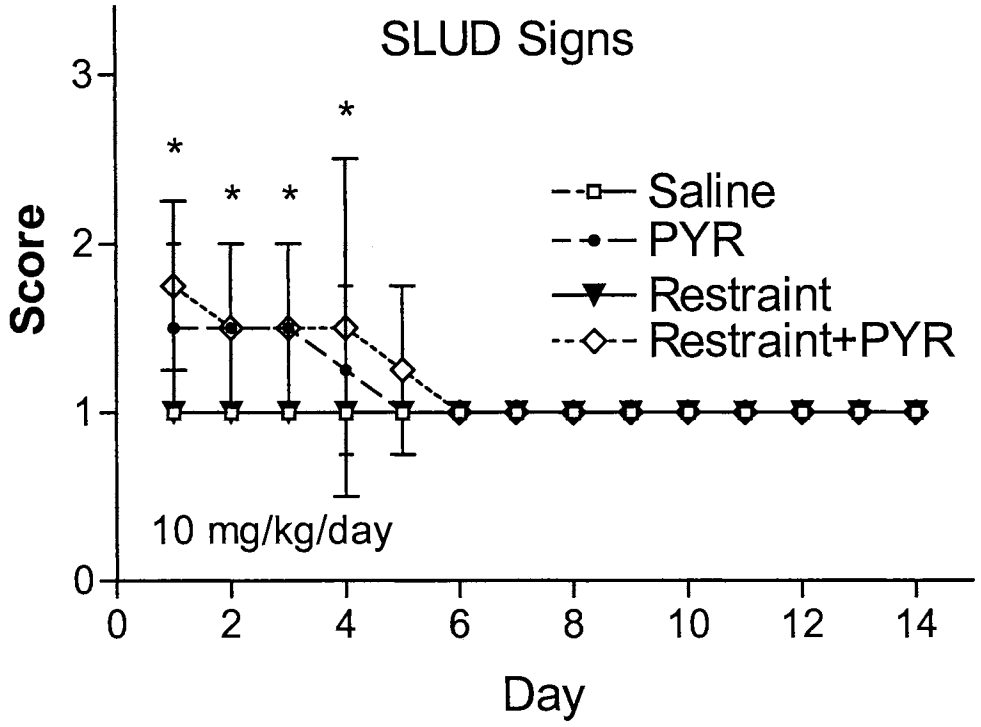

Figure 18: Effects of repeated restraint stress on PYR-induced SLUD signs of toxicity. Rats (n=6/treatment group) were given PYR (10 mg/kg/day, p.o.) or saline each day immediately prior to placement into restraint tubes or acclimation cage for 60 min over 14 consecutive days. SLUD signs were recorded at the end of the 60 min restraint session each day and reported as median ± IQR. Asterisk indicates significant ($p<0.05$) difference from control group.

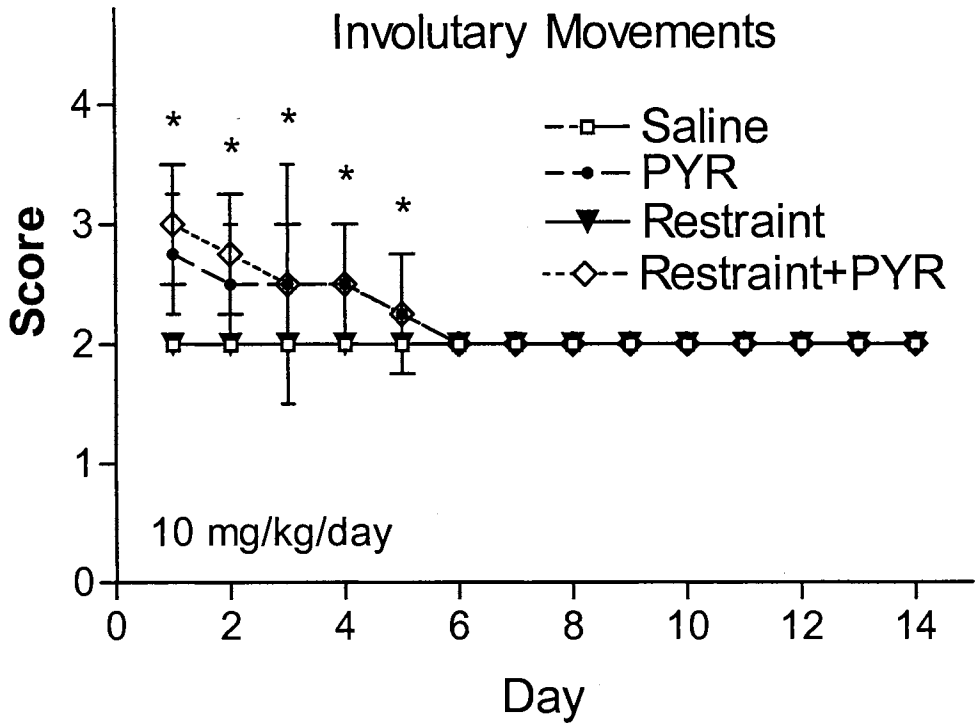

Figure 19: Effects of repeated restraint stress on PYR-induced IM signs of toxicity. Rats (n=6/treatment group) were given PYR (10 mg/kg/day, p.o.) or saline each day immediately prior to placement into restraint tubes or acclimation cage for 60 min over 14 consecutive days. IM signs were recorded at the end of the 60 min restraint session each day and reported as median ± IQR. Asterisk indicates significant ($p<0.05$) difference from control group.

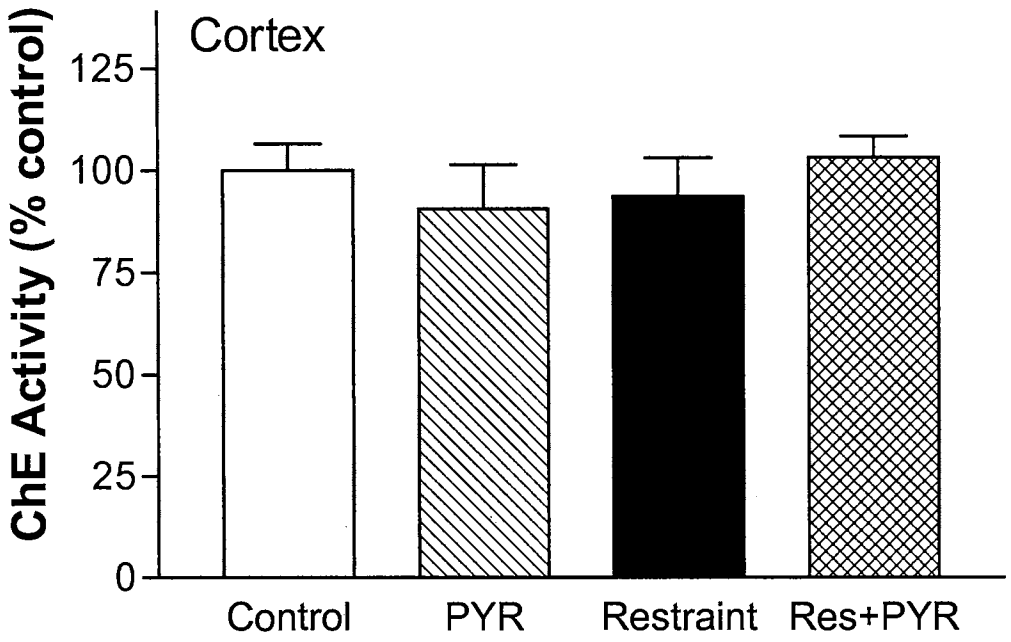

Figure 20: Effects of acute restraint stress protocol 1 on PYR-induced ChE inhibition in frontal cortex. Rats (n=6/treatment) were immobilized (or not restrained) for 90 min and then given PYR (or saline) for 60 min. Rats were sacrificed and frontal cortex was dissected for ChE activity. ChE activity was calculated as nmol/min/mg protein and expressed as mean percentage of control group ± SEM.

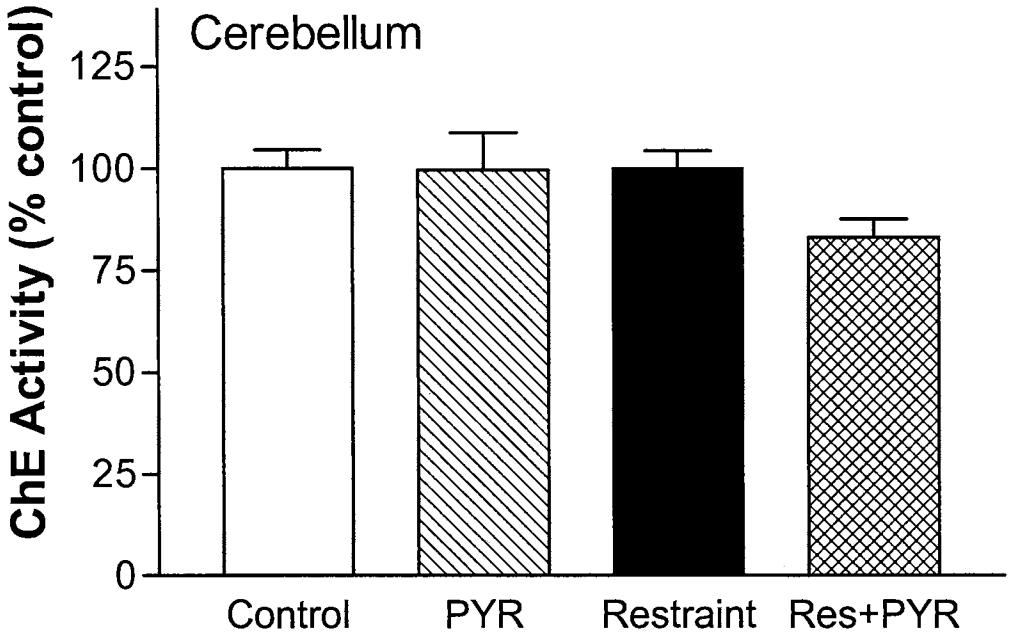

Figure 21: Effects of acute restraint stress protocol 1 on PYR-induced ChE inhibition in cerebellum. Rats (n=6/treatment) were immobilized (or not restrained) for 90 min and then given PYR (or saline) for 60 min. Rats were sacrificed and cerebellum was dissected for ChE activity. ChE activity was calculated as nmol/min/mg protein and expressed as mean percentage of control group ± SEM.

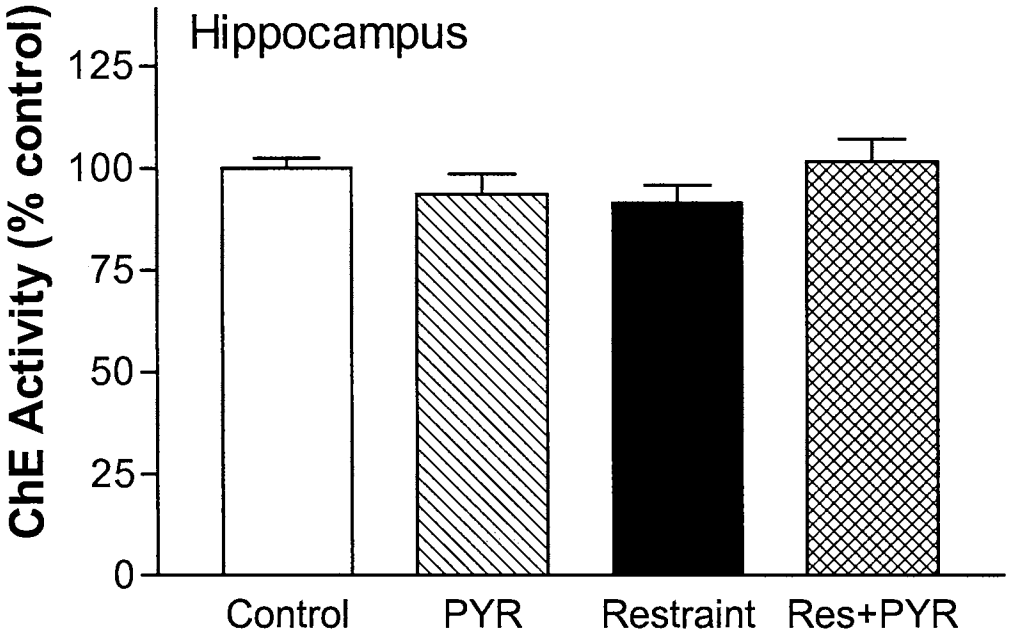

Figure 22: Effects of acute restraint stress protocol 1 on PYR-induced ChE inhibition in cerebellum. Rats (n=6/treatment) were immobilized (or not restrained) for 90 min and then given PYR (or saline) for 60 min. Rats were sacrificed and cerebellum was dissected for ChE activity. ChE activity was calculated as nmol/min/mg protein and expressed as mean percentage of control group ± SEM.

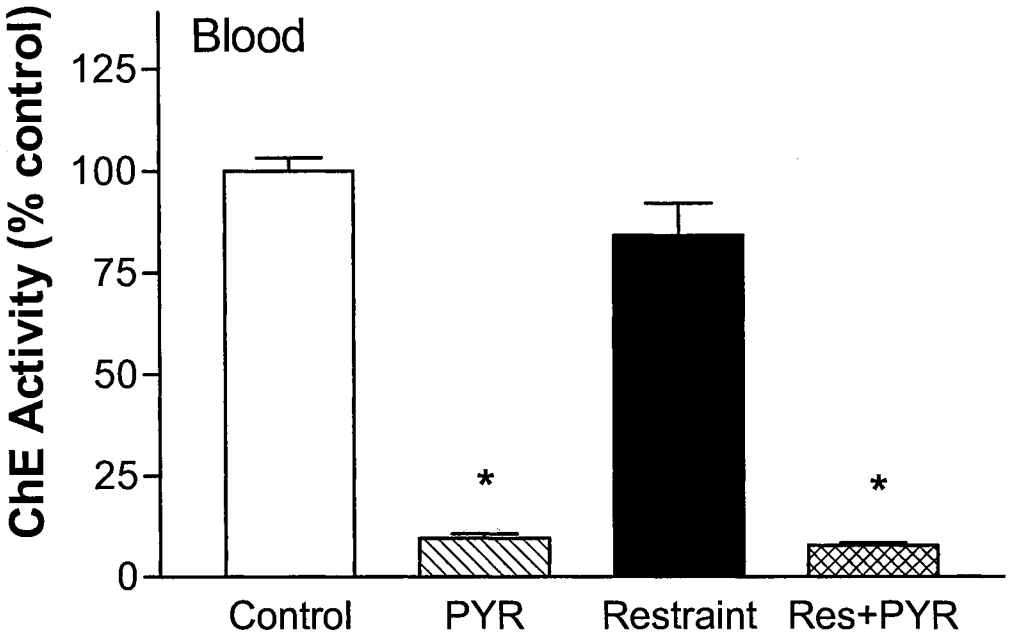

Figure 23: Effects of acute restraint stress protocol 1 on PYR-induced ChE inhibition in whole blood. Rats (n=6/treatment) were immobilized (or not restrained) for 90 min and then given PYR (or saline) for 60 min. Rats were sacrificed and blood was collected for ChE activity. ChE activity was calculated as nmol/min/μl blood and expressed as mean percentage of control group ± SEM. Asterisk indicates significant ($p<0.05$) difference from control group.

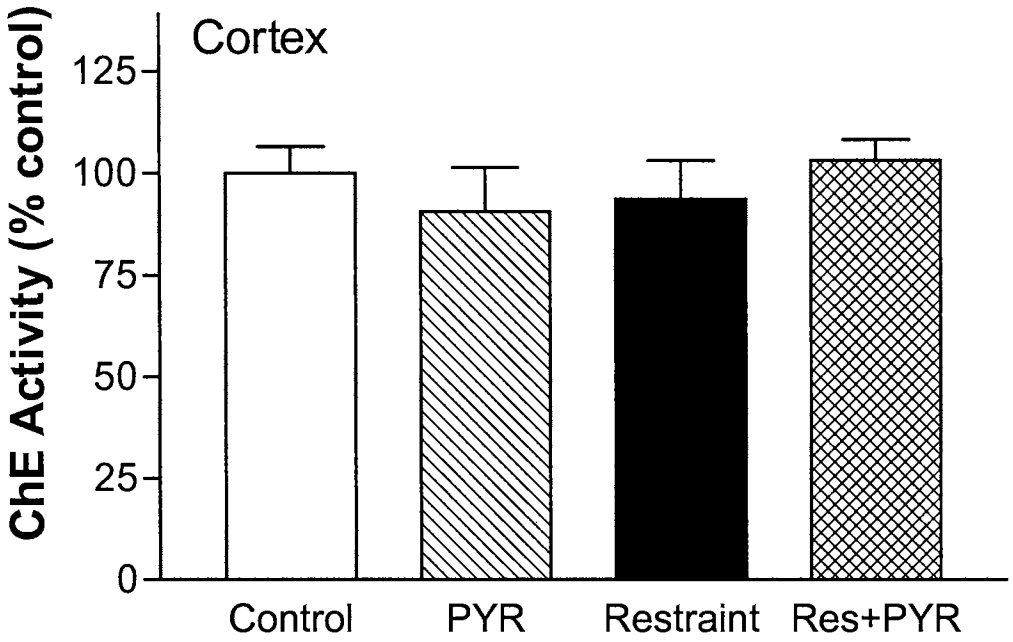

Figure 24: Effects of acute restraint stress protocol 2 on PYR-induced ChE inhibition in frontal cortex. Rats (n=6/treatment) were given PYR (or saline) and immediately placed in restraint tubes (or acclimation cage) for 60 min. Rats were sacrificed and frontal cortex was dissected for ChE activity. ChE activity was calculated as nmol/min/mg protein and expressed as mean percentage of control group ± SEM.

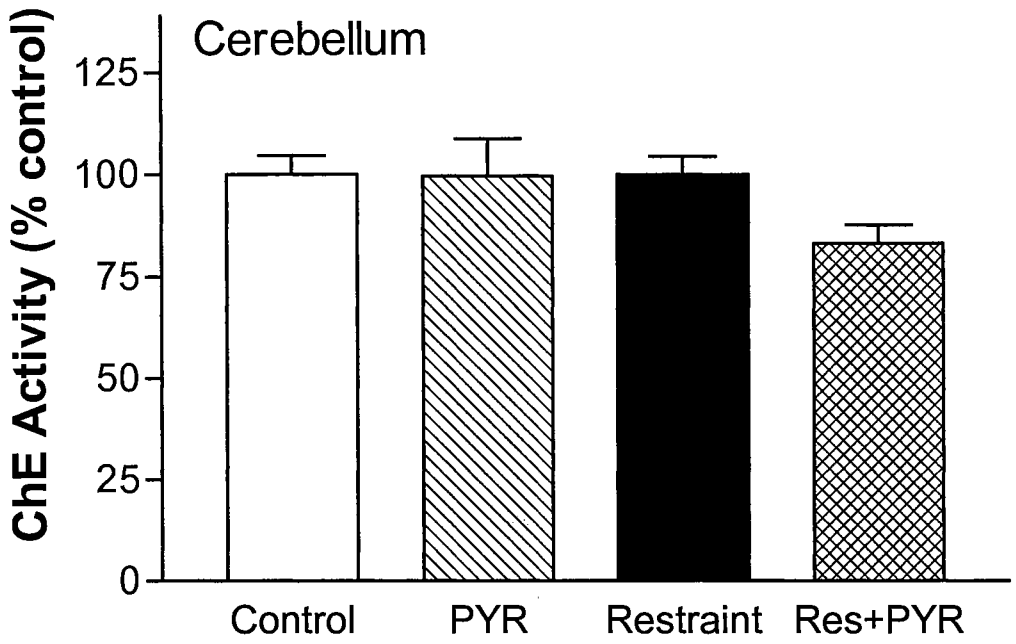

Figure 25: Effects of acute restraint stress protocol 2 on PYR-induced ChE inhibition in cerebellum. Rats (n=6/treatment) were given PYR (or saline) and immediately placed in restraint tubes (or acclimation cage) for 60 min. Rats were sacrificed and cerebellum was dissected for ChE activity. ChE activity was calculated as nmol/min/mg protein and expressed as mean percentage of control group ± SEM.

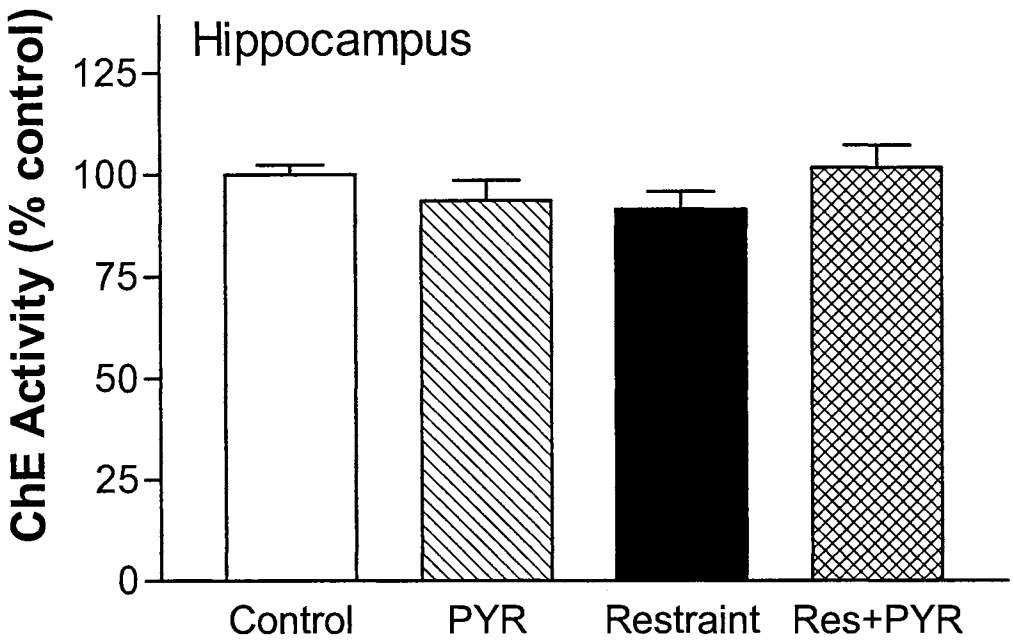

Figure 26: Effects of acute restraint stress protocol 2 on PYR-induced ChE inhibition in hippocampus. Rats (n=6/treatment) were given PYR (or saline) and immediately placed in restraint tubes (or acclimation cage) for 60 min. Rats were sacrificed and hippocampus was dissected for ChE activity. ChE activity was calculated as nmol/min/mg protein and expressed as mean percentage of control group ± SEM.

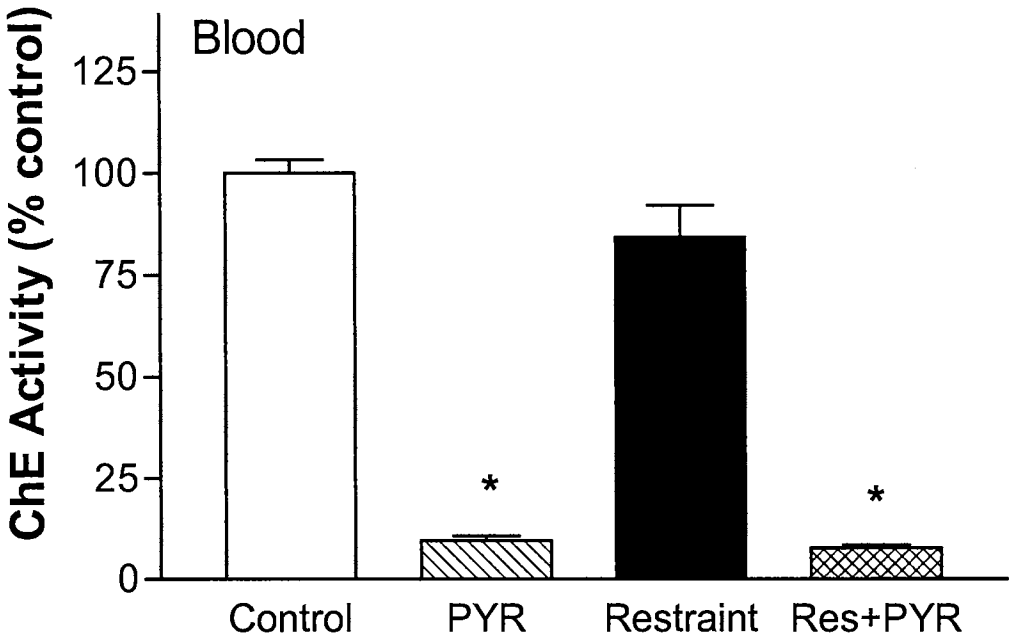

Figure 27: Effects of acute restraint stress protocol 2 on PYR-induced ChE inhibition in whole blood. Rats (n=6/treatment) were given PYR (or saline) and immediately placed in restraint tubes (or acclimation cage) for 60 min. Rats were sacrificed and blood was collected for ChE activity. ChE activity was calculated as nmol/min/µl blood and expressed as mean percentage of control group ± SEM. Asterisk indicates significant ($p<0.05$) difference from control group.

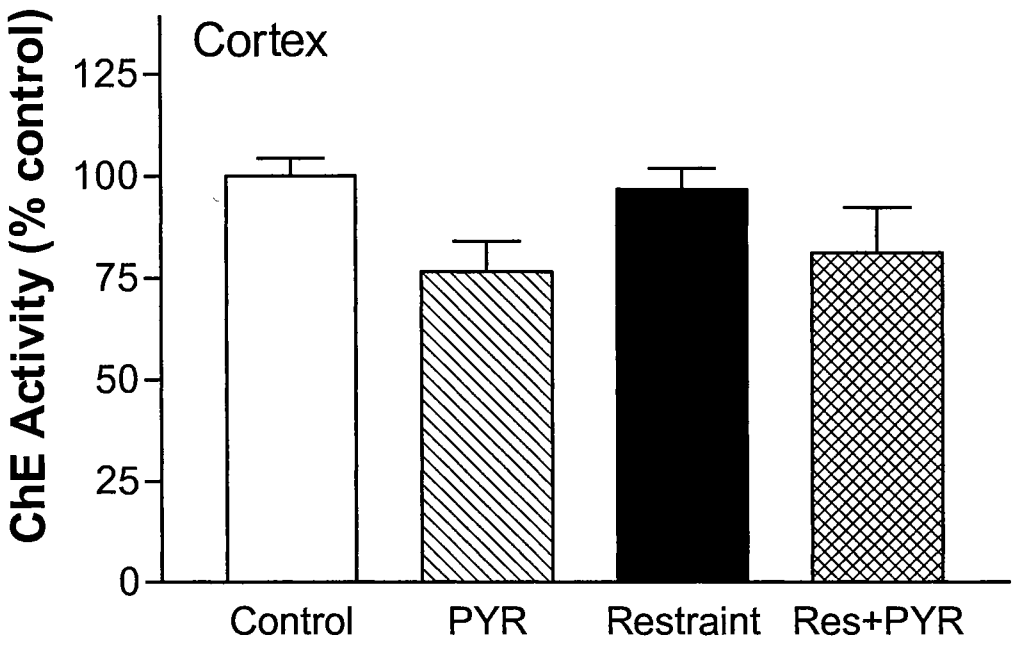

Figure 28: Effects of acute restraint stress protocol 3 on PYR-induced ChE inhibition in frontal cortex. Rats (n=6/treatment) were immobilized (or not restrained) for 3 hrs, given PYR (or saline) and replaced into the restraint tubes (or acclimation cage) for an additional 60 min. Rats were sacrificed and frontal cortex was dissected for ChE activity. ChE activity was calculated as nmol/min/mg protein and expressed as mean percentage of control group ± SEM.

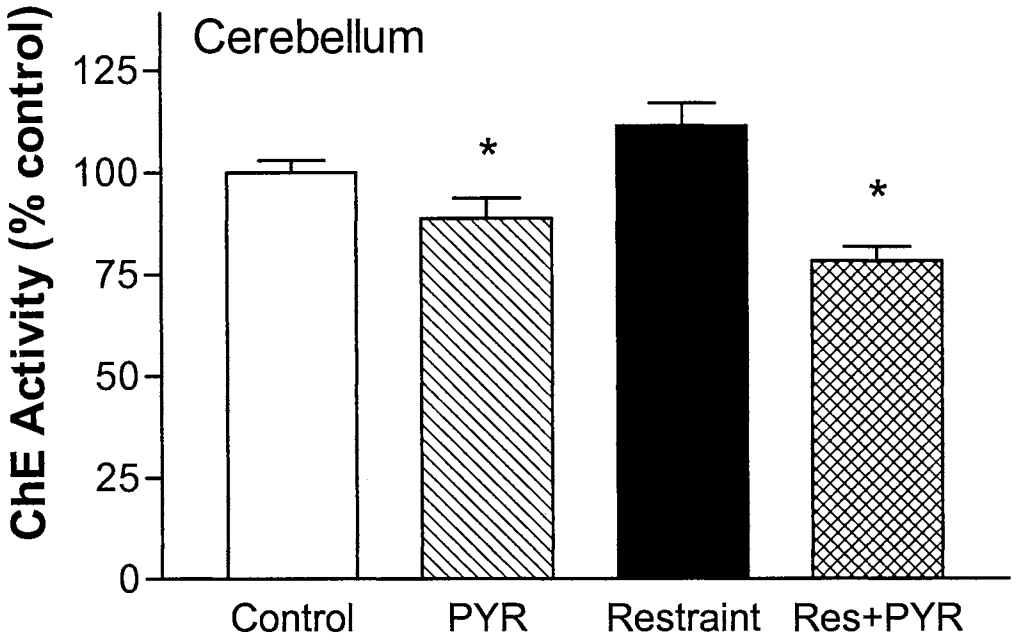

Figure 29: Effects of acute restraint stress protocol 3 on PYR-induced ChE inhibition in cerebellum. Rats (n=6/treatment) were immobilized (or not restrained) for 3 hrs, given PYR (or saline) and replaced into the restraint tubes (or acclimation cage) for an additional 60 min. Rats were sacrificed and cerebellum was dissected for ChE activity. ChE activity was calculated as nmol/min/mg protein and expressed as mean percentage of control group ± SEM. Asterisk indicates significant ($p<0.05$) difference from control group.

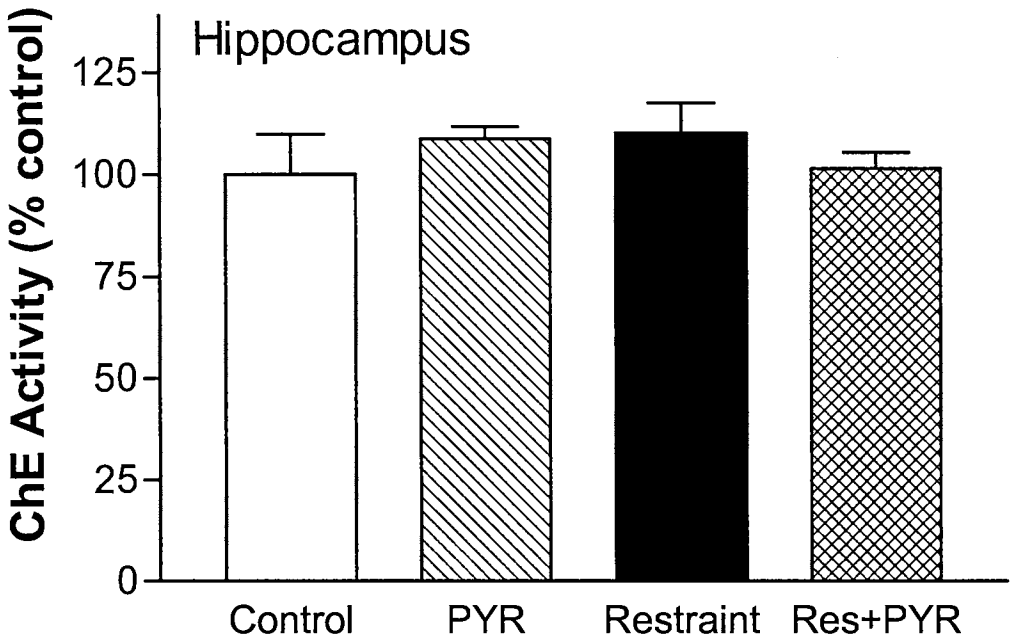

Figure 30: Effects of acute restraint stress protocol 3 on PYR-induced ChE inhibition in hippocampus. Rats (n=6/treatment) were immobilized (or not restrained) for 3 hrs, given PYR (or saline) and replaced into the restraint tubes (or acclimation cage) for an additional 60 min. Rats were sacrificed and hippocampus was dissected for ChE activity. ChE activity was calculated as nmol/min/mg protein and expressed as mean percentage of control group ± SEM.

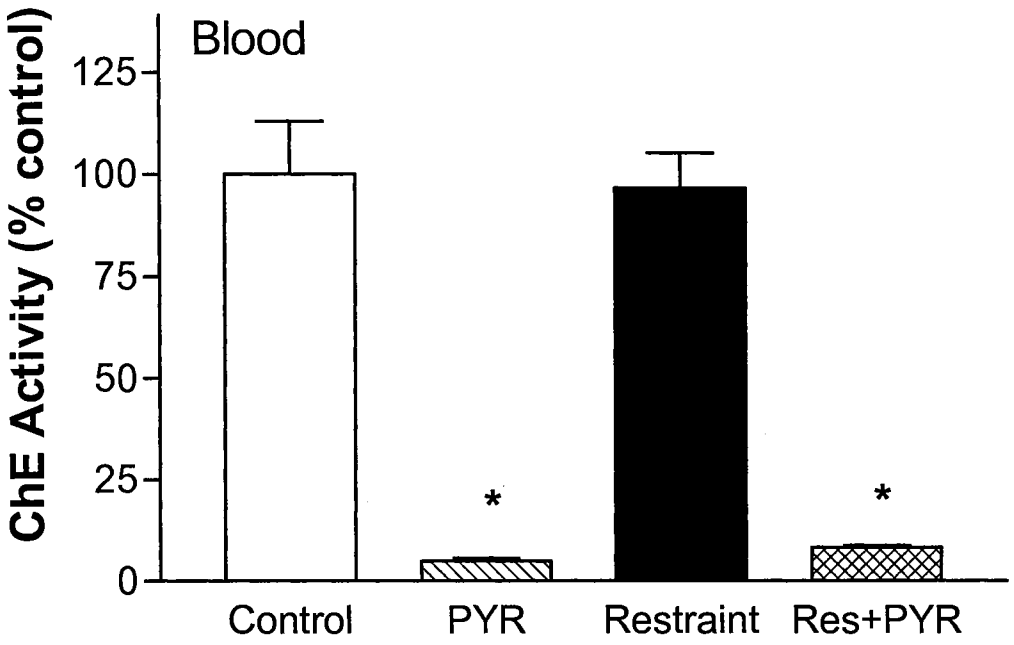

Figure 31: Effects of acute restraint stress protocol 3 on PYR-induced ChE inhibition in whole blood. Rats (n=6/treatment) were immobilized (or not restrained) for 3 hrs, given PYR (or saline) and replaced into the restraint tubes (or acclimation cage) for an additional 60 min. Rats were sacrificed and blood was collected for ChE activity. ChE activity was calculated as nmol/μl blood and expressed as mean percentage of control group ± SEM. Asterisk indicates significant ($p<0.05$) difference from control group.

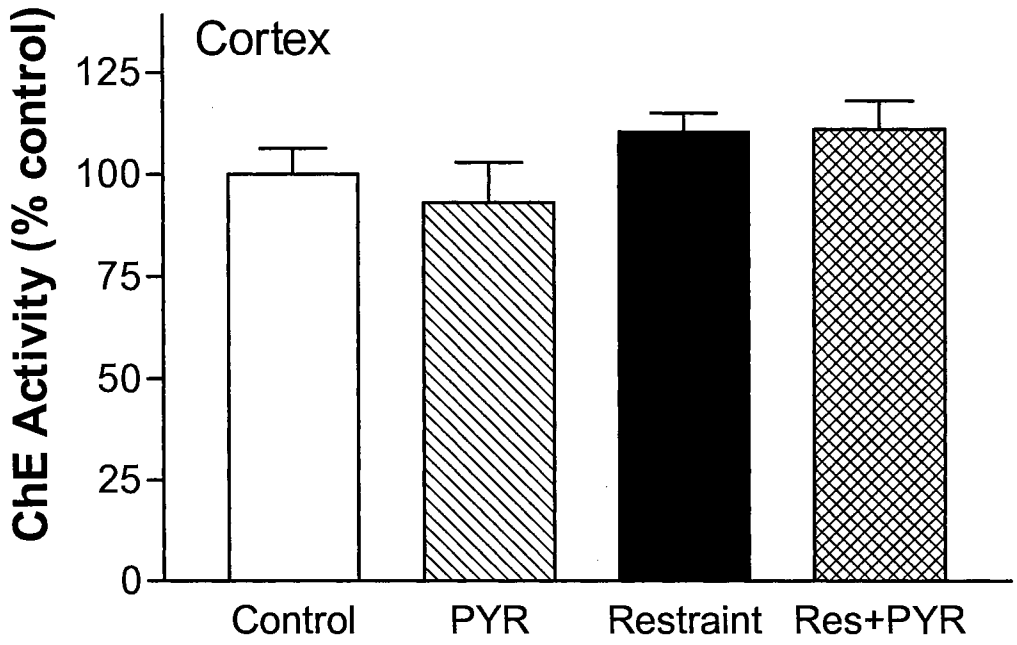

Figure 32: Effects of repeated restraint stress on PYR-induced ChE inhibition in frontal cortex. Rats (n=6/treatment group) were given PYR (3 mg/kg/day, p.o.) or saline each day immediately prior to placement into restraint tubes or acclimation cage for 60 min over 14 consecutive days. Rats were sacrificed and frontal cortex was dissected for ChE activity. ChE activity was calculated as nmol/min/mg protein and expressed as mean percentage of control group ± SEM.

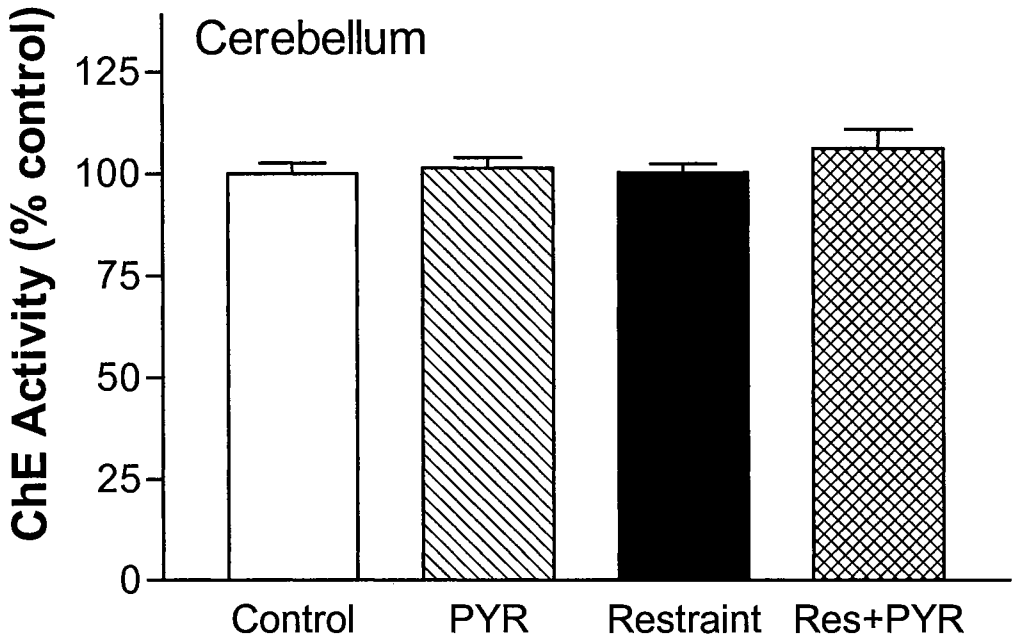

Figure 33: Effects of repeated restraint stress on PYR-induced ChE inhibition in cerebellum. Rats (n=6/treatment group) were given PYR (3 mg/kg/day, p.o.) or saline each day immediately prior to placement into restraint tubes or acclimation cage for 60 min over 14 consecutive days. Rats were sacrificed and cerebellum was dissected for ChE activity. ChE activity was calculated as nmol/min/mg protein and expressed as mean percentage of control group ± SEM.

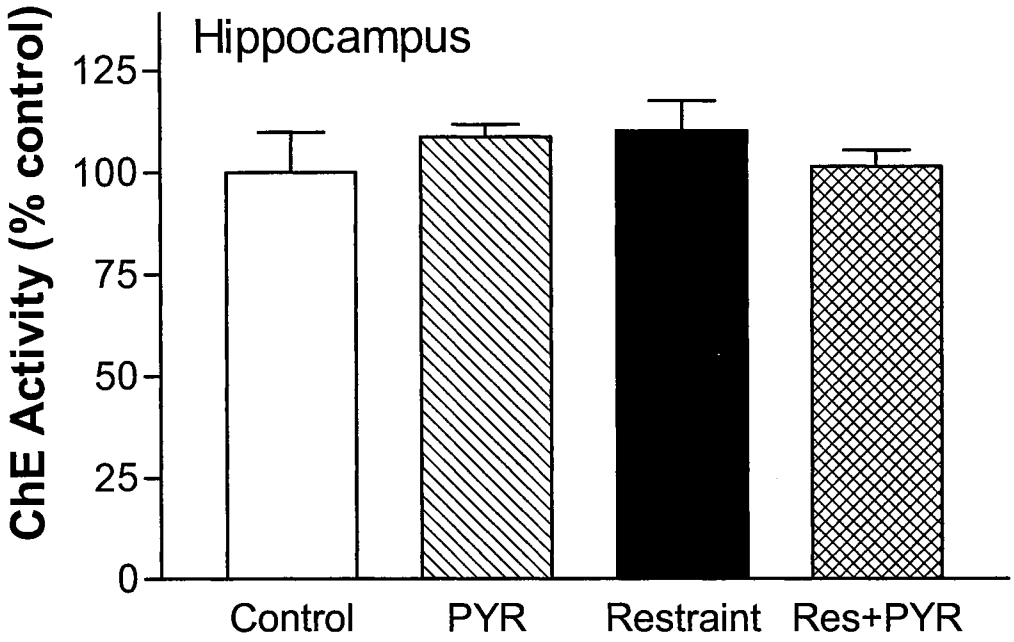

Figure 34: Effects of repeated restraint stress on PYR-induced ChE inhibition in hippocampus. Rats (n=6/treatment group) were given PYR (3 mg/kg/day, p.o.) or saline each day immediately prior to placement into restraint tubes or acclimation cage for 60 min over 14 consecutive days. Rats were sacrificed and hippocampus was dissected for ChE activity. ChE activity was calculated as nmol/min/mg protein and expressed as mean percentage of control group ± SEM.

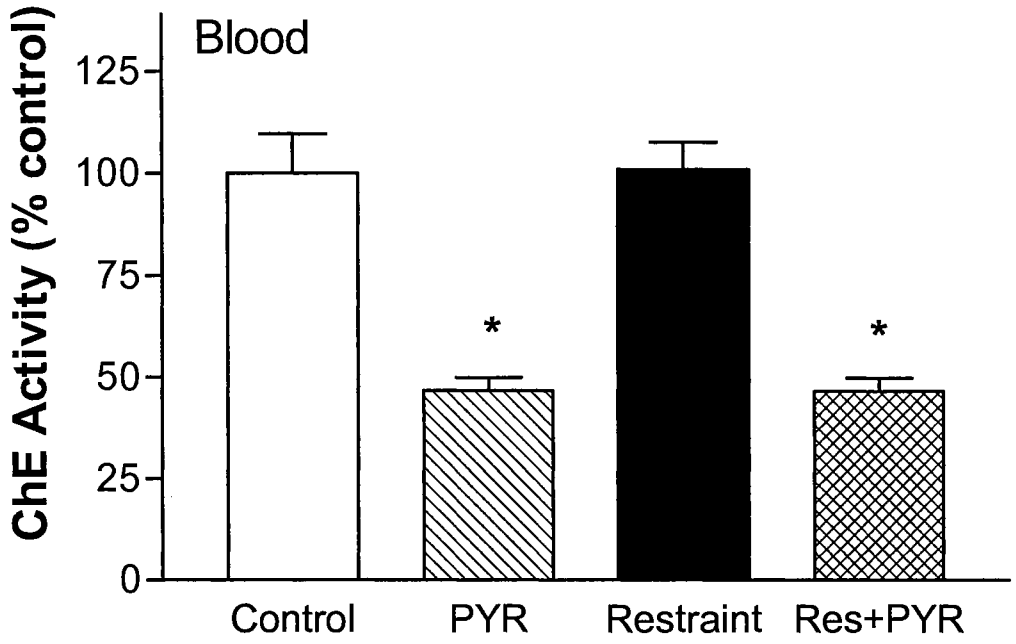

Figure 35: Effects of repeated restraint stress on PYR-induced ChE inhibition in whole blood. Rats (n=6/treatment group) were given PYR (3 mg/kg/day, p.o.) or saline each day immediately prior to placement into restraint tubes or acclimation cage for 60 min over 14 consecutive days. Rats were sacrificed and blood was collected for ChE activity. ChE activity was calculated as nmol/min/μl blood and expressed as mean percentage of control group ± SEM. Asterisk indicates significant ($p<0.05$) difference from control group.

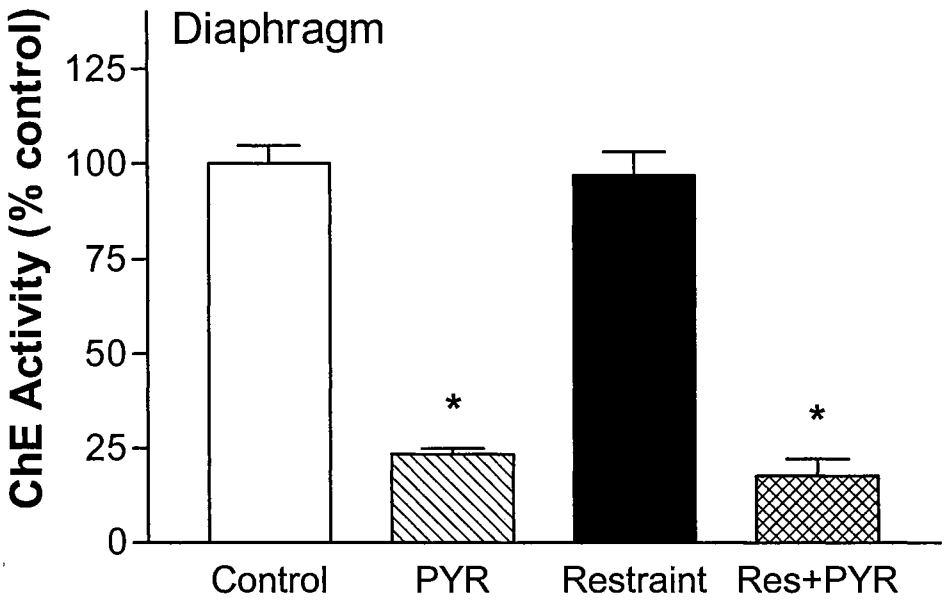

Figure 36: Effects of repeated restraint stress on PYR-induced ChE inhibition in diaphragm. Rats (n=6/treatment group) were given PYR (3 mg/kg/day, p.o.) or saline each day immediately prior to placement into restraint tubes or acclimation cage for 60 min over 14 consecutive days. Rats were sacrificed and frontal cortex was dissected for ChE activity. ChE activity was calculated as nmol/min/mg protein and expressed as mean percentage of control group ± SEM. Asterisk indicates significant ($p<0.05$) difference from control group.

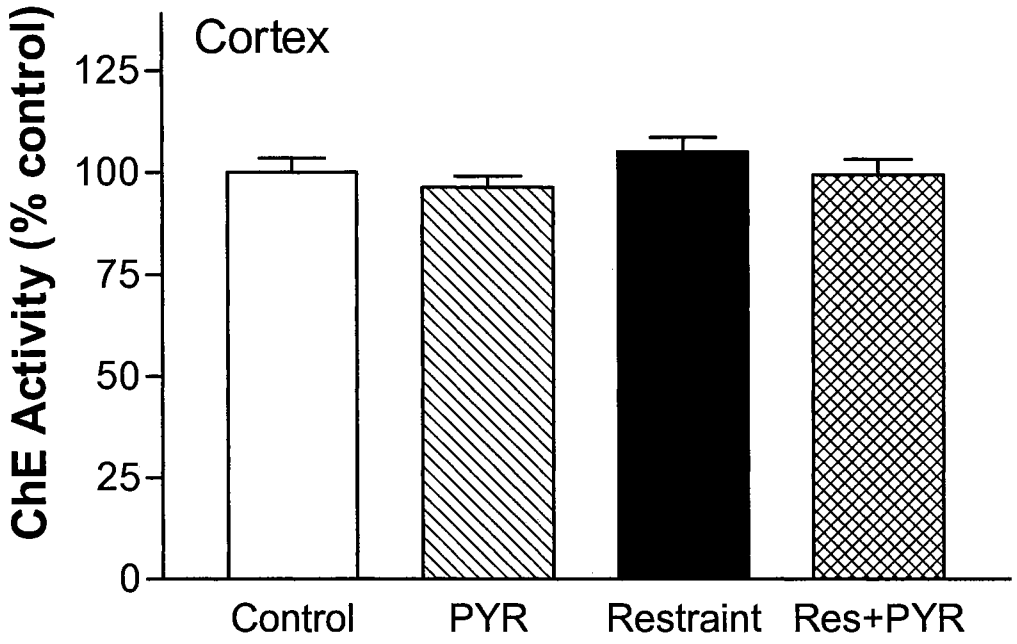

Figure 37: Effects of repeated restraint stress on PYR-induced ChE inhibition in frontal cortex. Rats (n=6/treatment group) were given PYR (10 mg/kg/day, p.o.) or saline each day immediately prior to placement into restraint tubes or acclimation cage for 60 min over 14 consecutive days. Rats were sacrificed and frontal cortex was dissected for ChE activity. ChE activity was calculated as nmol/min/mg protein and expressed as mean percentage of control group ± SEM.

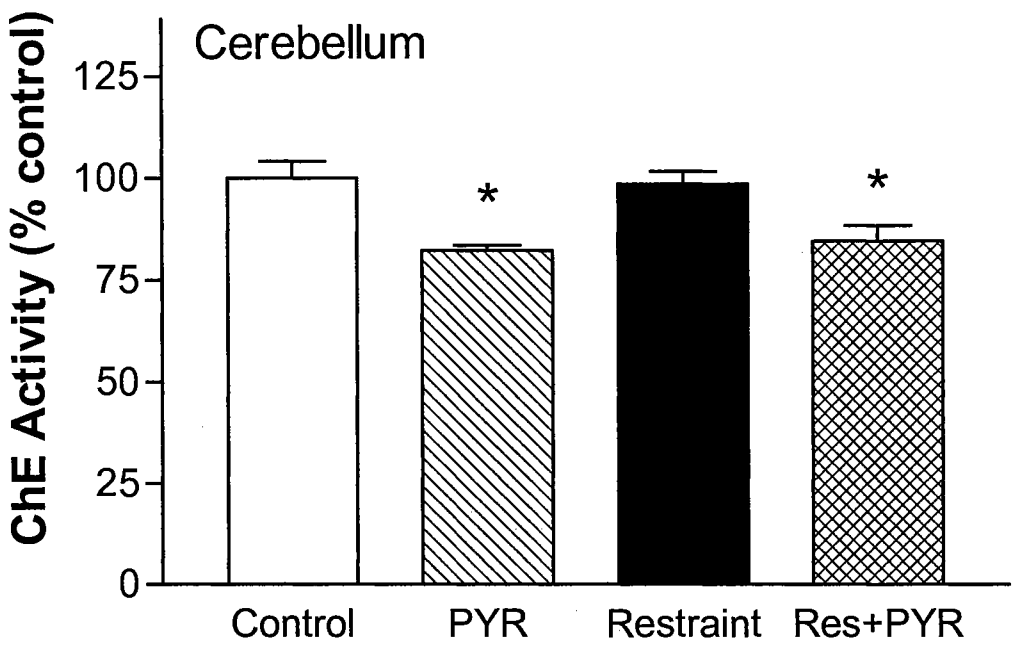

Figure 38: Effects of repeated restraint stress on PYR-induced ChE inhibition in cerebellum. Rats (n=6/treatment group) were given PYR (10 mg/kg/day, p.o.) or saline each day immediately prior to placement into restraint tubes or acclimation cage for 60 min over 14 consecutive days. Rats were sacrificed and cerebellum was dissected for ChE activity. ChE activity was calculated as nmol/min/mg protein and expressed as mean percentage of control group ± SEM. Asterisk indicates significant ($p<0.05$) difference from control group.

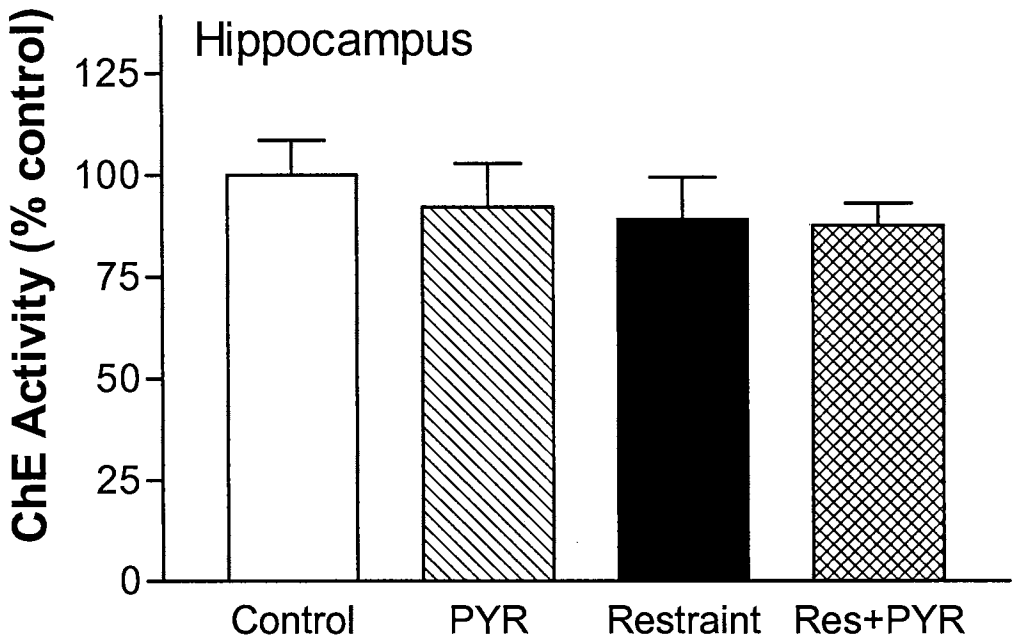

Figure 39: Effects of repeated restraint stress on PYR-induced ChE inhibition in hippocampus. Rats (n=6/treatment group) were given PYR (10 mg/kg/day, p.o.) or saline each day immediately prior to placement into restraint tubes or acclimation cage for 60 min over 14 consecutive days. Rats were sacrificed and hippocampus was dissected for ChE activity. ChE activity was calculated as nmol/min/mg protein and expressed as mean percentage of control group ± SEM.

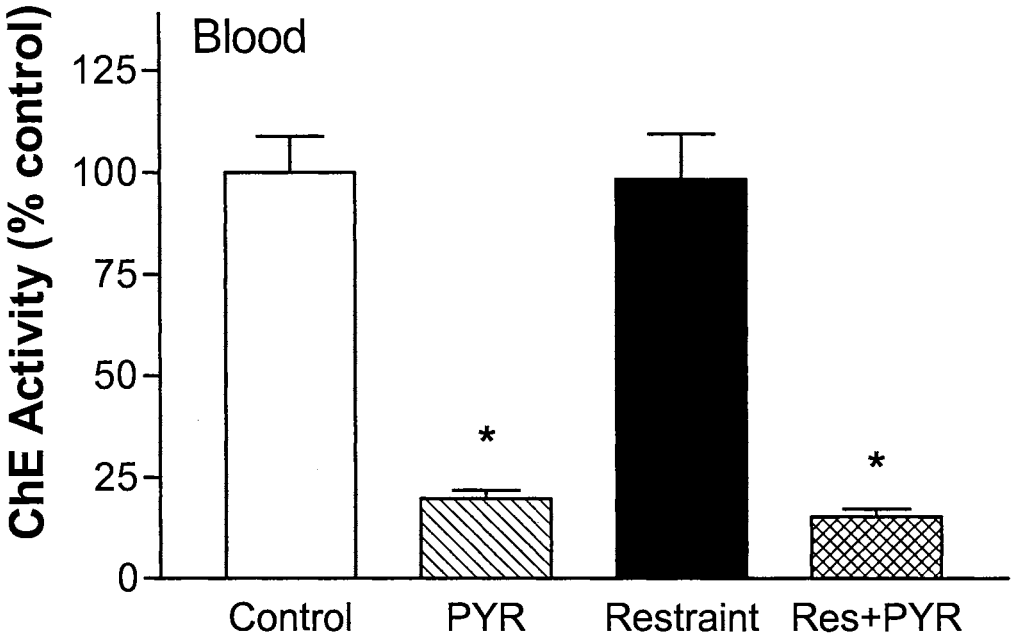

Figure 40: Effects of repeated restraint stress on PYR-induced ChE inhibition in whole blood. Rats (n=6/treatment group) were given PYR (10 mg/kg/day, p.o.) or saline each day immediately prior to placement into restraint tubes or acclimation cage for 60 min over 14 consecutive days. Rats were sacrificed and blood was collected for ChE activity. ChE activity was calculated as nmol/min/μl blood and expressed as mean percentage of control group ± SEM. Asterisk indicates significant ($p<0.05$) difference from control group.

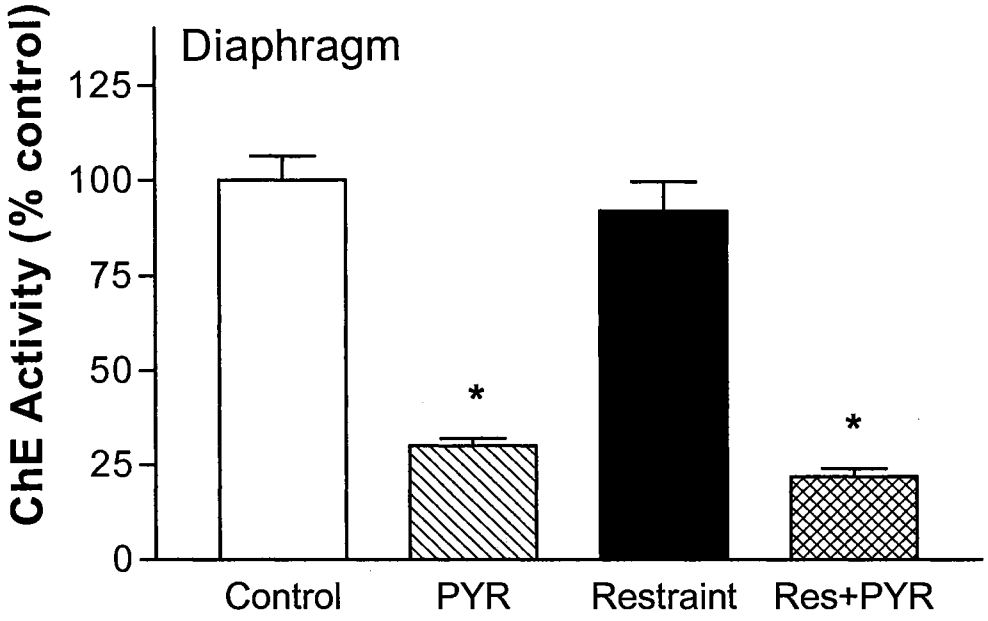

Figure 41: Effects of repeated restraint stress on PYR-induced ChE inhibition in diaphragm. Rats (n=6/treatment group) were given PYR (10 mg/kg/day, p.o.) or saline each day immediately prior to placement into restraint tubes or acclimation cage for 60 min over 14 consecutive days. Rats were sacrificed and frontal cortex was dissected for ChE activity. ChE activity was calculated as nmol/min/mg protein and expressed as mean percentage of control group ± SEM. Asterisk indicates significant ($p<0.05$) difference from control group.

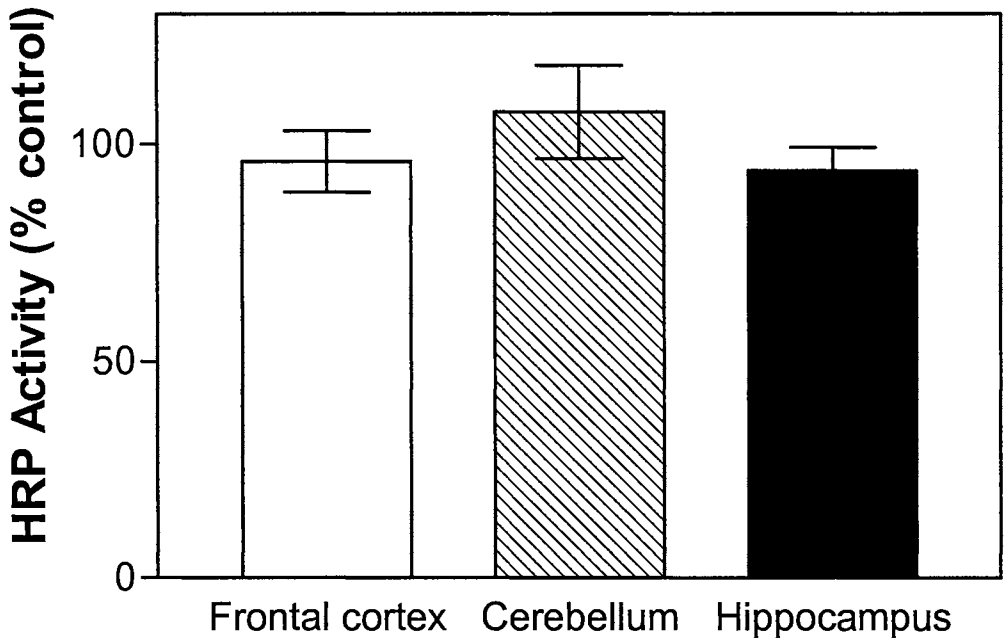

Figure 42: Accumulation of HRP in brain regions following acute immobilization stress. Rats (n=5-9/treatment group) were restrained for 60 minutes as described in Methods, immediately anesthetized with pentobarbital (50 mg/kg, i.p. injection) and horseradish peroxidase (in vehicle containing Evans blue dye) was injected by cardiac puncture 10 minutes later. Rats were sacrificed 10 min after cardiac injection and brain regions (frontal cortex, cerebellum and hippocampus) dissected for measurement of peroxidase activity (expressed as mean ± SEM). Only tissues from rats exhibiting systemic blue discoloration were used in the analyses.

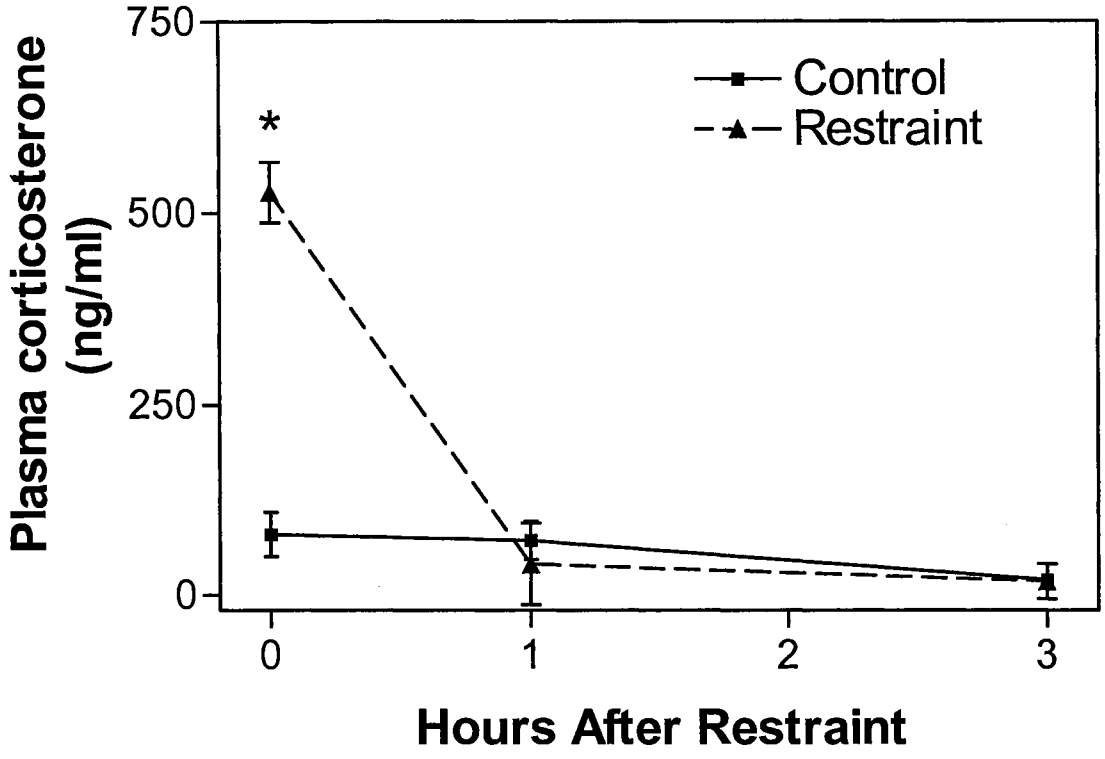

Figure 43: Plasma corticosteroids following acute restraint stress. Rats (n=4/treatment) were restrained for 60 min. Plasma samples were collected from rats at 0, 1 or 3 hrs after termination of immobilization. Corticosteroids were measured by radioimmunoassay as described in Methods and calculated as ng/ml plasma. Asterisk indicates significant ($p<0.05$) difference from control group.

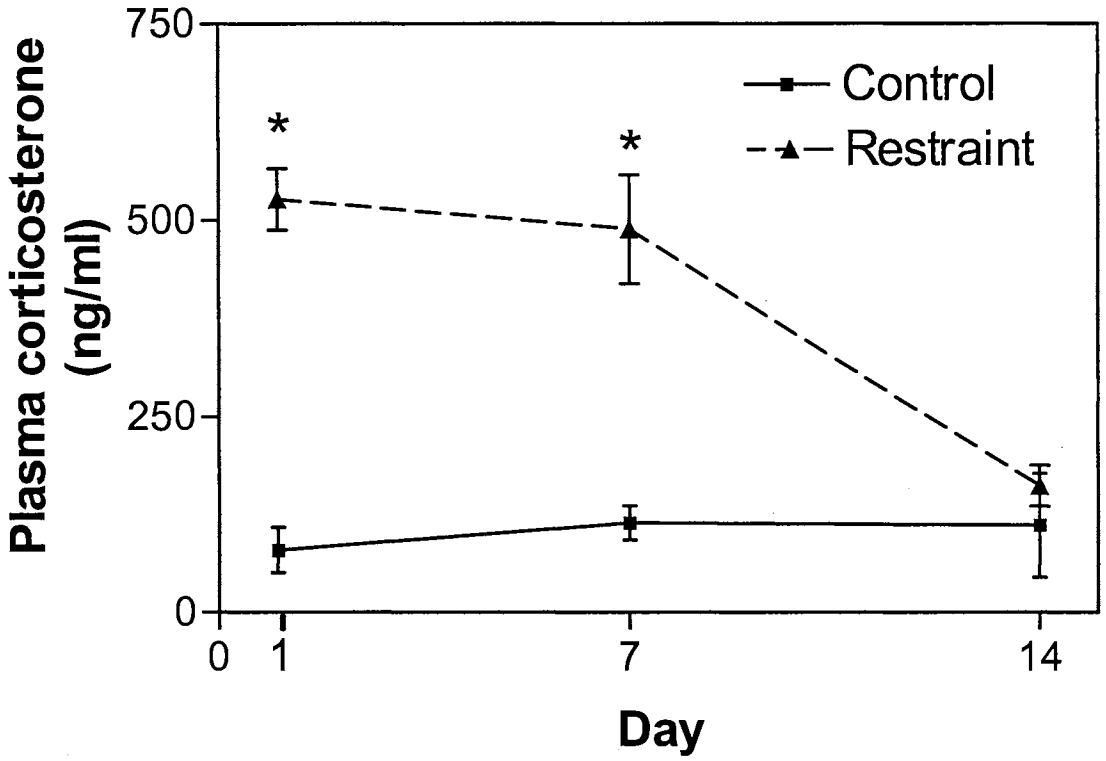

Figure 44: Plasma corticosteroids following repeated restraint stress. Rats (n=4/treatment) were restrained for 60 min each day for 14 consecutive days. Plasma samples were collected from rats immediately following 60 minutes immobilization on days 1, 7 and 14. Corticosteroids were measured by radioimmunoassay as described in Methods and calculated as ng/ml plasma. Asterisk indicates significant ($p<0.05$) difference from control group.

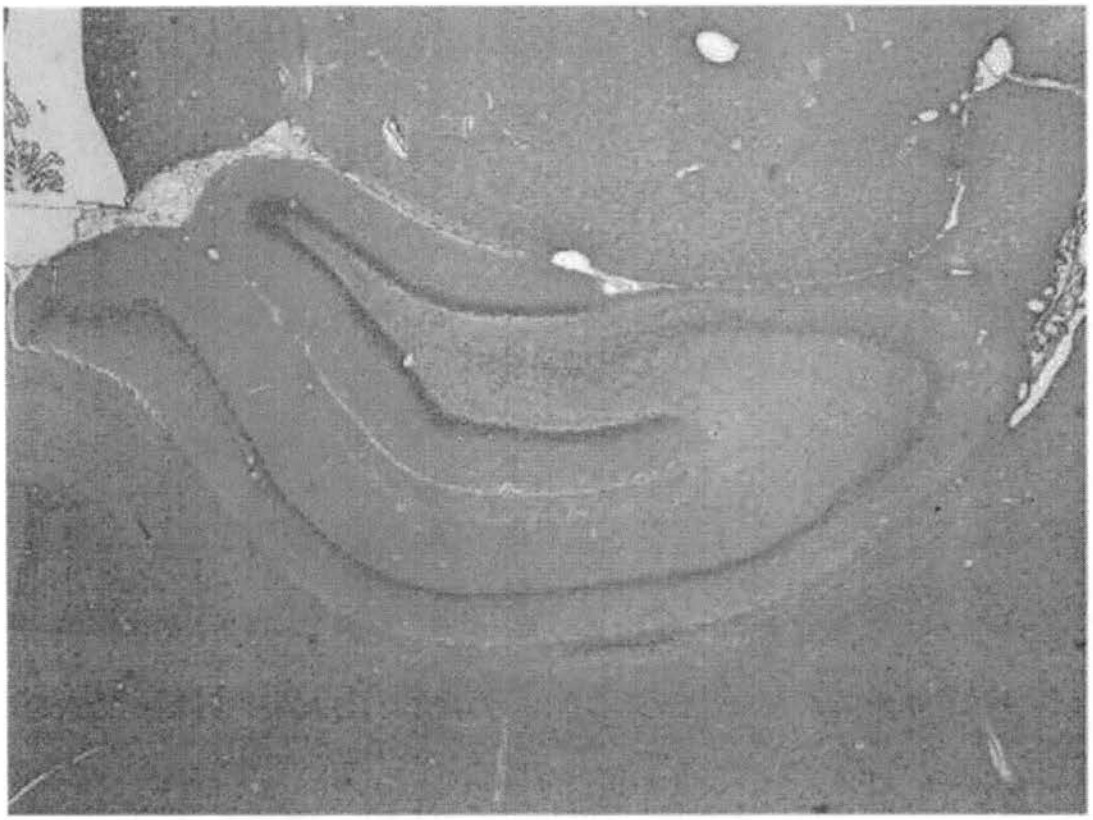

Figure 45: Light micrograph (40 X magnification) of neurons in hippocampus of rats following PYR exposure. Rats were injected with PYR (1.85 mg/kg, i.p.) every 12 hrs for 4 days. Three hrs after last dosing, rats were purfused and fixed. Brains were removed, histochemically processed and evaluated for cell death by Hematoxylin-Eosin staining.

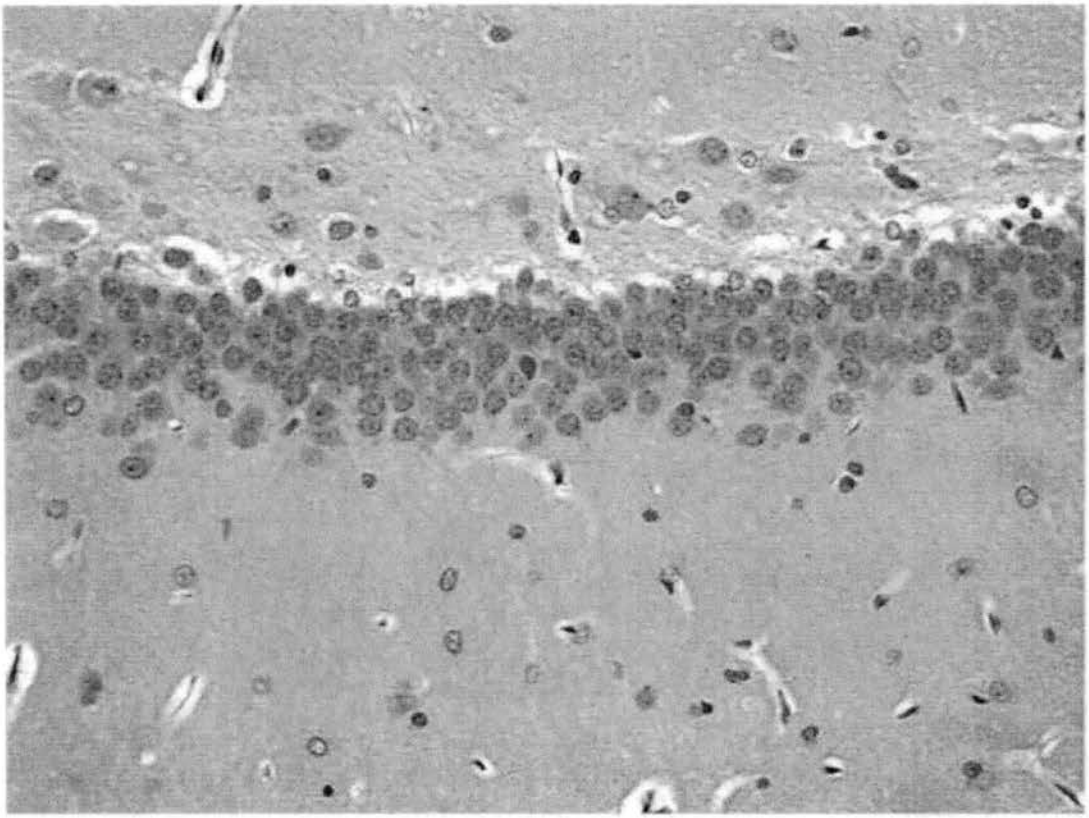

Figure 46: Light micrograph (400 X magnification) of neurons in brain of rats following PYR exposure. Rats were injected with PYR (1.85 mg/kg, i.p.) every 12 hrs for 4 days. Three hrs after last dosing, rats were purfused and fixed. Brains were removed, histochemically processed and evaluated for cell death by Hematoxylin-Eosin staining.

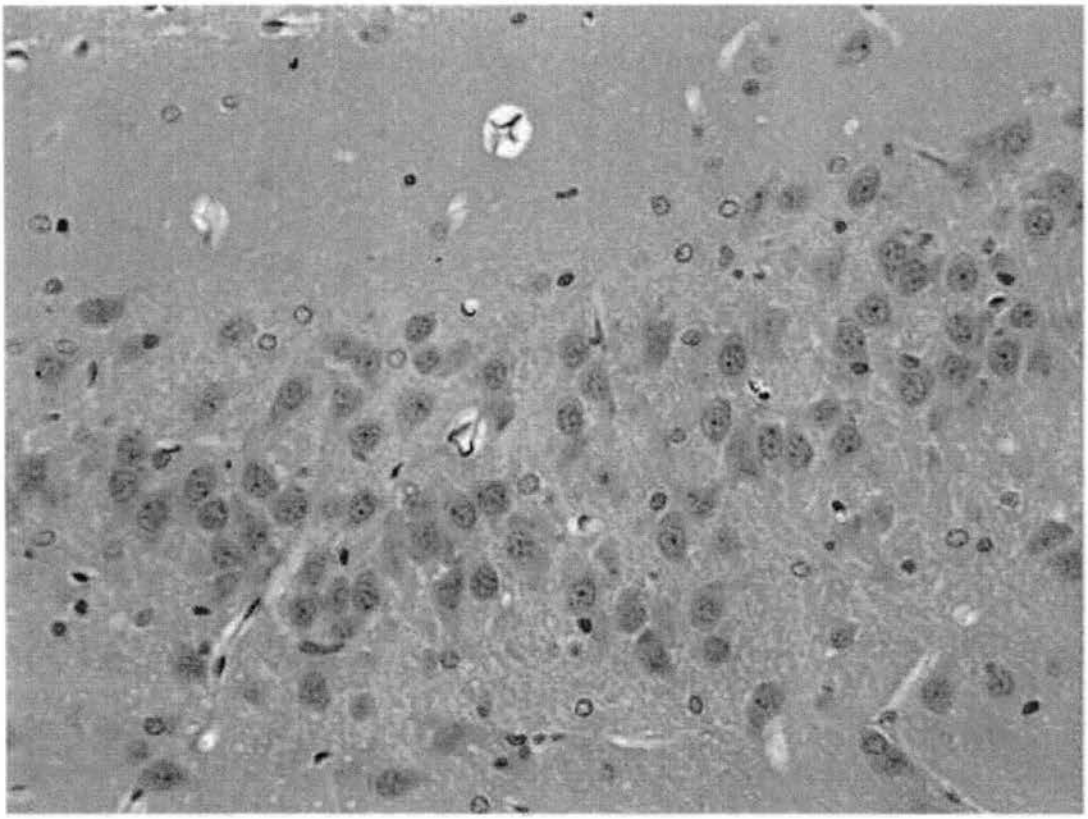

Figure 47: Light micrograph (600 X magnification) of neurons in brain of rats following PYR exposure. Rats were injected with PYR (1.85 mg/kg, i.p.) every 12 hrs for 4 days. Three hrs after last dosing, rats were purfused and fixed. Brains were removed, histochemically processed and evaluated for cell death by Hematoxylin-Eosin staining.

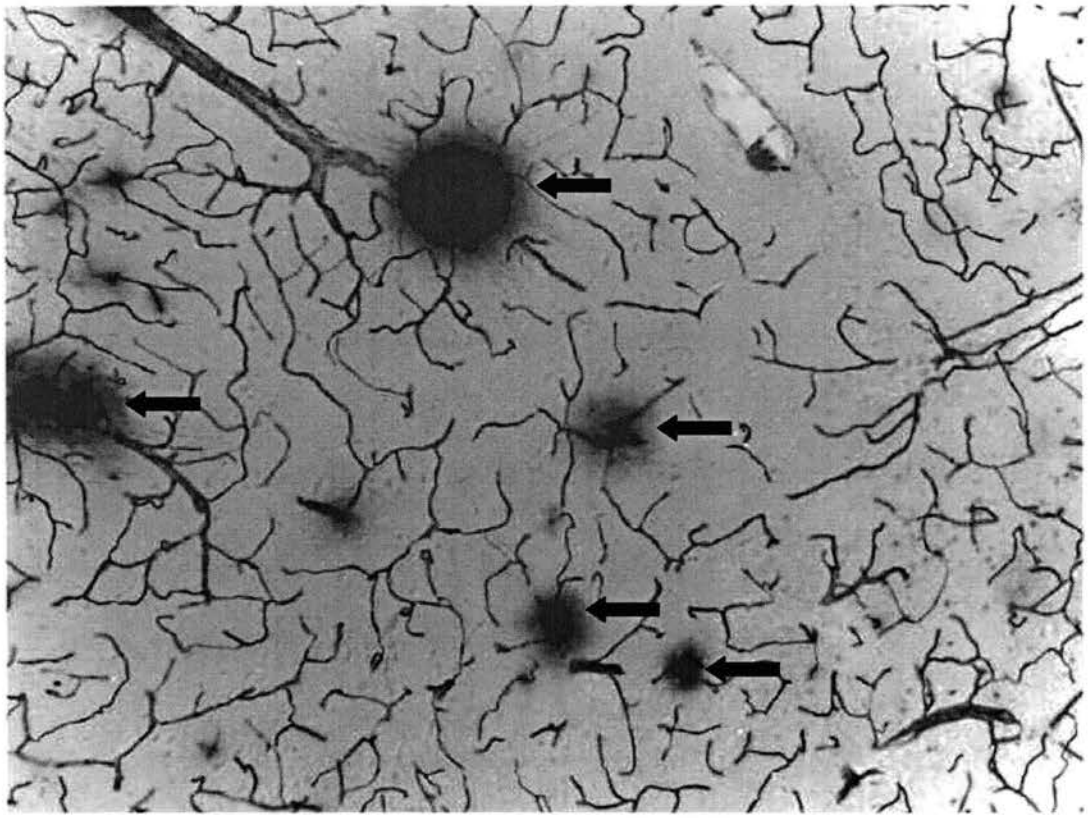

Figure 48: Light micrograph of BBB disruption as visualized by HRP leakage (as indicated by black arrows) after chemical stress (paraoxon) exposure. Rats were given paraoxon (10 μg/kg, i.m.). Ten minutes later, rats were injected HRP through the left ventricle and circulation was allowed 1 min before sacrificing the rats. Brain were removed and fixed in 2.5% glutaraldehyde and sectioned at 70 μm. HRP was visualized by reaction with DAB.

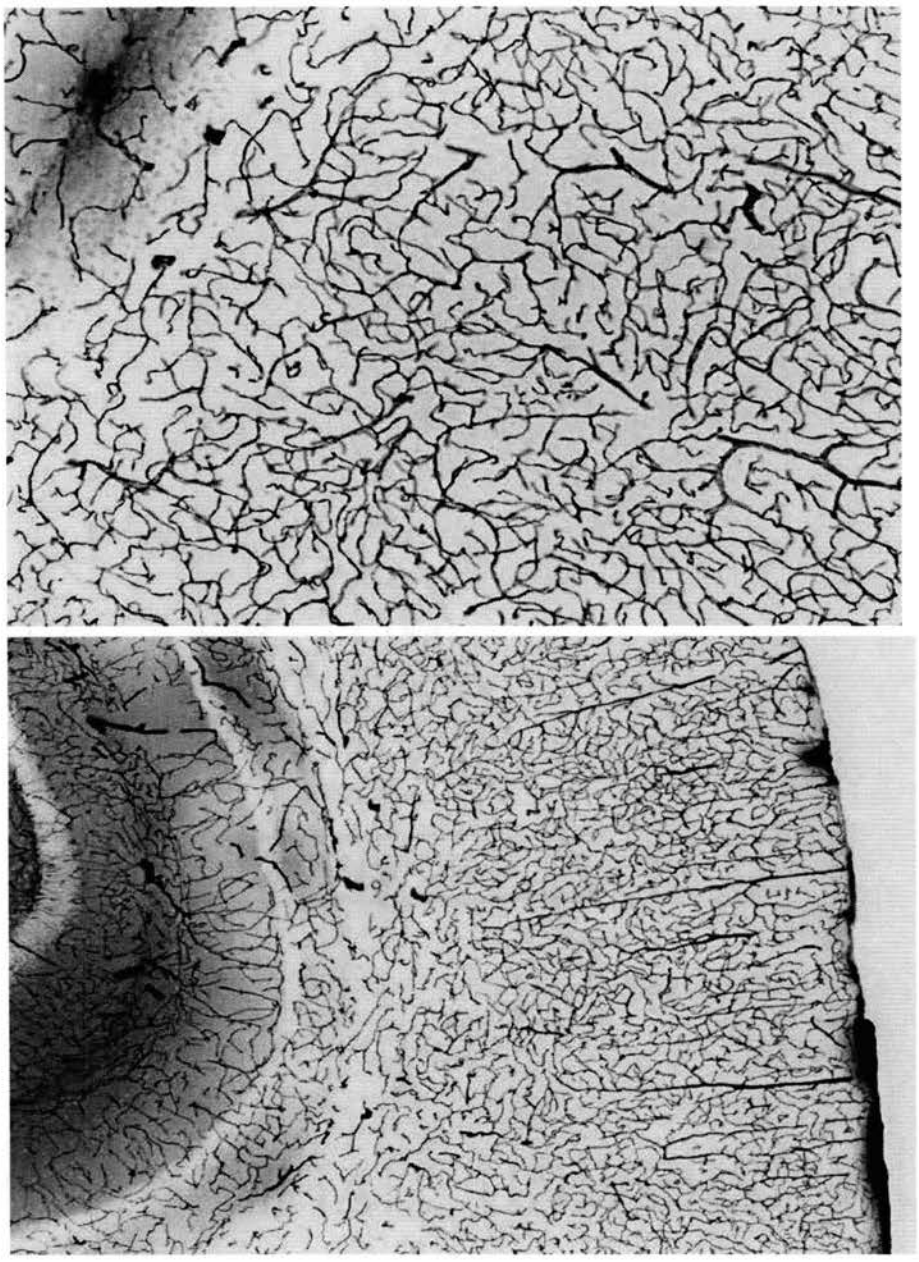

Figure 49: Light micrographs of HRP leaks in the coronal cortex sections of the control rats. Rats were given either vehicle or PYR (30mg/kg, p.o.) for 50 min followed by either vehicle or paraoxon (100 μg/kg, i.m.). Ten minutes later, rats were injected HRP through the left ventricle and circulation was allowed 1 min before sacrificing the rats. Brain were removed and fixed in 2.5% glutaraldehyde and sectioned at 70 μm. HRP was visualized by reaction with DAB.

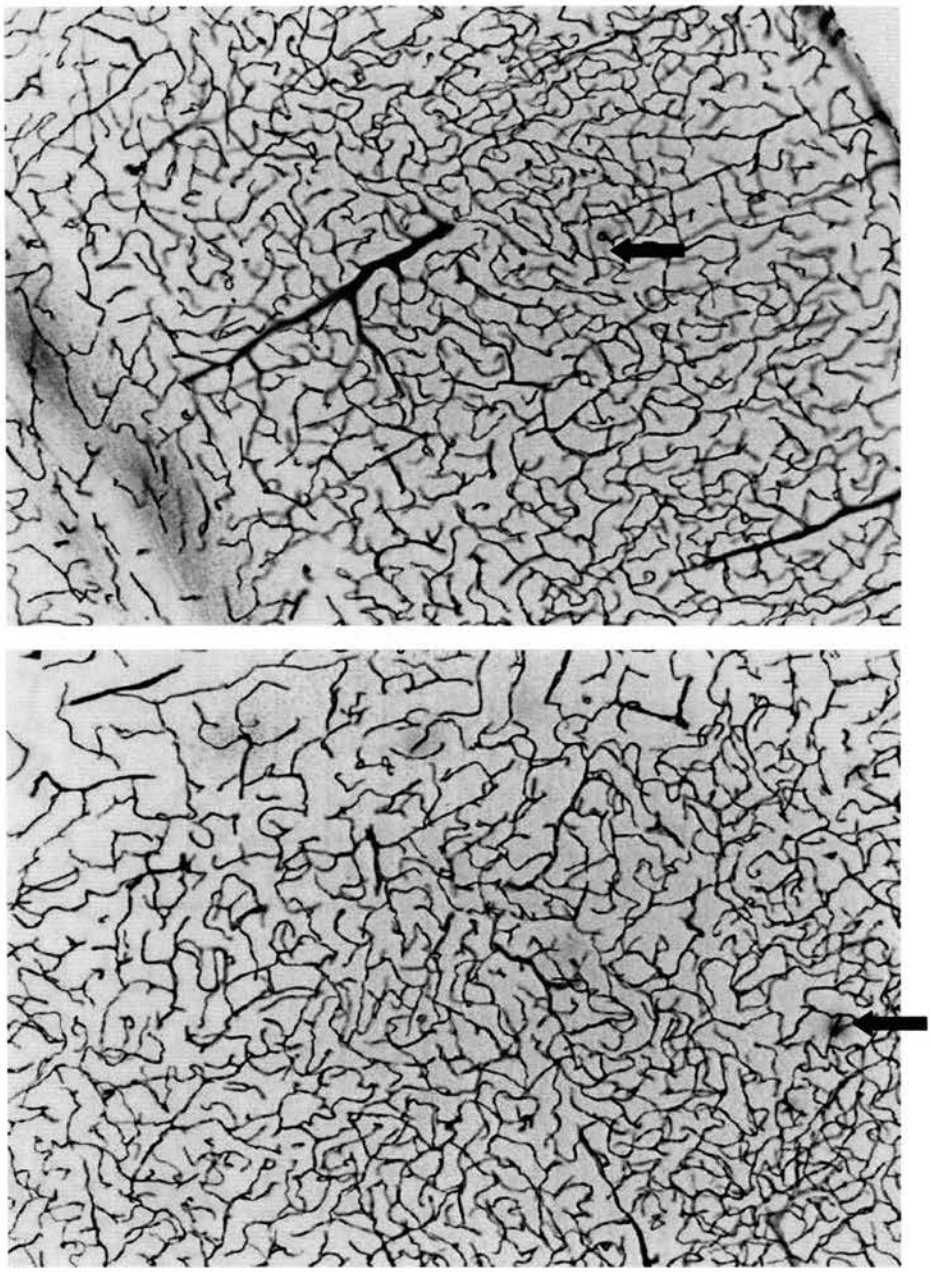

Figure 50: Light micrographs of HRP leaks (indicated with arrows) in the coronal cortex sections of the rats treated with single PYR. Rats were given either vehicle or PYR (30mg/kg, p.o.) for 50 min followed by either vehicle or paraoxon (100 μg/kg, i.m.). Ten minutes later, rats were injected HRP through the left ventricle and circulation was allowed 1 min before sacrificing the rats. Brain were removed and fixed in 2.5% glutaraldehyde and sectioned at 70 μm. HRP was visualized by reaction with DAB.

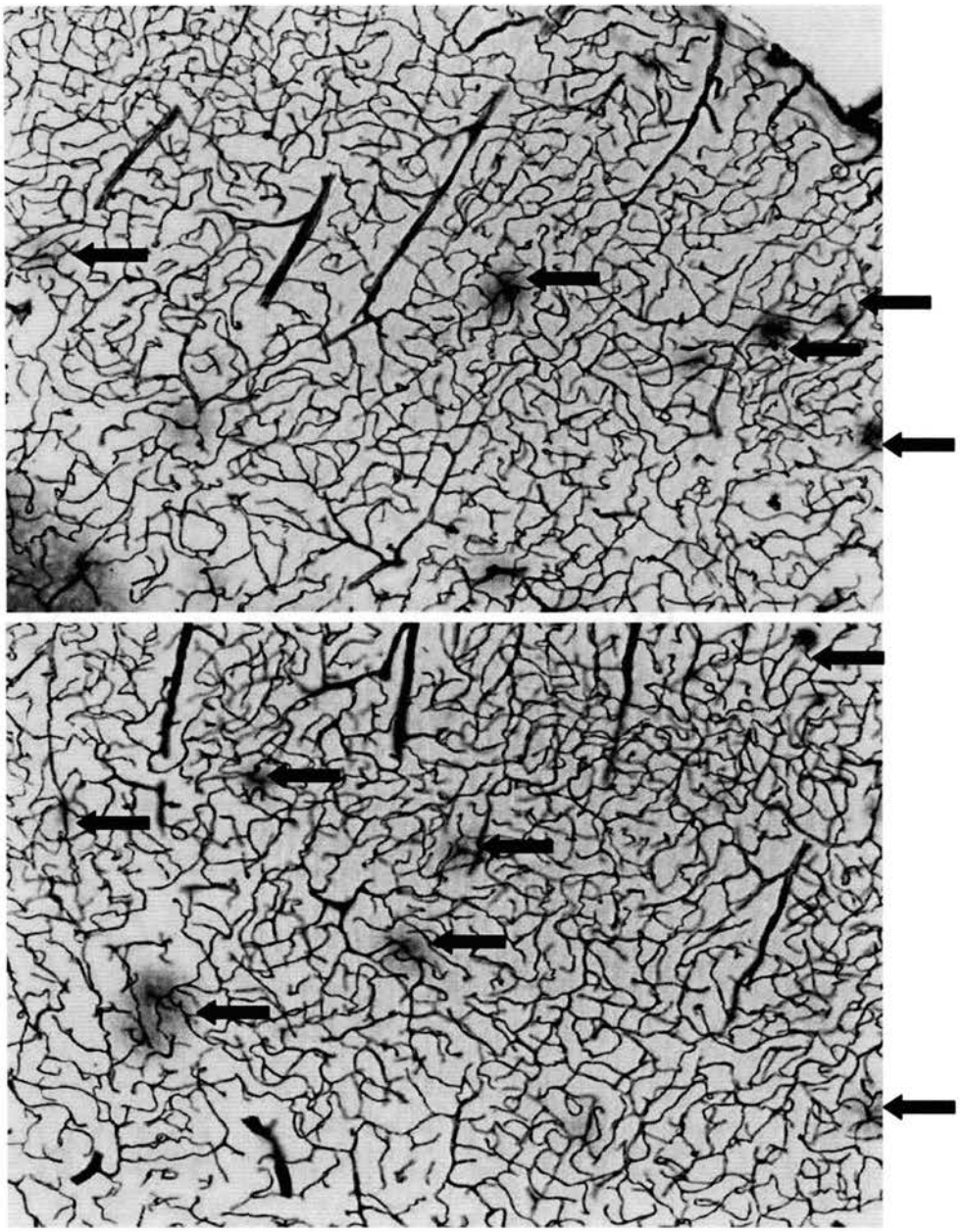

Figure 51: Light micrographs of HRP leaks (indicated with arrows) in the coronal cortex sections of the rats treated with single paraoxon. Rats were given either vehicle or PYR (30mg/kg, p.o.) for 50 min followed by either vehicle or paraoxon (100 μg/kg, i.m.). Ten minutes later, rats were injected HRP through the left ventricle and circulation was allowed 1 min before sacrificing the rats. Brain were removed and fixed in 2.5% glutaraldehyde and sectioned at 70 μm. HRP was visualized by reaction with DAB.

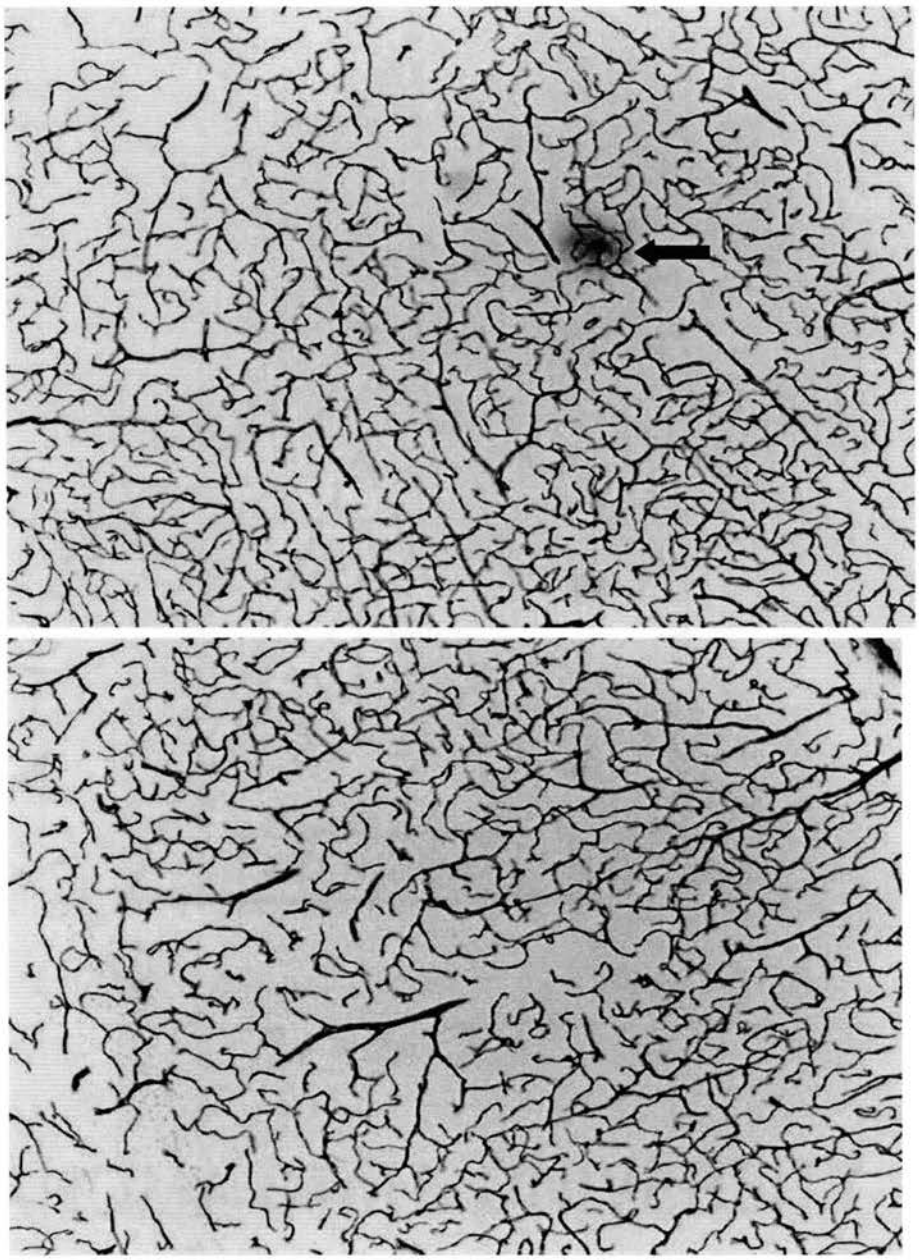

Figure 52: Light micrographs of HRP leaks (indicated with arrows) in the coronal cortex sections of the rats treated with both PYR and paraoxon. Rats were given either vehicle or PYR (30mg/kg, p.o.) for 50 min followed by either vehicle or paraoxon (100 μg/kg, i.m.). Ten minutes later, rats were injected HRP through the left ventricle and circulation was allowed 1 min before sacrificing the rats. Brain were removed and fixed in 2.5% glutaraldehyde and sectioned at 70 μm. HRP was visualized by reaction with DAB.

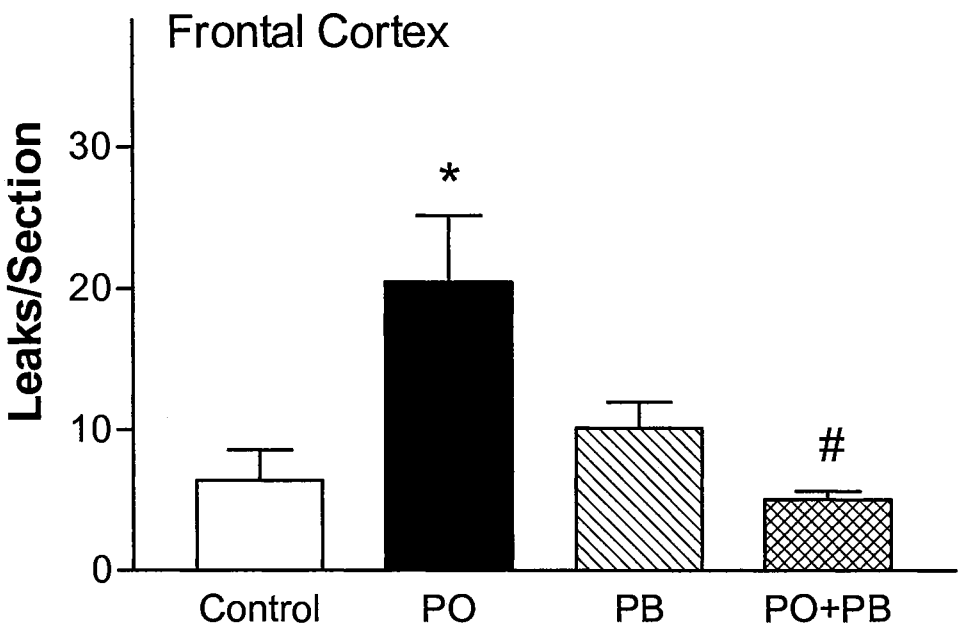

Figure 53: Blood-brain barrier disruption quantified by the number of HRP leaks in the frontal cortex after PYR, paraoxon or both. Rats were given either saline or PYR (30 mg/kg, oral gavage) for 50 min and followed by either saline or paraoxon (100 μg/kg, i.m.) for 10 min. Rats were then injected with HRP (40 mg/kg) from the left ventricle and allowed circulation for 1 min before sacrifice. HRP leaks from the brain vessels into the brain parenchyma in each of 8 sections of frontal cortex slice were counted and calculated the mean leaks per section. Asterisk indicates significant difference ($p < 0.05$) from control rats while number sign indicates significant difference ($p < 0.05$) from paraoxon-treated rats.

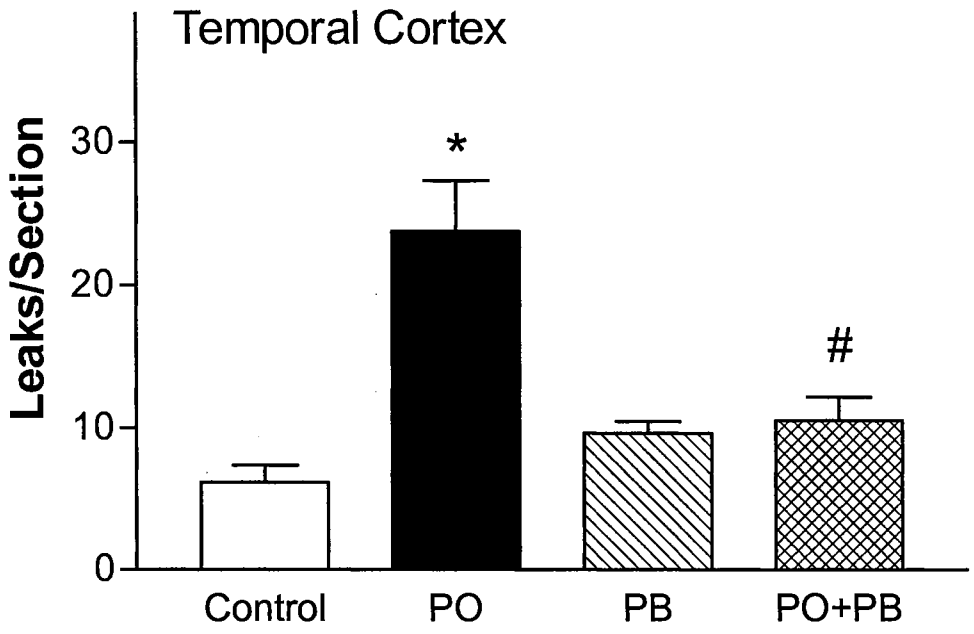

Figure 54: Blood-brain barrier disruption quantified by the number of HRP leaks in the temporal cortex after PYR, paraoxon or both. Rats were given either saline or PYR (30 mg/kg, oral gavage) for 50 min and followed by either saline or paraoxon (100 μg/kg, i.m.) for 10 min. Rats were then injected with HRP (40 mg/kg) from the left ventricle and allowed circulation for 1 min before sacrifice. HRP leaks from the brain vessels into the brain parenchyma in each of 8 sections of frontal cortex slice were counted and calculated the mean leaks per section. Asterisk indicates significant difference ($p < 0.05$) from control rats while number sign indicates significant difference ($p < 0.05$) from paraoxon-treated rats.

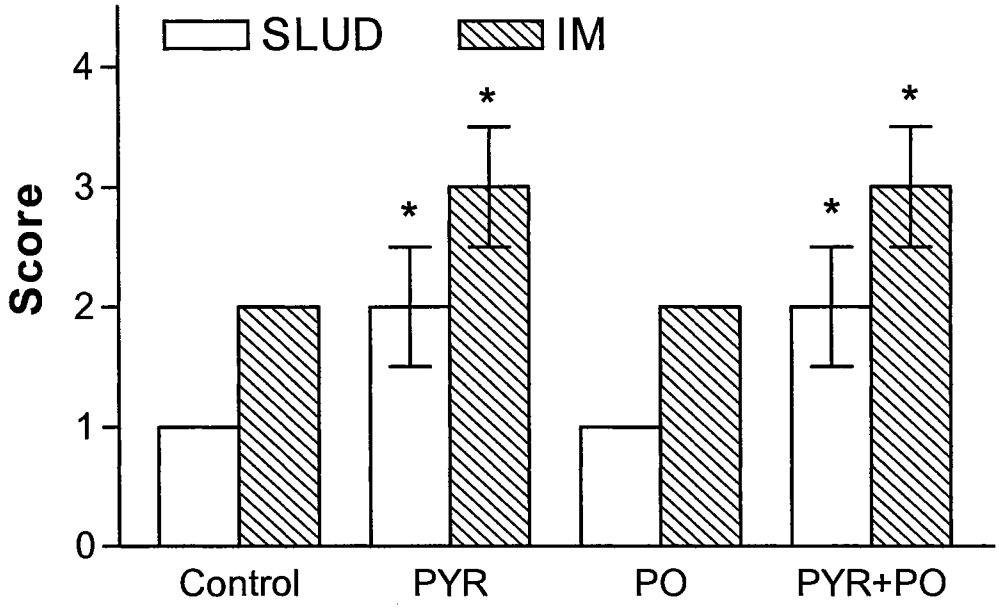

Figure 55: Score of the cholinergic signs of toxicity following PYR, paraoxon or both. Rats (n=14/treatment) were given either saline or PYR (30 mg/kg) for 50 min and then given either saline or paraoxon (100 μg/kg) for 10 min. Rats were observed for SLUD signs and involuntary movement (IM) before sacrifice. Scores were expressed as median ± interquartile range. Asterisk indicates statistically significant ($p < 0.05$) relative to control rats.

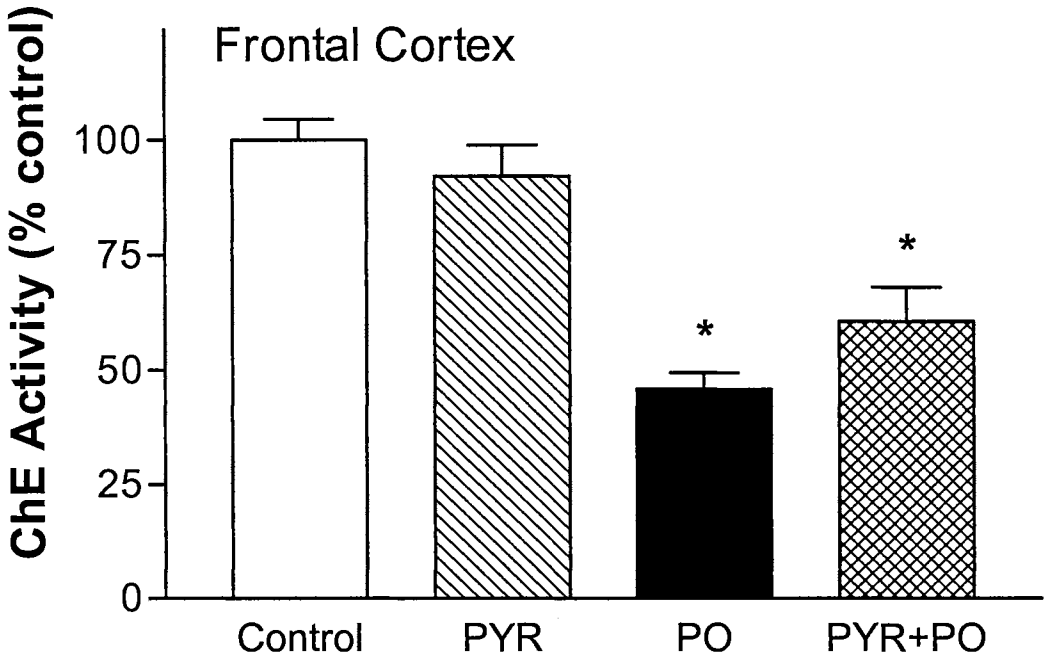

Figure 56: ChE activity in the frontal cortex after PYR, paraoxon or both in chemical stress protocol 1. Twenty-eight rats at age of 25 to 30-day old were divided into 4 groups, control (rats given vehicle of PYR + vehicle of paraoxon), PYR (rats given PYR + vehicle of paraoxon), PO (rats given vehicle of PYR + paraoxon), and PYR + PO (rats given PYR + PO). Rats (n = 7/treatment) were given either saline or PYR (30 mg/kg) for 50 min. Rats were then given either saline or paraoxon (100 μg/kg) for 10 min before sacrifice. ChE activity was radiometrically measured and calculated as nmol/min/mg protein. ChE activity was expressed in the graph as mean percentage of control group ± standard error of the mean. Asterisk indicates significant ($p<0.05$) difference from control group.

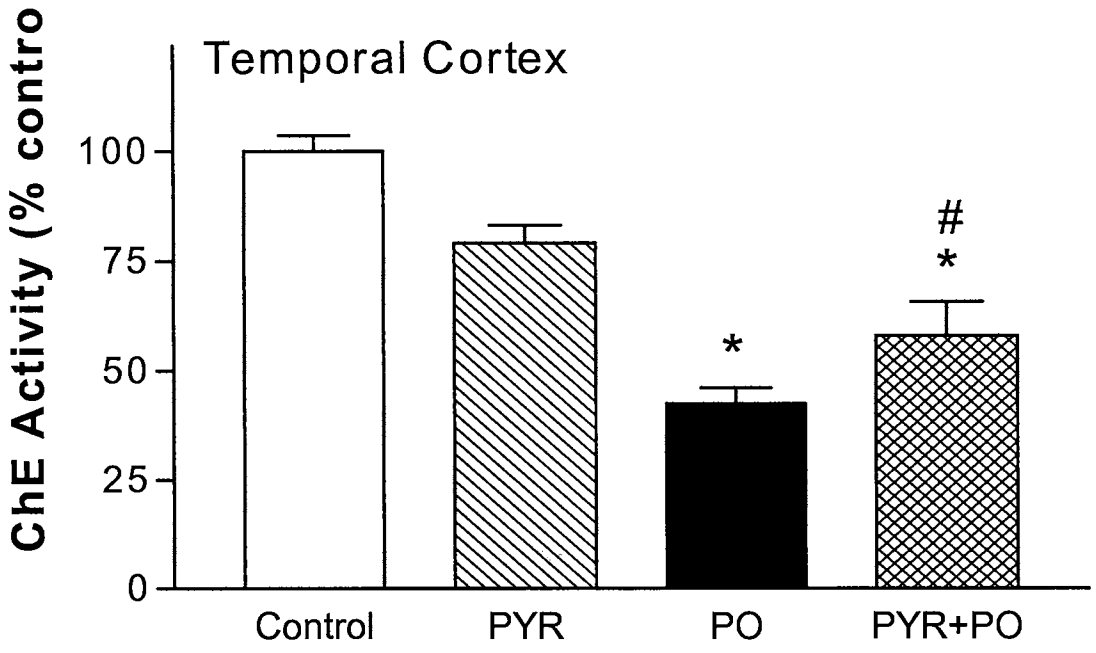

Figure 57: ChE activity in the temporal cortex after PYR, paraoxon or both in chemical stress protocol 1. Twenty-eight rats at age of 25 to 30-day old were divided into 4 groups, control (rats given vehicle of PYR + vehicle of paraoxon), PYR (rats given PYR + vehicle of paraoxon), PO (rats given vehicle of PYR + paraoxon), and PYR + PO (rats given PYR + PO). Rats (n = 7/treatment) were given either saline or PYR (30 mg/kg) for 50 min. Rats were then given either saline or paraoxon (100 μg/kg) for 10 min before sacrifice. ChE activity was radiometrically measured and calculated as nmol/min/mg protein. ChE activity was expressed in the graph as mean percentage of control group ± standard error of the mean. Asterisk indicates significant ($p<0.05$) difference from control group. Number sign indicates significant ($p<0.05$) difference from PO group.

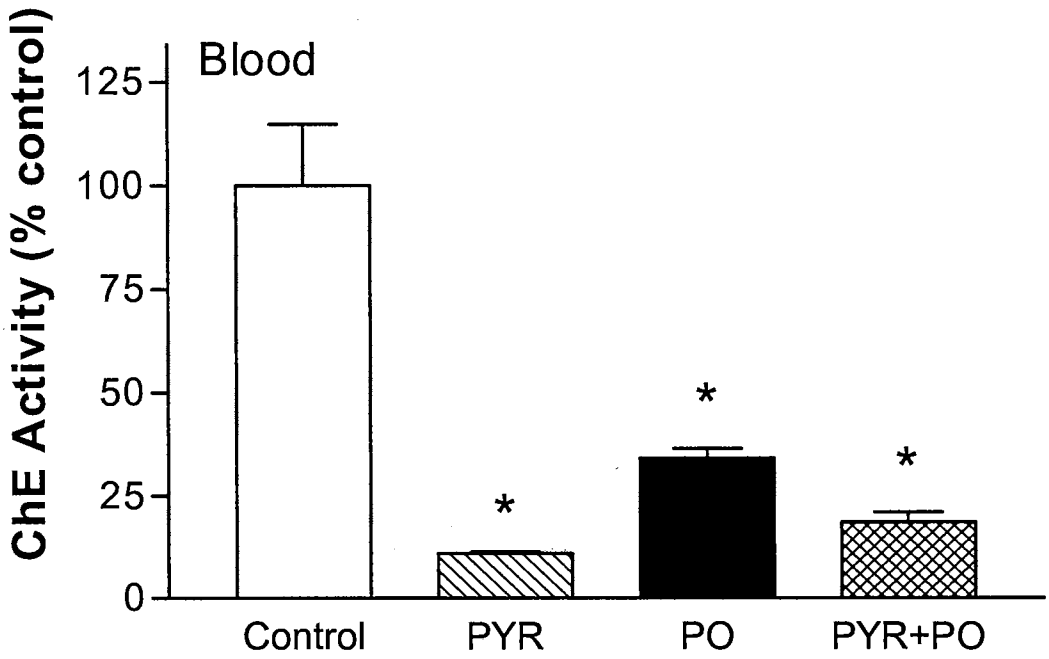

Figure 58: ChE activity in the blood after PYR, paraoxon or both in chemical stress protocol 1. Twenty-eight rats at age of 25 to 30-day old were divided into 4 groups, control (rats given vehicle of PYR + vehicle of paraoxon), PYR (rats given PYR + vehicle of paraoxon), PO (rats given vehicle of PYR + paraoxon), and PYR + PO (rats given PYR + PO). Rats (n = 7/treatment) were given either saline or PYR (30 mg/kg) for 50 min. Rats were then given either saline or paraoxon (100 μg/kg) for 10 min before sacrifice. ChE activity was radiometrically measured and calculated as nmol/min/ml blood. ChE activity was expressed in the graph as mean percentage of control group ± standard error of the mean. Asterisk indicates significant ($p<0.05$) difference from control group.

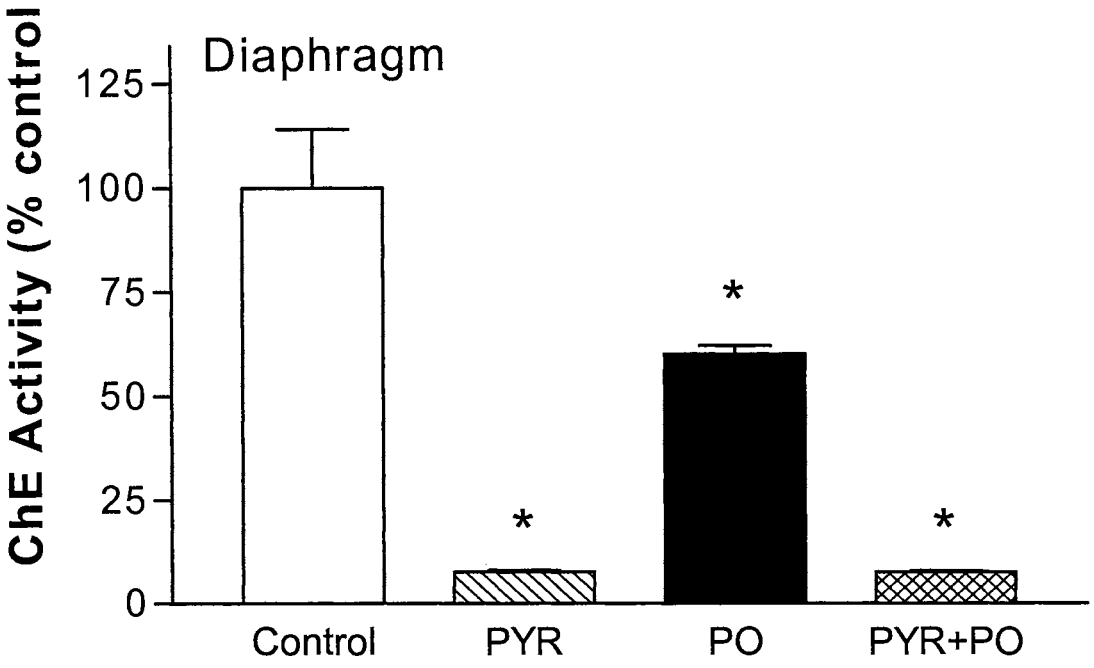

Figure 59: ChE activity in the diaphragm after PYR, paraoxon or both in chemical stress protocol 1. Twenty-eight Long Evans rats at age of 25 to 30-day old were divided into 4 groups, control (rats given vehicle of PYR + vehicle of paraoxon), PYR (rats given PYR + vehicle of paraoxon), PO (rats given vehicle of PYR + paraoxon), and PYR + PO (rats given PYR + PO). Rats (n = 7/treatment) were given either saline or PYR (30 mg/kg) for 50 min. Rats were then given either saline or paraoxon (100 μg/kg) for 10 min before sacrifice. ChE activity was radiometrically measured and calculated as nmol/min/mg protein. ChE activity was expressed in the graph as mean percentage of control group ± standard error of the mean. Asterisk indicates significant ($p<0.05$) difference from control group.

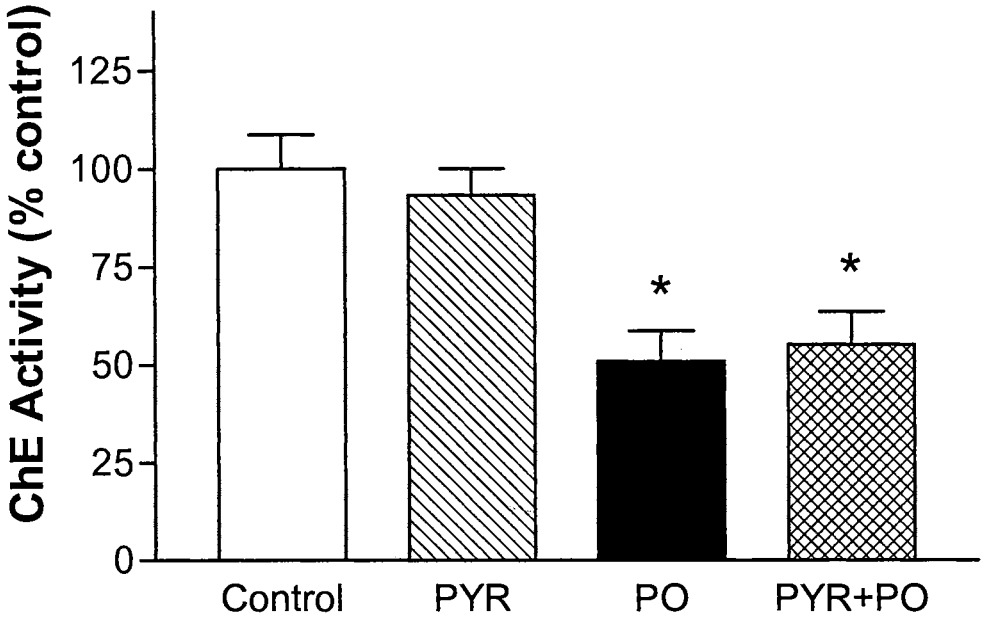

Figure 60: ChE activity in the frontal cortex after PYR, paraoxon or both in chemical stress protocol 2. Twenty-four Sprague Dawley rats at age of 6-week old were divided into 4 groups, control (rats given vehicle of PYR + vehicle of paraoxon), PYR (rats given PYR + vehicle of paraoxon), PO (rats given vehicle of PYR + paraoxon), and PYR + PO (rats given PYR + PO). Rats (n = 6/treatment) were given either saline or PYR (30 mg/kg) for 50 min. Rats were then given either saline or paraoxon (220 μg/kg) for 10 min before sacrifice. ChE activity was radiometrically measured and calculated as nmol/min/mg protein. ChE activity was expressed in the graph as mean percentage of control group ± standard error of the mean. Asterisk indicates significant ($p<0.05$) difference from control group.

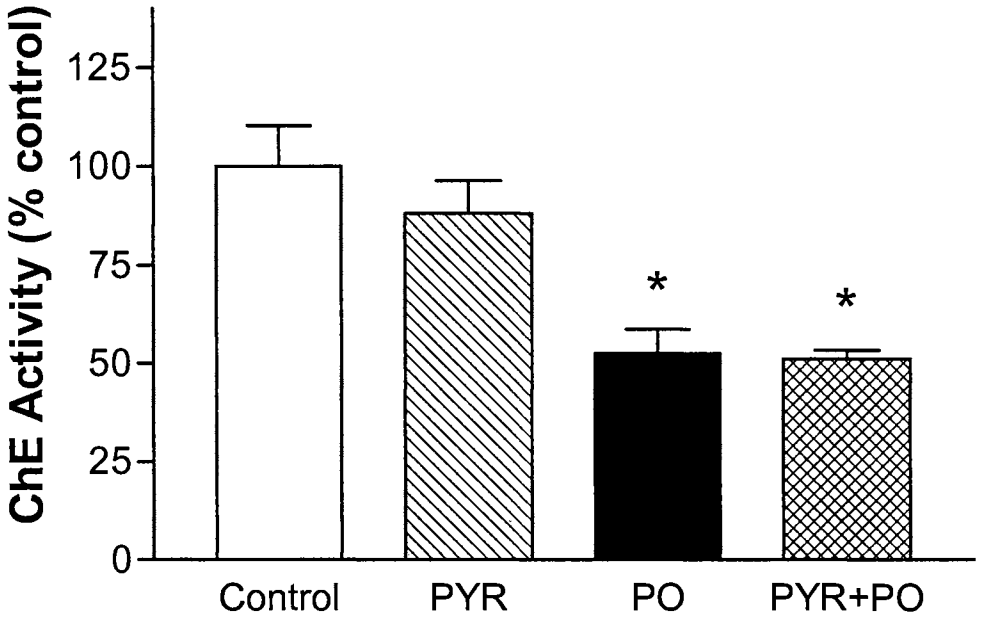

Figure 61: ChE activity in the cerebellum after PYR, paraoxon or both in chemical stress protocol 2. Twenty-four Sprague Dawley rats at age of 6-week old were divided into 4 groups, control (rats given vehicle of PYR + vehicle of paraoxon), PYR (rats given PYR + vehicle of paraoxon), PO (rats given vehicle of PYR + paraoxon), and PYR + PO (rats given PYR + PO). Rats (n = 6/treatment) were given either saline or PYR (30 mg/kg) for 50 min. Rats were then given either saline or paraoxon (220 μg/kg) for 10 min before sacrifice. ChE activity was radiometrically measured and calculated as nmol/min/mg protein. ChE activity was expressed in the graph as mean percentage of control group ± standard error of the mean. Asterisk indicates significant ($p<0.05$) difference from control group.

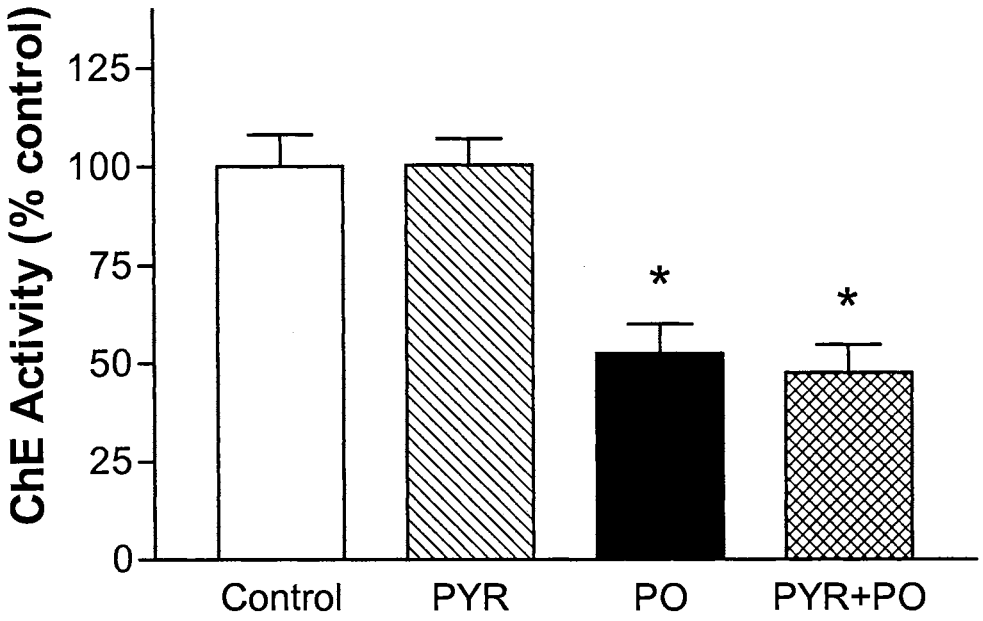

Figure 62: ChE activity in the hippocampus after PYR, paraoxon or both in chemical stress protocol 2. Twenty-four Sprague Dawley rats at age of 6-week old were divided into 4 groups, control (rats given vehicle of PYR + vehicle of paraoxon), PYR (rats given PYR + vehicle of paraoxon), PO (rats given vehicle of PYR + paraoxon), and PYR + PO (rats given PYR + PO). Rats (n = 6/treatment) were given either saline or PYR (30 mg/kg) for 50 min. Rats were then given either saline or paraoxon (220 μg/kg) for 10 min before sacrifice. ChE activity was radiometrically measured and calculated as nmol/min/mg protein. ChE activity was expressed in the graph as mean percentage of control group ± standard error of the mean. Asterisk indicates significant ($p<0.05$) difference from control group.

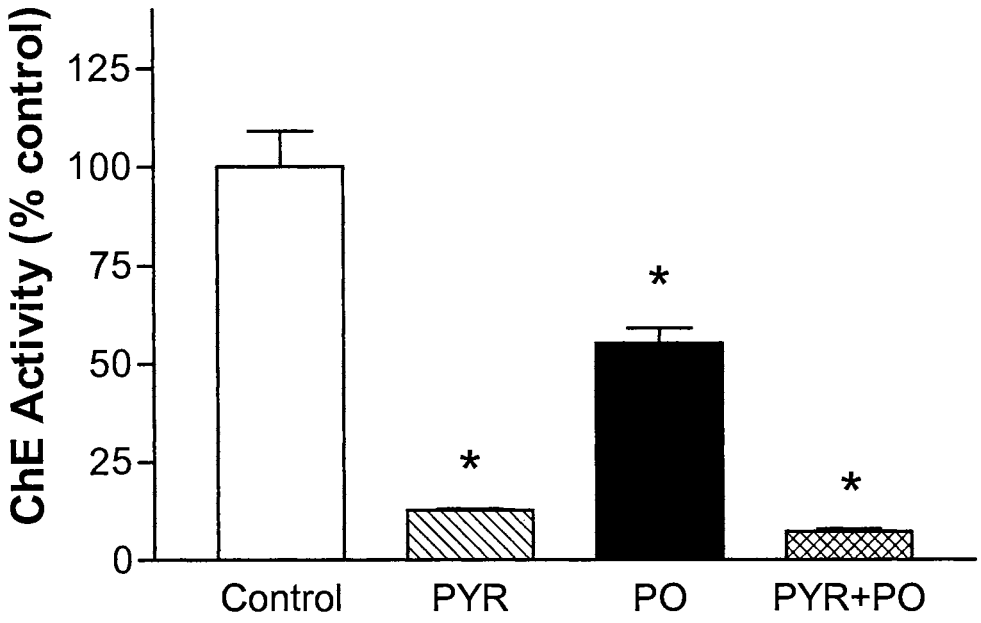

Figure 63: ChE activity in the whole blood after PYR, paraoxon or both in chemical stress protocol 2. Twenty-four Sprague Dawley rats at age of 6-week old were divided into 4 groups, control (rats given vehicle of PYR + vehicle of paraoxon), PYR (rats given PYR + vehicle of paraoxon), PO (rats given vehicle of PYR + paraoxon), and PYR + PO (rats given PYR + PO). Rats (n = 6/treatment) were given either saline or PYR (30 mg/kg) for 50 min. Rats were then given either saline or paraoxon (220 μg/kg) for 10 min before sacrifice. ChE activity was radiometrically measured and calculated as nmol/min/ml blood. ChE activity was expressed in the graph as mean percentage of control group ± standard error of the mean. Asterisk indicates significant ($p<0.05$) difference from control group.

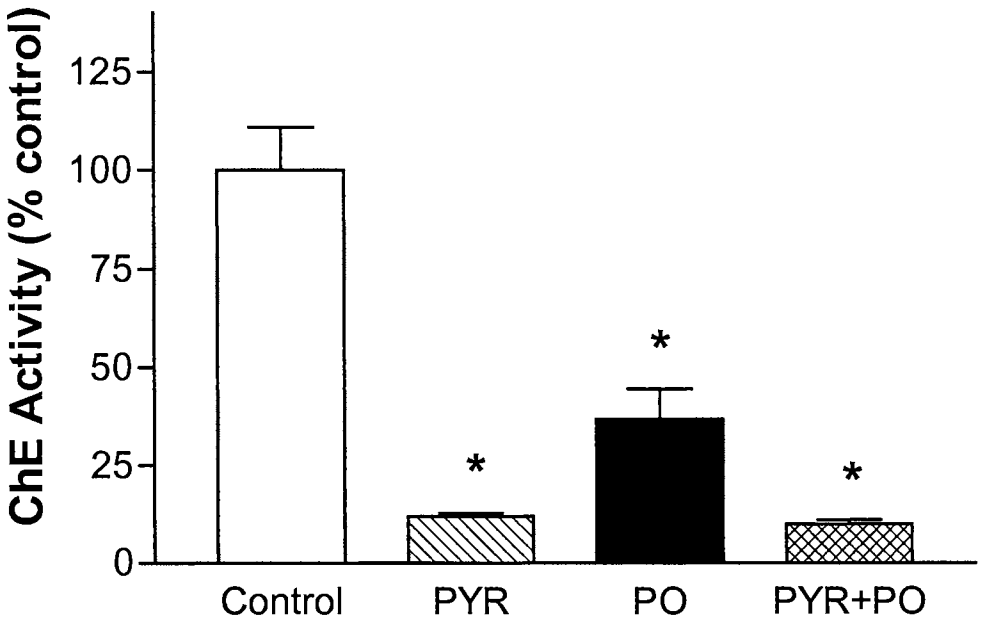

Figure 64: ChE activity in the diaphragm after PYR, paraoxon or both in chemical stress protocol 2. Twenty-four Sprague Dawley rats at age of 6-week old were divided into 4 groups, control (rats given vehicle of PYR + vehicle of paraoxon), PYR (rats given PYR + vehicle of paraoxon), PO (rats given vehicle of PYR + paraoxon), and PYR + PO (rats given PYR + PO). Rats (n = 6/treatment) were given either saline or PYR (30 mg/kg) for 50 min. Rats were then given either saline or paraoxon (220 μg/kg) for 10 min before sacrifice. ChE activity was radiometrically measured and calculated as nmol/min/mg protein. ChE activity was expressed in the graph as mean percentage of control group ± standard error of the mean. Asterisk indicates significant ($p<0.05$) difference from control group.

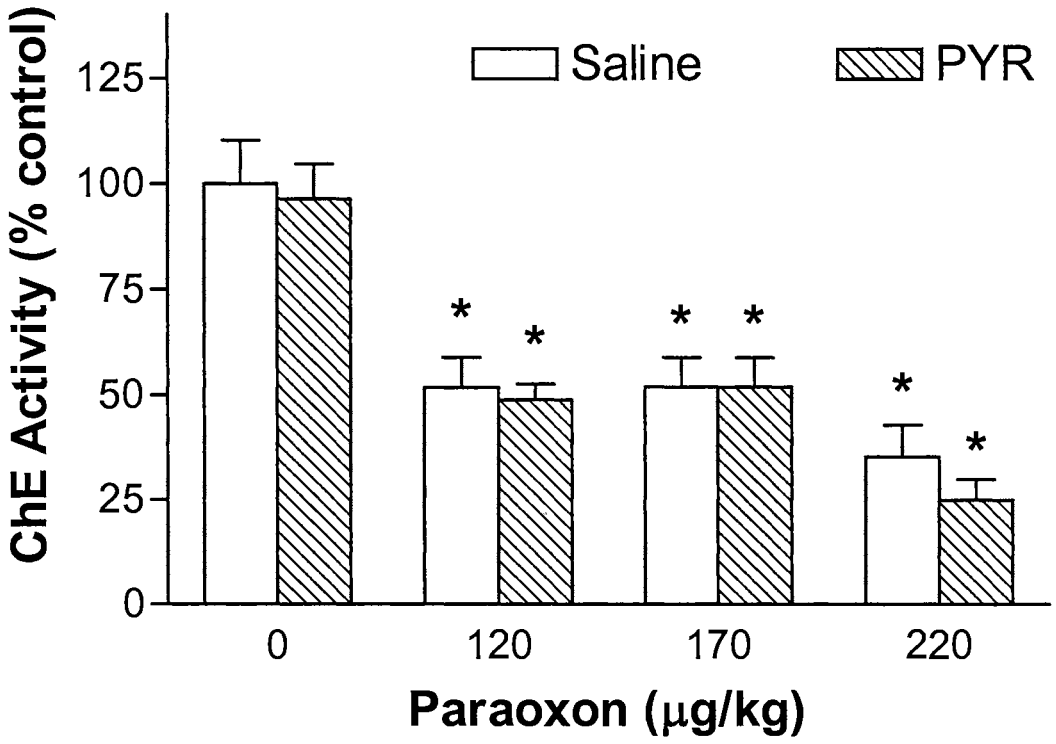

Figure 65: ChE activity in the frontal cortex after PYR, paraoxon or both in chemical stress protocol 3. Sprague Dawley rats (25 to 30-day old, n = 6/treatment) were given either saline or PYR (30 mg/kg) for 50 min. Rats were then given either saline or paraoxon (120, 170 or 220 μg/kg) for 10 min before sacrifice. ChE activity was radiometrically measured and calculated as nmol/min/mg protein. ChE activity was expressed in the graph as mean percentage of control group ± standard error of the mean. Asterisk indicates significant ($p<0.05$) difference from control group.

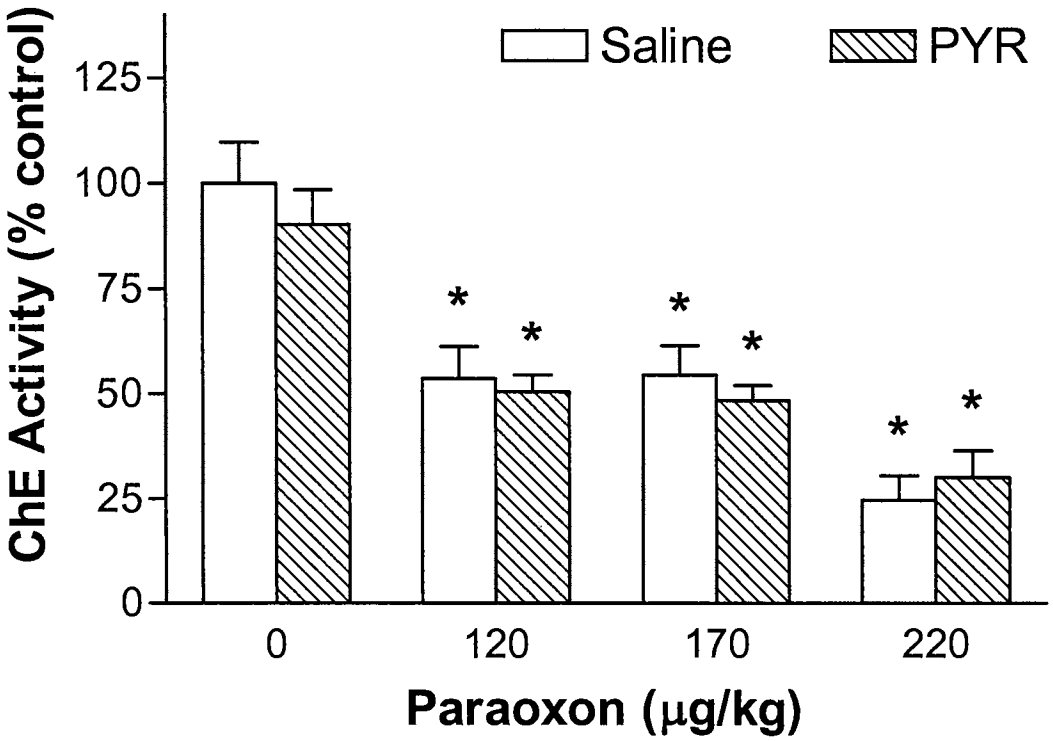

Figure 66: ChE activity in the cerebellum after PYR, paraoxon or both in chemical stress protocol 3. Sprague Dawley rats (25 to 30-day old, n = 6/treatment) were given either saline or PYR (30 mg/kg) for 50 min. Rats were then given either saline or paraoxon (120, 170 or 220 µg/kg) for 10 min before sacrifice. ChE activity was radiometrically measured and calculated as nmol/min/mg protein. ChE activity was expressed in the graph as mean percentage of control group ± standard error of the mean. Asterisk indicates significant ($p<0.05$) difference from control group.

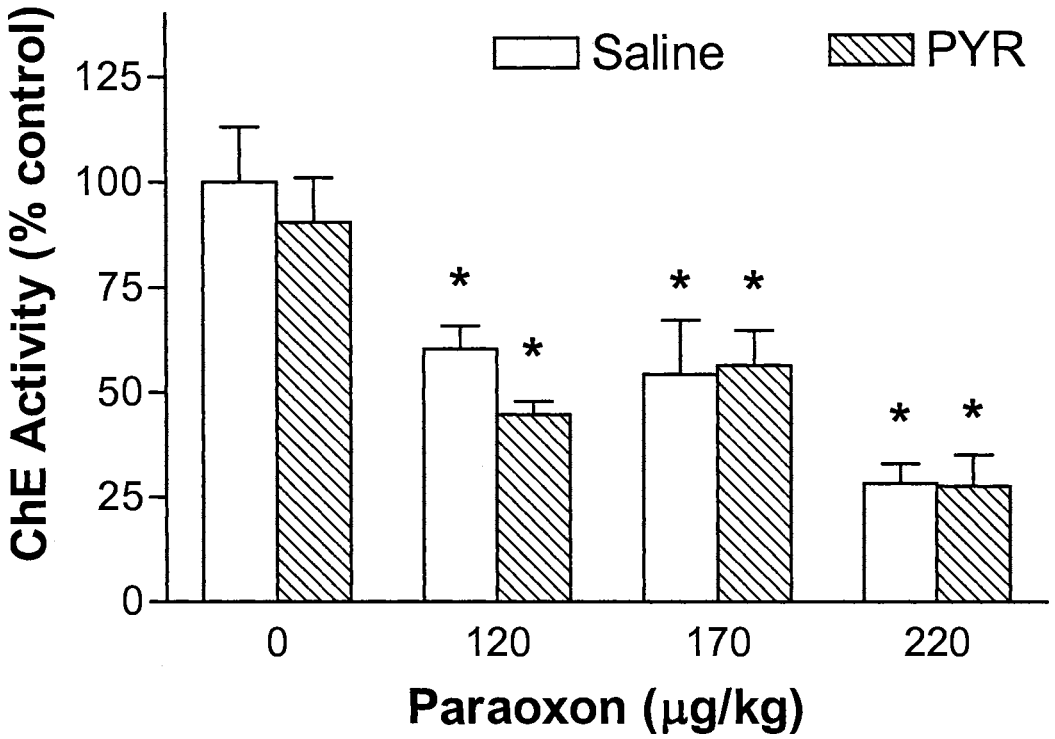

Figure 67: ChE activity in the hippocampus after PYR, paraoxon or both in chemical stress protocol 3. Sprague Dawley rats (25 to 30-day old, n = 6/treatment) were given either saline or PYR (30 mg/kg) for 50 min. Rats were then given either saline or paraoxon (120, 170 or 220 µg/kg) for 10 min before sacrifice. ChE activity was radiometrically measured and calculated as nmol/min/mg protein. ChE activity was expressed in the graph as mean percentage of control group ± standard error of the mean. Asterisk indicates significant ($p<0.05$) difference from control group.

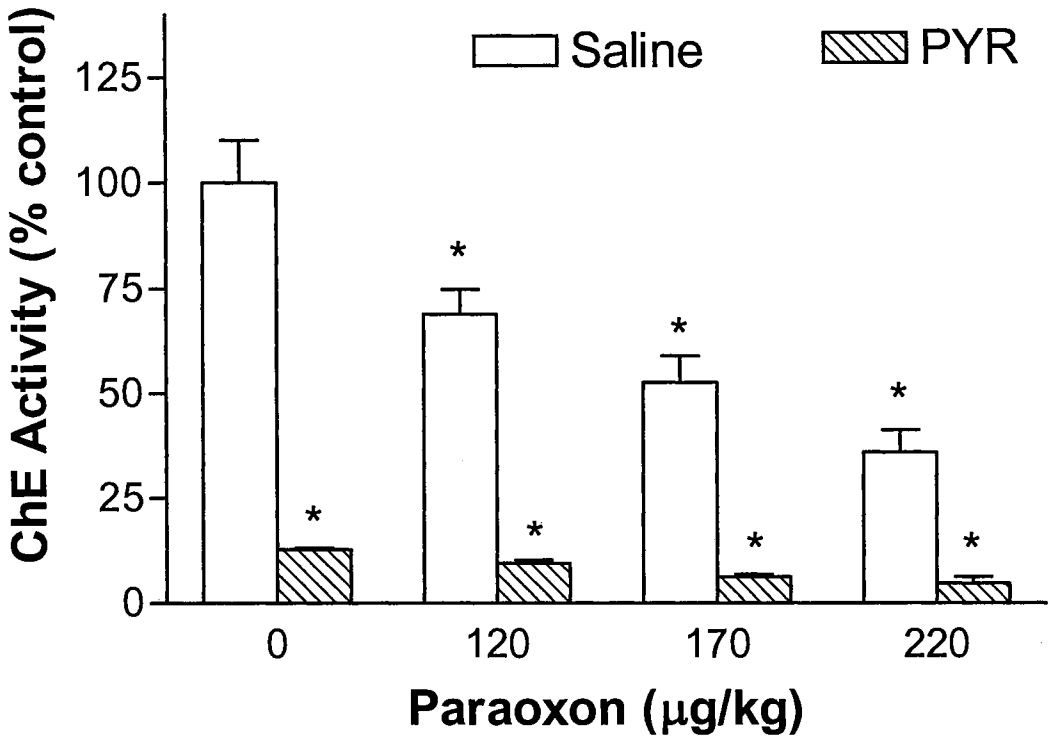

Figure 68: ChE activity in the whole blood after PYR, paraoxon or both in chemical stress protocol 3. Sprague Dawley rats (25 to 30-day old, n = 6/treatment) were given either saline or PYR (30 mg/kg) for 50 min. Rats were then given either saline or paraoxon (120, 170 or 220 μg/kg) for 10 min before sacrifice. ChE activity was radiometrically measured and calculated as nmol/min/ml blood. ChE activity was expressed in the graph as mean percentage of control group ± standard error of the mean. Asterisk indicates significant ($p<0.05$) difference from control group.

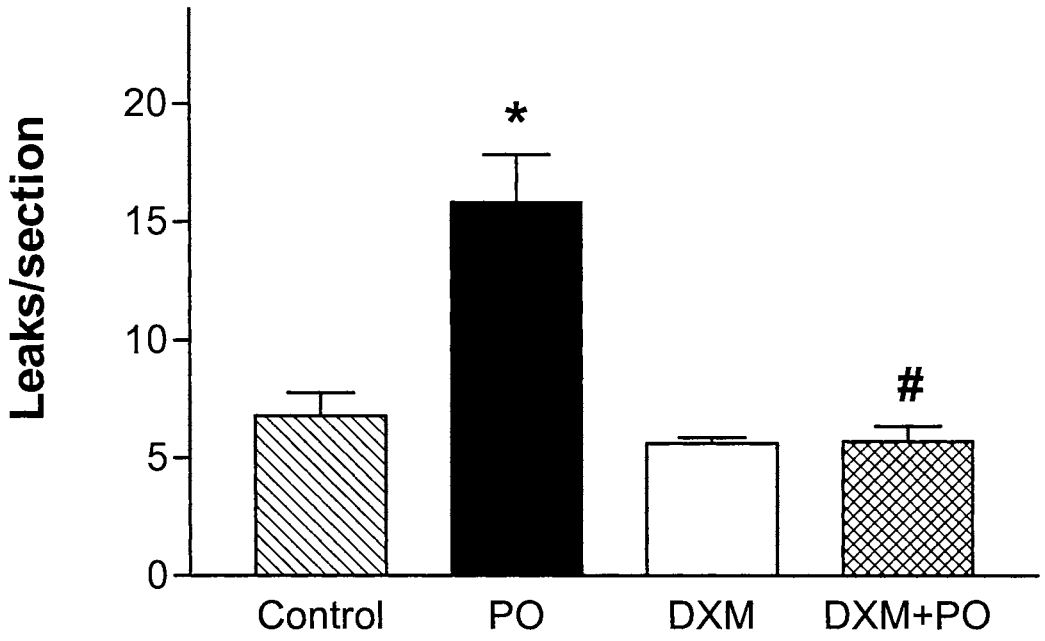

Figure 69: Effects of dexamethasone (DXM) on paraoxon (PO)-elicited BBB disruption. Rats were given 2 injections of DXM (2 mg/kg, i.p.) with 24 hrs between the 2 treatments. Three hrs after the last DXM injection, rats were given PO (100 μg/kg, i.m.) for 10 min followed by intra-cardiac injection of HRP. Brains were collected 1 min after HRP perfusion and evaluated for HRP leakage. Leaks were counted under the light microscope and calculated the mean leaks per section. Asterisk indicates significant difference ($p < 0.5$) from control rats while number sign indicates significant difference ($p < 0.5$) from PO-treated rats.

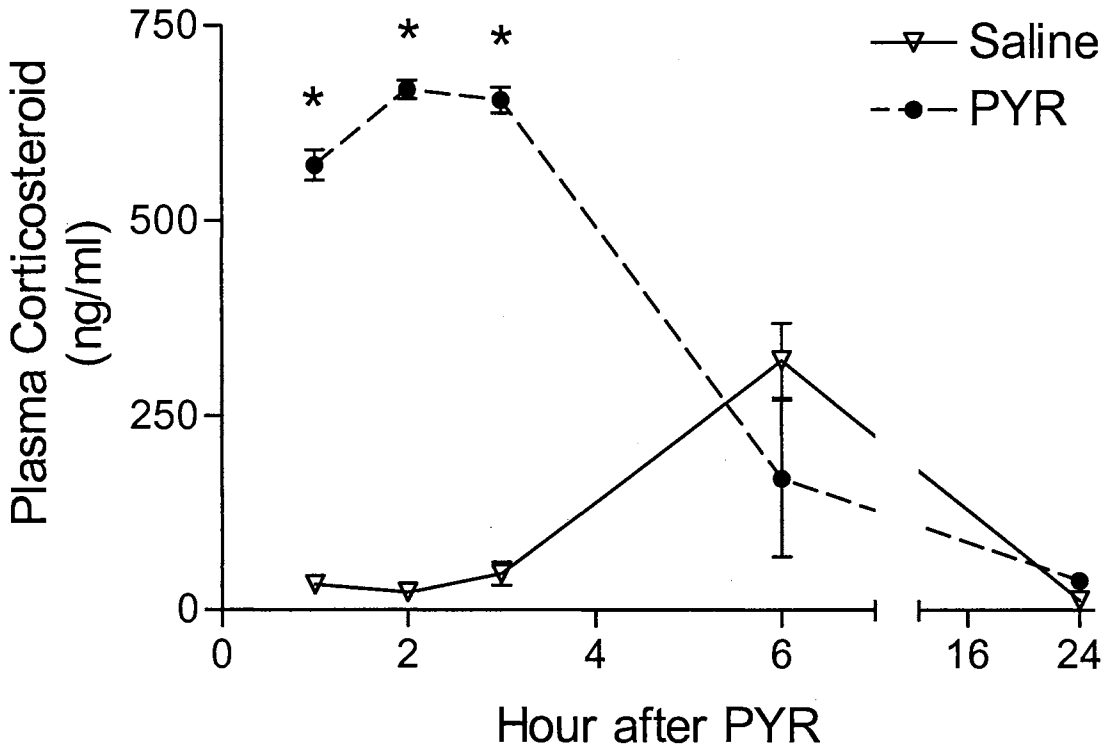

Figure 70: Time-dependent corticosteroid secretion after MTD of PYR treatment. Rats were treated with PYR at a dose of 30 mg/kg. Whole blood was collected at 1, 2, 3, 6, and 24 hrs after PYR administration. Plasma was separated and measured for the corticosteroid levels. Asterisk indicates significant difference from control ($p < 0.05$).

Chapter 4

DISCUSSION

Effects of Physical Stress on PYR Toxicity

The hypothesis that physical stress-induced changes in permeability of the BBB allowed PYR to enter the central nervous system and lead to persistent neurological signs and symptoms in GW veterans has attracted considerable attention in the past decade. In the present studies, we evaluated the effects of physical (restraint) or chemical (paraoxon) stressors on PYR cholinergic toxicity and BBB permeability.

Physical stressors stimulate the hypothalamic-pituitary-adrenal axis resulting in the release of corticotropin releasing hormone leading to secretion of glucocorticoids and other hormones (Chrousos, 1995). In our physical stress studies, acute restraint and repeated restraint models elicited substantial elevation of plasma corticosteroids (see Figures 43 and 44), indicating the general validity of the stress models utilized. However, under a variety of stress-PYR interactive conditions (e.g., simultaneous stress and PYR, stress followed by PYR, short-term vs. long-term stress, acute stress/acute PYR vs. repeated stress/repeated PYR), we found little evidence of stress-related increased toxicity following PYR exposure. The results suggest that neither acute nor repeated restraint stress enhance ChE inhibition or cholinergic signs of toxicity following high

level PYR exposures. While the dosage of PYR utilized in acute studies was the highest level that could be tolerated without lethality and caused substantial ChE inhibition in peripheral tissues (i.e., diaphragm, whole blood), PYR had little direct anti-cholinesterase action in the brain. We did on occasion notice a slight reduction (11-22%) in ChE activity in some brain regions in our studies, e.g., frontal cortex of rats simultaneously restrained and given PYR for 1 hr, cerebellum of the rats exposed to 3 hrs restraint followed by 1 hr restraint and PYR co-treatment, and cerebellum of the rats exposed to daily 1 hr restraint and 10 mg/kg PYR co-treatment for 14 consecutive days (see Table 1). Although statistically significant, this degree of reduction in ChE activity would generally be considered to have little functional consequence (Nostrandt et al., 1997). More importantly, however, in those cases where minimal brain regional ChE inhibition was noted, the degree of inhibition was not increased by restraint stress.

Besides using ChE inhibition as the biomarker for BBB permeability to PYR entrance, HRP accumulation in brain regions following systemic administration was measured to directly evaluate possible BBB permeability changes following acute restraint stress. While increased plasma corticosteroids levels following restraint stress suggested the procedures were indeed stressful to the animals, acute immobilization stress had no apparent effects on brain regional HRP activity (see Figure 42). Another study from our lab (Tian *et al.*, 2002) obtained similar findings in studies evaluating treadmill running and forced swimming stressors. Together, these results suggest that physical stressors in rats have little effect on BBB permeability changes to either protein tracer (HRP) or a peripherally-acting ChE inhibitor (PYR).

Friedman and coworkers (1996) first reported a substantial effect of forced swimming stress on PYR-induced ChE inhibition in the brain of mice, showing that the anti-cholinesterase potency of PYR was increased over 100-fold by brief swimming stress episodes. Other indicators of enhanced entry of PYR into the brain (e.g., increased brain levels of c-fos and AChE mRNA) were also reported. The discrepancies between our findings and those of Friedman and colleagues (1996) regarding the PYR-elicited ChE inhibition after stress could arise from a variety of factors, e.g., age of the animals and thus the maturational integrity of the BBB, strain of the animals, intensity or conditions of stress, etc. Among these considerations, the age of the animal model has been noted as a very important factor in sensitivity to possible BBB disruption by environmental factors. In previous studies showing BBB disruption after physical stress (Sharma and Dey, 1986; Ben-Nathan et al., 1989; Wijsman and Shivers, 1993), the animals used in the experiment were relatively young, with stress-associated BBB disruptions not noted in older animals. Of interest, while we found little BBB change under stressful conditions in young rats (5-7 weeks of age, an age group previously shown sensitive to BBB disruption with environmental stressors, Sharma and Dey, 1986), Friedman and colleagues (1996) reported BBB leakage after forced swim stress in adult mice.

Intensity and duration of stress may also influence the change of BBB permeability. Previous studies showed a period of 30 min forced swim stress increased BBB permeability to Evans blue and ^{131}iodide sodium, but 5 or 15 min forced swim stress failed to elicit the BBB leakage (Sharma et al., 1991). These results suggested certain stress conditions need to be met to exert effects on BBB permeability.

A strain-related difference in BBB integrity is another important factor in the controversy of the BBB response to stress and PYR exposure. The FVB/N mice that showed BBB leakage to forced swim stress in the previous study by Friedman and co-workers (1996) was reported to have a "special" BBB, more permeable to penetrating agents compared to other strains (Wong et al., 1991; Telang et al., 1999). After inoculated with the ts1 mutant of Moloney murine leukemia virus TB, all FVB/N mice developed hindlimb paralysis at 30-60 days post-inoculation, whereas only 33% of BALB/c mice developed hindlimb paralysis with a much longer latency period (Wong et al., 1991). Results suggested a strain-related difference in BBB resistance to environmental disruption.

A result from Friedman's (1996) publication itself suggested the FVB/N mice have unusual BBB maturation. It was reported in the study that the ED_{50} of PYR for brain ChE inhibition (dosage of PYR causing 50% ChE inhibition in the brain) is 100 fold less in stressed mice than in non-stressed mice. The presumption of the findings was that a single PYR exposure could indeed cause significant ChE inhibition (>=50%) in the brain of mice under un-stressed conditions, which is contrary to the well-established concept that PYR has little effect on brain ChE activity (Xia et al., 1981; Grauer et al., 2000; Li et al., 2000; Song et al., 2002; Tian et al., 2002). A number of studies have demonstrated that PYR should not enter the brain and elicit ChE inhibition to an extent of 50% even if administered at lethal dosage causing extensive ChE inhibition in peripheral tissues (Xia et al., 1981; Song et al., 2002; Tian et al., 2002). The PYR-elicited 50% ChE inhibition in the brain in Friedman's study (1996) suggested the mouse strain used in their experiments may have underdeveloped BBB.

Strain-related differences in sensitivity to PYR have been reported. Servatius and colleagues (1998) conducted an experiment to study exaggerated startle responses following PYR bromide exposure in Sprague Dawley and Wistar Kyoto rats. Both strains of rats were given PYR through the drinking water for 7 days. Sprague Dawley rats showed no response while Wistar Kyoto rats consistently demonstrated delayed exaggerated startle responses from 15 to 22 days after the last PYR treatment. Wistar Kyoto rats displayed a "come-sooner-go-faster" exaggerated startle response when they were treated with PYR for a second time. The remarkable difference between Sprague Dawley and Wistar Kyoto rats in response to PYR exposure was correlated with different levels of butyrylcholinesterase (BuChE) activity in the 2 strains, i.e., BuChE levels were significantly higher in Sprague Dawley than Wistar Kyoto rats. The enzyme was considered to serve as a scavenger or buffer for PYR and remarkably decreased the availability of PYR for possible penetration through the BBB. Therefore, the low level of BuChE activity in Wistar Kyoto rats may have increased the effective concentration of PYR in the circulation and thus the risk of PYR transport into the CNS and subsequent neurotoxicity.

Another study (Loewenstein-Lichtenstein et al., 1995) suggested a genetic predisposition with abnormal plasma BuChE might render those soldiers more susceptible to the adverse effects of PYR. These authors reported that an Israeli soldier that was homozygous for the "atypical" BuChE showed severe neurological symptoms following PYR exposure. Atypical BuChE, the most common type of variant BuChE, was shown to be far less sensitive to PYR and other carbamate inhibitors than the wild type. It was proposed that reduced enzymatic hydrolyzing capability of the variant

BuChE resulted in higher levels of circulating PYR than in "normal" soldiers, which may subsequently cause adverse effects. The findings that less active BuChE may contribute to unexpected PYR toxicity may provide at least some information to the etiology of GWI. Those PYR pretreated soldiers who express atypical BuChE could have decreased detoxification of PYR and potentially higher plasma levels of PYR. To test this hypothesis, it would be necessary to conduct an epidemiological study to screen the BuChE in the soldiers given PYR and deployed in the Operation Desert Storm and Desert Shield and match the genetic screening results to the GWI symptom study. Studies need to address if soldiers have a significantly higher proportion of atypical BuChE variants and if there is a correlation between BuChE deficiency and the undiagnosed symptoms. However, disagreement with this hypothesis exists. Lotti and Moretto (1995) argued that the dose of PYR used in GW would be too low to saturate the scavenger capacity of the "atypical" BuChE to cause CNS dysfunction in soldiers with that variant enzyme. To clarify the possibility that genetic differences among BuChE variants cause adverse responses to ChE inhibitors, further investigations need to be conducted.

Epstein and colleagues (1990) studied the effects of PYR on the thermoregulatory responses in human volunteers during exercise and heat stress. Given either placebo or PYR, young adult men were exposed to 170 min exercise (in order of 60 min sitting, 50 min walking, 10 min rest and 50 min walking) and heat stress (33°C, relative humidity 60%). The physiological responses and heat balance parameters were similar between the placebo and PYR treated groups after exercise/stress exposure.

Arad and co-workers (1992) evaluated the effects of heat-exercise stress and PYR exposure on CNS functions of human volunteers. Results suggested there were no significant interactive effects between pyridostigmine and several stressors.

Lallement and co-workers (1998) reported PYR did not penetrate into the brain of guinea pigs exposed to extreme heat stress. In their studies, guinea pigs were subjected to either a moderately or an extremely hot environment to maintain core temperature at 41.5°C and 42.6°C respectively. PYR or tritiated PYR was injected 90 min after the heat stress. ChE activity in brain regions (cortex, striatum and hippocampus) and radioactivity in brains (septum, caudate nuclei, hippocampus, thalamus and cortices) were measured to evaluate PYR penetration into the CNS. Results demonstrated 1) no brain ChE inhibition occurred regardless of the treatment or brain region, and 2) radioactive PYR was not detected in the CNS by autoradiographical analysis.

Teland and co-workers (1999) also studied the penetration of radioactive PYR into the brains of mice under stressful conditions. Swiss-Webster male mice were subjected to 2 periods of 4-min forced swim stress with a 4 min interval between the stresses similar to study by Friedman and colleagues (1996). One microcurie of ^{11}C-labeled PYR was injected into the tail vein 10 min after the termination of the forced swim. Brains were taken out 10 min after the PYR injection and radioactivity was measured. The amount of ^{11}C-labeled PYR in the brain following intravenous administration was not increased in mice following forced swimming stress.

Grauer and coworkers (2000) investigated the effect of PYR on central ChE activity in mice after several stress models. In one experiment, male or female CD-1 albino mice were subjected to either swimming stress (two 4-min periods of swim with a

2-min rest between the swimming sessions) or cold stress (a 5 min period with the mice feet placed in ice-cold water). PYR was given to the mice 10 min after the termination of the stress. Brains were collected 5 min after PYR exposure and ChE activity was measured. In another experiment, male FVB/n albino mice, the same mice strain used in Friedman et al (1996) and obtained from the same provider, were used to evaluate the interaction between stress and PYR. Results showed brain ChE activity was relatively similar in groups treated with PYR under either stressful or non-stressful conditions.

Sinton and colleagues (2000) studied the interactions between PYR and physical stress in 2 rat strains using multiple stress protocols. Adult male Long Evans rats were exposed to forced swim stress (two 10-min sessions of forced swim with a 2-min rest interval), restraint stress (physically immobilized in restraint tubes for 30 min), or combined swim and restraint stress (in order of 10 min swim, 2 min rest, 10 min swim, 30 min restraint, and 5 min swim). Adult male Wistar rats were exposed to only combined stress as used in Long Evans rats. PYR was given to rats 10 min after termination of the stress. Brains were removed 30 min after PYR treatment and ChE activity was measured. Data were similar across rat strain and stress protocols and suggested that brain ChE activity in PYR treated rats was actually higher under some stress conditions. These results suggest immobilization stress, forced swimming or combined stressors did not increase but actually reduced brain ChE inhibition by PYR.

Ovadia and coworkers (2001) examined the effects of several stress models on the brain retention of circulating Evans blue dye as a biomarker of BBB disruption. Male Balb/c mice, FVB/n albinos, and male Sabra rats were used throughout the experiments. Animals were subjected to each of several stressors including the swim stress protocol

used in Friedman et al (1996) studies (i.e., two 4-min swim stress with a 4-min rest interval between), restraint stress (physical immobilization in restraint tube for 4 hrs), photic stimulus (flashes at the rate of 4/second for a 4 min period in the dark room), acoustic stress (bell ringing with the intensity of 109 dB for a 4-min period). Evans blue or ^{125}I-albumin was injected into the heart of the rats 10 min after the completion of the stress procedure. Brains were collected 10 min after injection of tracers. Evans blue in the brains was measured spectrophotometrically and radioactivity of the ^{125}I-albumin retained in brain tissues was counted in a gamma counter. The amount of circulatory tracers extravasated in the brains of the animals following various stress stimuli was similar to that of the non-stressed animals, suggesting none of the physical stress protocols influenced BBB permeability. Another important issue addressed in these studies is that insufficient washout of the blood vessels during the perfusion resulting in tracers left within the capillaries in the brains may cause misinterpretation of changes of BBB permeability. In other words, the apparent higher amount of tracers detected might be in fact the tracers in the cerebral vessels but not the tracers leaked into the brain parenchyma. The investigators also noted that at least 10 to 15 min washout is needed to ensure the cleanout of tracers from the cerebral capillaries while 1 min washing as that used in previous studies (Sharma et al., 1991) was not sufficient.

Kant and co-workers (2001) studied the effects of footshock stress on PYR entry into the brain. Adult male Sprague Dawley rats were exposed to a 3-day long-term intermittent footshock and implanted with a mini-pump allowing slow release of PYR into the body. Brain ChE inhibition by PYR was not affected by the pre-exposure of footshock stress that significantly increased plasma corticosteroid level. Tian and co-

workers (2002) investigated the effects of either forced swimming or treadmill running stress on acute PYR-induced functional toxicity and ChE inhibition in brain and blood of 6-week old male Sprague Dawley rats. For the treadmill and PYR interaction study, rats were 1) treated with PYR and forced to run on the treadmill fro 20 min, 2) forced to run for 60 min, given PYR followed by another 30 min running, 3) forced to run for 90 min and then given PYR. For the forced swimming study, rats were 1) treated with PYR followed by swim stress for 15 min, 2) forced to swim for 15 min and treated with PYR. In all stress studies, rats were sacrificed for brains 1, 2 or 4 hours after PYR treatment. Results suggested neither treadmill running nor forced swimming influenced PYR associated brain-regional ChE inhibition or cholinergic toxicity.

Therefore, a number of laboratories have evaluated the effects of a variety of physical stressors on the penetration of PYR or BBB passage tracers into the brain of multiple strains of mice, rats, or other species. The majority of these studies suggested that physical stressors generally have little influence on the ability of systemic PYR to interact directly with brain ChE or BBB permeability.

Following Friedman and co-workers' findings (1996), Fishman (1997) argued that the BBB breakdown caused by forced swimming stress may not be a direct consequence of swim stress but a result of the hypertension associated with the forced swim stress. The author proposed that PYR may penetrate into the brain through the enlarged clefts between endothelial cells of the BBB in response to the mechanical stretch induced by high blood pressure. The forced swim stress may mask the effects of hypertension caused by itself and was thought to be the responsible factor to the BBB disruption. In other words, hypertension, which was caused by forced swimming may facilitate the PYR

entry into the brain causing cholinergic toxicity, which may contribute to the development of GWI. If it were true that hypertension increases BBB permeability, it would be interesting and important to examine whether all physical stressors cause hypertension and in what animal models, and how to extrapolate the data from animals to humans.

We concluded that physical stress does not markedly increase the entry of PYR into the brain. Environmental stressors, however, could modify the peripheral actions of PYR and lead to unexpected toxicity. Our results similar to findings from other laboratories, suggest that restraint stress has little effect on overt functional signs of cholinergic toxicity following high dosages of PYR. It is possible that stress could influence responses to lower dose PYR exposures, however. Nobrega and colleagues (1999) reported that comparatively low PYR exposure (45 mg dose, <1 mg/kg) reduced the heart rate increase associated with a mental stressor (arithmetic test) in healthy human volunteers. In another study, this same group (Nobrega et al., 2001) reported that repeated PYR dosing (30 mg every 8 hours) reduced heart rate and increased heart rate variability in healthy human volunteers over a 24-hour period. Plasma cholinesterase inhibition in this study was reported to be $\leq$ 15% at 2 and 24 hours after initiation of dosing. Thus, more subtle peripheral effects may occur with very low dose PYR exposures associated with negligible inhibition of blood ChE. While blood ChE is generally more sensitive than AChE in "target" tissues, AChE in some autonomic pathways regulating cardiac function could possibly be more sensitive and inhibited by lower dosages of PYR. It may also be that another target macromolecule aside from AChE with higher sensitivity to PYR is responsible for the reported changes in cardiac

function. PYR has been shown to interact directly with nicotinic (Akaiki et al., 1984; Albuquerque et al., 1988) and muscarinic (Lockhart et al., 2001) receptors and such interactions could potentially be important in the observed modulation of cardiac function. Modulation of PYR-mediated chronotropic and heart rate variability changes by environmental stressors should be investigated.

Haley and coworkers (1997) grouped the GWI into 6 syndromes. Two of these syndromes "confusion ataxia" and "arthro-myo-neuropathy," were significantly associated with self-reported adverse responses to PYR. The complaints were from the adverse experience with using of PYR but not the side effects of PYR exposure, suggesting veterans that served in GW may have different responses to the treatment of PYR and lead to the development of the illnesses complained by those veterans after GW.

Substantial differences in PYR levels in plasma have been noted. First, the amount of PYR reaching the target enzyme is influenced by absorption and excretion. Individual differences in absorption of PYR result in a wide range of bioavailability of the drug. PYR-induced stimulation on gastrointestinal peristalsis influences its absorption. After PYR is absorbed, differences in the levels and types of metabolic enzymes influence the clearance of PYR. In summary, individual differences in ADME may lead to wide variations in the levels of PYR in the plasma (Cohan et al., 1977; Aquilonius et al., 1980).

Variable responses to PYR have been noted. Li and co-workers (2000) found that brain ChE activity was not significantly inhibited by intra-peritoneal PYR exposures, but some treated rats showed substantial ChE inhibition. In our studies, we occasionally

noted up to 3 fold differences in the whole blood ChE inhibition after oral 30 mg/kg PYR (for example most rats showed greater than 90% inhibition while some showed approximately 30% inhibition). Additional, different degrees of PYR-elicited clinical toxicity signs of the rats within the same experimental groups were noted in some cases. Other studies showed a different duration of action by PYR, different PYR hydrolysis by ChE in presence of similar PYR levels in the plasma, etc. All the findings pointed to the existence of individual response to PYR anti-Cholinesterase effects.

In summary, after PYR administration at the same dosage levels with same exposure route, it is likely that different responses occur with the different influence of one or more of the following events including absorption, metabolism of parent compound, clearance from the body, affinity and/or sensitivity to BuChE or AChE for hydrolysis. These factors could individually or cumulatively play a role in the development of differential toxicity, which may have developed as unexplained illnesses in more susceptible veterans.

Effects of Chemical Stress on PYR Toxicity

While the BBB appeared to be resistant to alteration by physical stressors, a number of studies reported BBB breakdown after exposure to chemical stressors, e.g. OP ChE inhibitor pre-exposure, or seizure eliciting agents. Ashani and Catravas (1981) reported soman or paraoxon exposure increased BBB permeability and allowed phospholine iodide, a peripherally-acting ChE inhibitor, to enter the brain and cause ChE inhibition. Carpentier and co-workers (1990) found that a high dosage of soman caused extravasation of injected Evans blue dye and endogenous immuno-protein IgG into the

brains of Wistar rats. Petrali and coworkers (1991) detected Evans blue, HRP and quaternary ^{3}H-hexamethonium leaks in the brain after 0.9 X LD_{50} of soman. The exact mechanism for the BBB disruption after high dosage OP exposure is not clear. However, OP-induced BBB disruptions mentioned above were associated with seizures, which suggested OPs may compromise BBB integrity through eliciting convulsions and seizures. Sokrab and co-workers (1990) observed leaks of endogenous plasma albumin, fibrinogen and fibronectin in the brain after the induction of experimental seizures by intravenous bicuculline. Furthermore, the seizure-associated BBB leakages were significantly blocked by pre-treatment with anti-convulsive agents, e.g., nembutal or atropine sulfate (Ashani and Catravas, 1981).

Our pilot findings suggested that relatively low dosage paraoxon (100 μg/kg, intra-muscular injection, 10 min exposure, causing about 50% brain ChE inhibition) could compromise BBB integrity, allowing the enzyme marker HRP to penetrate into the brain (See Figure 48). Using this "chemical stress" model, we investigated the effects of low dosage of paraoxon-elicited BBB opening on PYR toxicity. Interestingly, the results suggested that paraoxon-induced BBB leakage did not facilitate PYR entry into the brain. PYR elicited minimal signs of toxicity, but PYR-elicited toxicity was not increased by paraoxon (see Figure 55). Rather than a cumulative effect, combined pyridostigmine and paraoxon exposure resulted in lesser brain ChE inhibition than noted with paraoxon treatment alone. Relatively similar group-wise changes were noted in both frontal and temporal cortical regions. Moreover, paraoxon caused remarkable HRP leakage in the brain, but pre-exposure to PYR at least partially prevented the paraoxon-elicited BBB disruptions. One possible factor allowing PYR to block paraoxon-induced BBB

permeability might be related to the corticosteroid secretion in response to PYR exposure. Pretreatment with dexamathesone significantly reduced HRP leakage caused by paraoxon exposure (see Figure 69). Our current results confirmed several previous findings. Either a single injection or 3 daily treatments with dexamethasone significantly decreased the blood to brain transport of sucrose and alpha-aminoisobutyric acid in the brain of the rats (Ziylan et al., 1988, 1989). Dexamethasone was shown to decrease BBB permeability in rats experiencing ischemia, apparently through blocking the blood to brain transport of sodium ions (Betz and Coester, 1990). We found in our study that acute treatment with a high dosage of PYR caused remarkable elevation of corticosteroids in the plasma, and the stimulation lasted from 1 to 3 hrs after PYR challenge (See Figure 70). We propose that the increased levels of corticosteroids after PYR dosing may decrease the BBB permeability to HRP penetration as that was noted in our PYR and paraoxon interaction study. Further studies need to investigate the effects of corticosteroid antagonists on the modulation of paraoxon-induced BBB disruption by PYR. Additional studies in adrenalectomized rats would also be interesting. Data from these studies could provide additional information of the relationship between PYR, corticosteroid secretion, and BBB permeability.

Interaction of PYR and Some Pesticides Used During GW

Our results suggested pararoxon and PYR have little additive or synergistic effects on central cholinergic toxicity. However, evidence exists to support the hypothesis that interaction of PYR with other chemicals causing synergistic toxicities might develop into unexplained illnesses in soldiers deployed in the GW. Considerable attention has

been given to the interaction between PYR and pesticides used by GW veterans. These pesticides include permethrin, DEET and chlorpyrifos.

Permethrin (3-(2,2-dichloro-ethenyl)-2, 2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl) methyl ester) is a synthetic pyrethroid approved by the US EPA for use as an insecticide. Permethrin has been used to impregnate military uniforms. Permethrin affects multiple enzyme systems (e.g., monoamine oxidase A and AChE) in the brain (Rao and Rao, 1993, 1995) and elicits toxicity signs including hyperactivity, ataxia, and tremor (McDaniel and Moser, 1993). DEET (N, N-diethyl-m-toluamide), a widely-used commercial insect repellent, was used extensively by the US army during the GW. DEET may cause toxicities such as ataxia, hypotension, coma and seizures (de Garbino and Laborde, 1983; Tenenbein, 1987). Chlorpyrifos (*O, O*-diethyl *O*-3, 5, 6-trichloropyridinyl phosphorothioate), an OP pesticide with inhibitory effects on ChE activity, elicits toxicities including SLUD signs, involuntary movements, tremors, memory and cognitive problems (Kaplan et al., 1993; Zheng et al., 2000). These three pesticides were all used during the GW and therefore could have potentially interacted with PYR.

McCain and colleagues (1997) investigated acute toxicity after PYR, DEET and permethrin co-exposure. Rats were treated with two of the three compounds at LD16 dosages (additive LD32) and the 3rd compound at varied dose levels (LD0, 16, 30, 50, 70 and 84). PYR, permethrin, and DEET given concurrently caused a significantly higher lethality than the predicted. Results from this study suggested that exposure to a mixture of PYR, permethrin and DEET may lead to synergistic toxicity.

Abou-Donia and colleagues (1996a) investigated neurotoxicity after PYR, permethrin, and DEET co-exposure in chickens. Each of the compounds elicited limited toxicity alone. Binary dosing, however, induced elevated neurotoxicity relative to individual compounds. Concurrent exposure to all 3 agents further increased neurotoxicity. The mechanism postulated for these interactive effects was that agents concurrently administered compete with each other for metabolism and result in decreased breakdown, thus increasing the availability for brain uptake.

Abou-Donia and colleagues (1996b) also studied neurotoxicity after combined PYR, DEET and chlorpyrifos exposure in chickens. ChE inhibition following combined exposures was higher than that after single compound exposures. Brain neurotoxic esterase (NTE) activity was not inhibited by any single agent or PYR and DEET co-exposure, but was significantly inhibited by combined treatment with chlorpyrifos (PYR/chlorpyrifos, DEET/chlorpyrifos, and PYR/DEET/chlorpyrifos). These results suggested that PYR, DEET and chlorpyrifos co-exposure may lead to more extensive neurotoxicity.

Recently, Abou-Donia and coworkers (2001) reported the effects of PYR, DEET and permethrin on sensorimotor behavior and central cholingergic signal transduction pathways in rats. DEET or permethrin, as single treatment or co-exposure, had no effects on motor activity while PYR, single dosing or in combination with DEET and permethrin caused significant deficits in sensorimotor function. PYR and DEET concurrent exposure or in addition with permethrin significantly inhibited the ChE activity in selected brain regions. Interactive effects of PYR and sarin on sensorimotor function in rats were also reported by Abou-Donia and colleagues (2002). Sensorimotor impairments caused by

single PYR or sarin exposures were increased if rats were exposed to both agents concurrently.

Chaney and colleagues (2000) evaluated the effects of DEET on PYR-induced ChE inhibition in rats. While treatments of DEET or PYR alone had no effects on brain ChE activity, co-exposure of PYR and DEET induced significant ChE inhibition (40%) in the whole brain. Results suggested DEET potentiates PYR toxicity through facilitating PYR inhibition of brain ChE activity.

Hoy and colleagues (2000 a) studied the alteration of locomotor behavior in rats after PYR, permethrin and DEET exposures. Male and female Sprague Dawley rats were given PYR, permethrin and DEET either individually or in combination. Single chemical treatments had limited effects on locomotor behavior. Binary treatments of PYR and permethrin at half of the single-drug dosing, however, significantly affected the locomotion rate. Hoy and colleagues (2000 b) also studied changes in locomotor behavior in rats after repeated co-exposure to PYR, permethrin and DEET. Rats were given PYR, permethrin and DEET individually or concurrently (2 of 3 compounds or all 3) on a daily basis for 7 consecutive days. Binary compound treatments significantly reduced locomotor activity, e.g., repeated PYR and DEET co-exposures significantly decreased the locomotion rate in male and female rats while repeated PYR and permethrin co-exposure caused less thigmotaxis. These studies suggested an interactive effect between PYR, permethrin and DEET on locomotor behavior in rats.

Van Haaren and co-workers (2001) evaluated the effects of PYR, permethrin and DEET on operant (fixed-ratio and fixed-interval) behavior in rats. Results suggested the existence of synergistic effects on the fixed-interval response in rats concurrently

exposed to PYR and permethrin or PYR and DEET binary treatments, all at a relatively low dose.

Toxicity after PYR, permethrin and DEET exposure has been noted recently in the reproductive system (Abou-Donia et al., 2003). Rats were challenged with PYR, permethrin and DEET, either alone or concurrently for 4 weeks. Rats treated with all 3 agents exhibited changes in the reproductive system, e.g., arrested spermatogenesis, thickened interstitium, vacuolization, and edema. In situ studies demonstrated apoptosis in the germ cells and spermatocytes. These changes were increased by daily stress (restraint) in rats treated with all 3 compounds.

In general, a number of these studies suggest that PYR and the agents, e.g., DEET and permethrin, interact and could possibly contribute to the development of GWI. The mechanisms for the interactions and the extrapolation to humans remain to be addressed.

Measurements of BBB Permeability

Besides measuring and visualizing the intra-cardiac injected HRP in the brain, the classic and widely used method for evaluating BBB leakage, there are several other methods recently reported for the effective measurement of BBB permeability.

S-100β, a brain-specific protein, is 3 times more concentrated in the cerebrospinal fluid than in the plasma. This protein leaks out of the brain and increases in plasma in the early stages of BBB disruption (Kapural et al, 2002). Agents causing a compromise of BBB integrity may increase S-100β protein transport across the BBB, in the opposite direction to HRP penetration as found in our study. Therefore, measurements of plasma

S-100β following either paraoxon alone or paraoxon plus PYR co-exposure could provide more information on the BBB permeability alterations following exposure to these toxicants.

Sokrab and coworkers (1990) detected leakage of plasma protein e.g. albumin, fibrinogen and fibronectin in the brains after BBB disruption caused by a seizure-eliciting agent. Therefore measurement of plasma proteins in the brain could be employed to evaluate BBB integrity.

Expression of endothelial barrier antigen (EBA) or blood-brain barrier protein in the cerebral endothelial cells has been related to the change of the BBB permeability by several research groups (Sternberger and Sternberger, 1987; Ghabriel et al., 2000; Abdel-Rahman et al., 2002). EBA is selectively expressed in brain regions with endothelial barrier but not found in areas lacking the BBB such as median eminence, pineal gland and choroids plexus (Rosenstein et al., 1992; Sternberger and Sternberger, 1987). EBA is also absent in the endothelial cells of the peripheral organs including kidney, liver, and intestine, which have no blood to organ barrier. In general, EBA appears to be a marker antigen located at the luminal membranes of endothelial cells which express blood-organ barrier. Particularly, BBB disruption was associated with decreased level of EBA in the brain vasculature (Ghabriel et al., 2000; Abdel-Rahman et al., 2002). Therefore, immunocytochemical detection of EBA in brains could be an effective method used to "map" the BBB disruption after paraoxon/PYR exposure.

Grauer and co-workers (2001) designed a novel method to estimate BBB permeability. A noninvasive but neurovirulent Sindbis virus systemically injected in mice causes viremia without CNS infection. A high virus titer was detected in the brain of

mice with BBB disruption, however. Results suggested Sindbis virus strain invasion could be utilized as a sensitive and reliable marker for BBB integrity. Computer based technologies such as CT and MRI have also been employed and reported effective in evaluation of BBB disruption (Tomkins *et al.*, 2001; Harris *et al.*, 2002).

Multiple methods utilized to evaluate BBB permeability are considered important to provide accurate and confirmatory information forleakage following stressor exposure. The current methodologies for the measurement of BBB permeability after stress, however, were not consistently effective and sensitive for all animal models. Some may even be potentially problematic. Wijsman and Shivers (1993) reported that extravasation of peripherally injected HRP occurred in the brain of the rats following acute heat stress but no leakage of plasma Evans blue albumin complex was noted in the brain, which was contrary to the findings of many others (Sharma and Dey, 1986; Sharma et al., 1991, 1992, 1995). As noted before, Ovadia and co-workers (2001) raised a critical issue in correct interpretation of BBB permeability studies. Their findings suggested that in order to accurately evaluate the BBB integrity by measuring the systemic Evans blue retained by the brain tissues after stress, sufficient perfusion time is necessary to clear out the dye retained in the lumen of the cerebral vasculature. Otherwise the apparently increased Evans blue detected might be the dye in the blood vessels in the brain and not the real dye leaked out of the blood and retained by the brain parenchyma. Thus, changes in vascular distention could bias interpretation of such marker studies.

For the study of PYR inhibition of brain ChE activity, most of the findings suggest little inhibition in the brain of the animals under normal conditions or limited inhibition after physical stress exposure. In our studies, the MTD of PYR caused less than

20% ChE inhibition in brain regions (see Table 1). The limited ChE inhibition has insignificant effects on the functions related to cholinergic signal transduction (Nostrandt et al., 1997). On the other hand, if PYR penetration occurred in a small, confined area in the brain, measurement of ChE activity in large tissues could mask effects in small regions. This could have important consequences. There is precedence for sub-regional neurotoxicity, e.g., substantia nigra affected by MPTP, striatum by manganese (Zheng, 2001). It has been demonstrated that the BBB is not uniformly resistant in different brain regions, which allows xenobiotics to penetrate into the CNS in a regional dependent manner. If the penetration of PYR also occurs in a regional-selective manner, closer examination of ChE inhibition in discreet regions may be informative.

Besides inhibitory effects on ChE activity, PYR was also shown to cause apoptosis in the CNS or reproductive system (Li et al., 2000, 2001; Abou-Donia et al., 2003). Li and co-workers (2000) reported apoptosis in the brain after repeated intra-peritoneal injection of PYR (1.85 mg/kg, every 12 hrs for 4 days), but no significant ChE inhibition was noted in brain regions including cortex, hippocampus and striatum. The results suggested PYR entrance into the brain causing little ChE inhibition may elicit severe damage to neurons. It is therefore possible other injuries in the CNS could occur after PYR exposure without significant ChE inhibition in the brain. Therefore, a sensitive and specific method for measuring the change of PYR uptake across the BBB is needed.

Summary

Physical stress elicits stressful responses as indicated by increasing corticosteroid levels in plasma. Whether physical stress enhances BBB permeability and allows PYR entry into the brain is still controversial. Our studies and most of the recent publications suggest, however, that physical stress is unable to enhance BBB penetrability or brain ChE inhibition by PYR. A number of methods have been employed to study the change of BBB permeability. Appropriate and sensitive evaluations are warranted to avoid misinterpretation.

Paraoxon can compromise BBB integrity. Paraoxon-induced BBB leakage did not facilitate PYR entry into the brain. Interestingly, PYR pre-treatment blocked systemic HRP leakage into the brain caused by paraoxon. The mechanism for these novel findings may relate to PYR's ability to elevate corticosteroid levels. Further investigations remain to be conducted.

PYR toxicity in CNS can not be ruled out as a contributing factor to the development of GWI. PYR may interact with pesticides (e.g., DEET, permethrin and chlorpyrifos) and cause synergistic toxicity, which may potentially developed into unexplained GWI. PYR may cause alternative forms of injury, e.g., apoptotic cell death, in brain with little ChE inhibition. Therefore, future studies on PYR toxicity related to the negative health effects in veterans from GW would be interaction between PYR and other environmental agents, or toxicity caused by limited PYR in sensitive brain regions.

Chapter 5

CONCLUSIONS

1. The maximum tolerated dosage of PYR is 30 mg/kg (p.o.) for 6-week old male Sprague Dawley rats.
2. PYR is a peripheral ChE inhibitor having little effects on brain ChE activity. The ED_{50} of PYR on whole blood ChE activity was about 3 mg/kg.
3. Both acute and repeated restraint stress elicited stress response in Sprague Dawley rats as measured by elevated cortisol levels in plasma.
4. Acute and repeated restraint stress had little effect on PYR-elicited cholinergic signs of toxicity and ChE inhibition in central and peripheral tissues.
5. Acute restraint stress did not facilitate the penetration of horseradish peroxidase into the brain regions following systemic administration.
6. Repeated PYR did not induce cell death in the brain.
7. Low dosage paraoxon induced BBB leakage in young Long Evans rats.
8. Paraoxon-induced BBB leakage did not increase PYR-elicited cholinergic signs of toxicity or ChE inhibition in brain regions or peripheral tissues.
9. Paraoxon-induced BBB leakage was blocked by PYR pretreatment.
10. Paraoxon-induced BBB leakage was blocked by dexamethasone pretreatment.
11. PYR exposure significantly elevated corticosteroid levels in plasma.

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VITA 2

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