

## Effects of combined, multiple stressors on pyridostigmine-induced acute toxicity in rats

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**Abstract** A number of studies have evaluated the possibility that stress-induced changes in blood–brain barrier permeability enhanced the central effects of the carbamate acetylcholinesterase inhibitor, pyridostigmine. We previously found relatively little evidence of stress-induced changes in the acute toxicity of pyridostigmine in rats using a variety of restraint, forced running and forced swimming stress conditions. In this study, we evaluated the effects of sequential pre-exposure to multiple stressors on the acute toxicity of pyridostigmine. Rats ( $n = 8$  per treatment group) were either un-stressed or stressed by restraint (60 min), forced running (60 min, 15 m/min, 6° incline) and forced swimming (15 min), and then given either vehicle (saline, 1 ml/kg, po) or pyridostigmine (30 mg/kg, po) immediately after the final stressor. Functional signs of cholinergic toxicity (involuntary movements, autonomic dysfunction) were recorded at 0.5, 1 and 2 h after dosing. Body temperature was measured both before stress and 2 h after dosing. Rats were sacrificed immediately after 2-h functional observations to collect tissues (whole blood, diaphragm, frontal cortex, hippocampus and cerebellum) for measurement of cholinesterase activity. Stressed rats treated with pyridostigmine exhibited higher lethality (2/8) compared to unstressed rats given pyridostigmine (0/8). Pyridostigmine elicited classical signs of cholinergic toxicity, but the rats that died did not show increased cholinergic signs and no

significant differences in cholinergic signs were noted between treatment groups. Cholinesterase activity was significantly inhibited in blood (47–50%) and diaphragm (80%) following pyridostigmine exposure regardless of stress conditions. Slight but significant inhibition (11–15%) of cerebellar cholinesterase activity was observed following pyridostigmine exposure, but inhibition was not influenced by stress. We conclude that while acute lethality from pyridostigmine may be increased by combined, multiple stressors, increased lethality does not appear due to enhanced cholinergic toxicity or via increased cholinesterase inhibition in either central or peripheral tissues.

**Keywords** Cholinesterase inhibition · Blood–brain barrier · Restraint · Treadmill · Forced swimming · Functional toxicity

### Introduction

Organophosphorus (OP) nerve agents, e.g., sarin, continue to be of concern for use in chemical warfare. OP toxicants inhibit the enzyme acetylcholinesterase (AChE, EC 3.1.1.7) to elicit acute toxicity (for review, see Pope et al. 2005). Significant exposure to an OP agent is associated with impaired breakdown of synaptic acetylcholine (ACh), leading to accumulation of acetylcholine at nerve endings in the central and peripheral nervous systems. This excess ACh causes an undue stimulation of cholinergic receptors on post-synaptic terminals of neurons, myocytes or autonomic end-organs, ultimately leading to signs of cholinergic toxicity. Exposure to an OP agent can lead to devastating adverse health consequences including seizures and

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convulsions, neuropathology, respiratory depression, cardiac arrhythmias and others. Thus, strategies to protect soldiers from OP nerve agents are critical when a threat to exposure exists.

Pyridostigmine is a short acting, “reversible” inhibitor of cholinesterases. This carbamate anticholinesterase has been routinely used in the therapy of the autoimmune disorder, myasthenia gravis (LoVecchio and Jacobson 1997). Based partially on its long-term record of safety (Taylor 2001) as well as experimental studies indicating it could protect against toxicity of OP agents (Leadbeater et al. 1985; Husain et al. 1993; Tuovinen et al. 1999), pyridostigmine was used as a prophylactic drug in soldiers during the Persian Gulf War in the early 1990s (Layish et al. 2005). Essentially, this prophylactic strategy relied on the relatively short-term covalent modification of the active site serine of AChE by pyridostigmine such that those enzyme molecules were masked from binding by an OP toxicant if a subsequent exposure occurred. If exposure to an OP agent occurred after taking pyridostigmine, treatment with an antimuscarinic (e.g., atropine), an AChE enzyme reactivator (e.g., pralidoxime) and an anticonvulsant (e.g., diazepam) could limit expression of toxicity. Because of the high reactivity and typically rapid clearance of the nerve agents, the temporarily inhibited enzymes could spontaneously reactivate within a few hours and avoid long-term inactivation by the nerve agent. Without pyridostigmine, even though antidotal therapy could alleviate signs of cholinergic toxicity, biochemical recovery of those phosphorylated AChE molecules would be greatly protracted, necessitating more prolonged medical care.

Soldiers returning from the Persian Gulf War complained of a variety of health problems including headaches, joint and muscle pain, fatigue, respiratory difficulties, cognitive problems, sleep disorders, gastrointestinal symptoms and others which were collectively referred to as Gulf war illnesses (GWI) (Goldstein et al. 1996; Jamal et al. 1996; Haley et al. 1997; Haley and Kurt 1997; Landrigan 1997). A variety of possible causes for these illnesses have been postulated including physical stress, vaccinations, inhalation of smoke from burning oil wells, exposures to pesticides and chemical warfare agents, and pyridostigmine (Abou-Donia et al. 1996; Hanin 1996; Nisenbaum et al. 2000).

Pyridostigmine was intentionally given to military personnel to protect against nerve agent intoxication. Many of the unexplained illnesses reported by returning soldiers appeared to be mediated by the central nervous system whereas pyridostigmine crosses the blood–brain barrier with great difficulty, arguing against a role for this drug in the etiology of these symptoms.

However, Friedman et al. (1996) reported that forced swimming stress could markedly enhance the ability of pyridostigmine to inhibit brain acetylcholinesterase activity in mice, and that other indicators of central nervous system activation (e.g., cFos increases) were also amplified by swim stress. A number of laboratories have since evaluated the possible influence of a variety of stress conditions on pyridostigmine toxicity (Lallement et al. 1998; Grauer et al. 2000; Sinton et al. 2000; Somani et al. 2000; Abou-Donia et al. 2003; Abdel-Rahman et al. 2004). In our laboratory, we found relatively little effect of restraint stress (Song et al. 2002), forced running stress (Tian et al. 2002; Shaikh et al. 2003), or forced swimming stress (Tian et al. 2002) on the acute or subacute toxicity of pyridostigmine.

We hypothesized that multiple, combined stressors would disrupt blood–brain barrier integrity and lead to inhibition of brain cholinesterase activity and increased toxicity of pyridostigmine. Using stress models previously implemented in our laboratory (Song et al. 2002; Tian et al. 2002), we evaluated the effects of combined restraint, forced running and forced swimming stress in sequence on acute pyridostigmine toxicity in male rats. While stressed rats receiving pyridostigmine exhibited increased lethality, there was little evidence of increased entry of pyridostigmine into the CNS, alteration of cholinesterase inhibition or enhanced cholinergic signs of toxicity due to combined stress exposures.

## Materials and methods

### Chemicals

Pyridostigmine bromide (3-dimethylaminocarbonyloxy-*N*-methyl-pyridinium bromide, >99% purity) and acetylcholine iodide were purchased from Sigma Chemical Company (St. Louis, MO, USA). Radioactive [<sup>3</sup>H]acetylcholine iodide (specific activity 82.0 mCi/mmol) was purchased from Perkin Elmer, Boston, MA, USA. All other chemicals were reagent grade.

### Animals and treatments

Male Sprague–Dawley rats (6 weeks of age) were used in the present studies. Rats were obtained at 5 weeks of age and placed in individual plastic cages 1 week before initiating treatments. Each rat was handled daily for a few minutes by removal from the home cage and partial insertion of a gavage feeding tube. Rats were maintained on a commercial laboratory diet (PMI® Laboratory Rodent Diet 5001, PMI Feeds,

Richmond, IN, USA) and water was provided ad libitum. Rats were housed in a temperature controlled room ( $23^{\circ}\text{C} \pm 1^{\circ}\text{C}$ ) with a 12 h light:12 h dark cycle. Pyridostigmine was prepared freshly in normal saline (0.9% NaCl) on the day of dosing. We previously estimated the maximum tolerated dosage of oral pyridostigmine at 30 mg/kg (Song et al. 2002) and this dosage was used in the present study (1 ml/kg in saline). Control animals received only the vehicle.

### Experimental design

Rats in this study were divided into four experimental groups. Each group consisted of eight rats as follows:

- Group 1: unstressed rats given saline.
- Group 2: stressed rats given saline.
- Group 3: unstressed rats given pyridostigmine.
- Group 4: stressed rats given pyridostigmine.

For inducing stress, rats were subjected to forced running, restraint, and forced swimming essentially as described before (Song et al. 2002; Tian et al. 2002; Shaikh and Pope 2003; Shaikh et al. 2003). For forced running, rats were placed in a 4-lane treadmill (OmniPacer Model No. LC4/R-MA, AccuScan Instruments, Columbus, USA). The speed was set at 15 m/min and the treadmill was set to an incline of  $6^{\circ}$  similar to conditions described by Gunderson et al. (1996). For restraint, rats were placed in cylindrical plexiglass tubes (Model 51336, Stoelting Research Instruments, Wood Dale, IL, USA) for 60 min. For forced swimming, rats were individually placed in water tanks (40 cm high, 45 cm diameter) containing water to a depth of 25 cm for 15 min. The water temperature was maintained at  $20 \pm 1^{\circ}\text{C}$ . Restraint stress was conducted first, followed by forced running and finally forced swimming.

Upon removal from the water, all rats were rapidly dried with an absorbent cotton towel prior to treatment with vehicle or pyridostigmine. Rats were then replaced into their home cages and were observed for functional signs of toxicity which included involuntary movements (IM), and autonomic dysfunction, i.e., excess salivation/lacrimation, urination, and diarrhea (SLUD signs) at 0.5, 1 and 2 h after dosing according to method of Moser et al. (1988) as described before (Tian et al. 2002). Observers were “blind” to treatment condition. Scores for involuntary movements were given as follows: 2 = normal quivering of vibrissae, head and limbs; 3 = mild, fine tremor typically seen in the forelimb and head; 4 = whole body tremor; 5 = myoclonic jerks and 6 = clonic convulsions. Scores for autonomic dysfunction were as follows: 1 = normal, no excessive secretions; 2 = slight, one SLUD sign or

very mild multiple signs; 3 = moderate, multiple overt SLUD signs and 4 = severe multiple, extensive SLUD signs. A thermistor probe (inserted to a depth of approximately 3 cm) connected to a tele-thermometer (Yellow Springs Instrument Co. Inc., Yellow Springs, OH, USA) was used to measure rectal body temperature. Body temperature was measured just prior to initiating stress treatments and then immediately following evaluation of functional signs of toxicity 2 h after pyridostigmine treatment.

Immediately after measuring body temperature at the 2 h time-point, each rat was decapitated and trunk blood was collected into 1.5 ml polypropylene microcentrifuge tubes containing 20  $\mu\text{l}$  of a heparin solution (10,000 units/ml). The tubes were inverted several times to ensure mixing of whole blood and anticoagulant. Brain was removed and placed on a glass sheet on ice to dissect frontal cortex, hippocampus and cerebellum essentially as described by Glowinski and Iversen (1966). The entire diaphragm was dissected, and rinsed with ice-cold saline and blotted with paper to remove blood. All tissues were frozen at  $-70^{\circ}\text{C}$  for later assay of cholinesterase activity.

### Cholinesterase assay

Cholinesterase activity was measured by the modified radiometric method of Johnson and Russell (1975) using [ $^3\text{H}$ ]acetylcholine iodide as the substrate as described before (Tian et al. 2002). As carbamate-inhibited cholinesterases can potentially reactivate within a relatively short time period (compared to organophosphorus-inhibited enzyme), tissues were individually assayed by rapid thawing, homogenization and immediate addition of substrate. Under such conditions, time-dependent reactivation of the enzyme activity is limited (Padilla and Hooper 1992). Thawed tissues were homogenized in five volumes of buffer (50 mM potassium phosphate, pH 7.0) using a Polytron PT 3000 homogenizer (Brinkman Instruments, Westbury, NY, USA) at 28,000 rpm for 20 s. Similarly, blood samples were individually thawed and diluted with five volumes of buffer immediately prior to assay. Triton X-100 (0.6%) was included in each reaction to solubilize tissue fragments. Preliminary assays determined appropriate incubation time and tissue concentration to achieve linear rates of substrate hydrolysis. Protein concentrations in the tissues were measured as described by Lowry et al. (1951), using bovine serum albumin as the standard protein. ChE activity in brain tissues and diaphragm was expressed as nanomoles/min mg protein, whereas activity in blood was expressed as nanomoles/min  $\mu\text{l}$  blood.

## Statistical analyses

Biochemical data are reported as mean  $\pm$  SEM and were analyzed using two-way ANOVA (JMP statistical package). Functional signs were reported as median  $\pm$  interquartile range (IQR) and analyzed using MANOVA (JMP statistical package). Body temperature data were analyzed using paired *t* tests. Lethality data were analyzed using LOG RANK TEST by Chi square analysis. Where appropriate, post hoc comparisons were made with Tukey–Kramer HSD test.

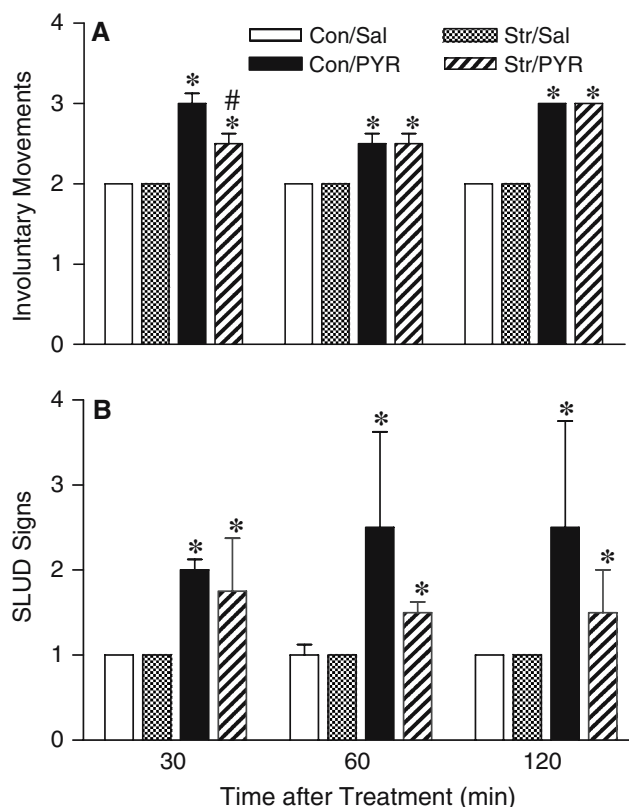
## Results

Rats exposed to all three stress conditions exhibited significantly higher lethality than unstressed rats following pyridostigmine dosing (lethality: unstressed rats, 0/8; stressed rats, 2/8). Both rats that died did so between the 1 and 2 h observation period. Figure 1 shows the effects of multiple, combined stressors and pyridostigmine on (a) involuntary movements and (b) SLUD signs. While pyridostigmine elicited signs of toxicity, stress did not increase functional signs of toxicity. There was actually a significantly lower involuntary movement score in stressed rats given pyridostigmine at 1 h after treatment compared to unstressed, pyridostigmine-treated rats (Fig. 1a). Table 1 shows the effects of stress and pyridostigmine on body temperature. There was a significant reduction in body temperature 2 h after pyridostigmine exposure in both unstressed and stressed rats.

Figure 2 shows ChE activity in diaphragm and blood in the four treatment groups. There was marked inhibition of ChE activity both in blood (47–51%) and diaphragm (80%) in pyridostigmine treated animals, but no statistical differences were noted between unstressed and stressed animals. Table 2 shows ChE activities in the selected brain regions. A slight but significant inhibition of cerebellar cholinesterase activity (11–15%) was noted following pyridostigmine exposure, but stress had no apparent influence on the degree of inhibition. No significant differences in cholinesterase activity were noted in other brain regions.

## Discussion

Previous work in our laboratory demonstrated that each of the individual stressors used in this study markedly increased rat plasma corticosterone levels, a biochemical indicator of stress, but had relatively little effect on acute or subacute pyridostigmine toxicity



**Fig. 1** Effects of combined multiple stressors and pyridostigmine on functional signs of cholinergic toxicity. Rats were unstressed (Con) or subjected to sequential restraint, forced running and forced swimming stress (Str) as described in [Materials and methods](#) and then treated with either vehicle (Sal) or pyridostigmine (PYR, 30 mg/kg, po). **a** Involuntary movements and **b** SLUD signs were recorded at 30, 60 and 120 min after dosing as described in [Materials and methods](#). Data are reported as median  $\pm$  IQR. An asterisk indicates a significant difference compared to Con/Sal group. A pound sign indicates a significant difference between unstressed and stressed rats treated with pyridostigmine (i.e., Con/PYR and Str/PYR groups)

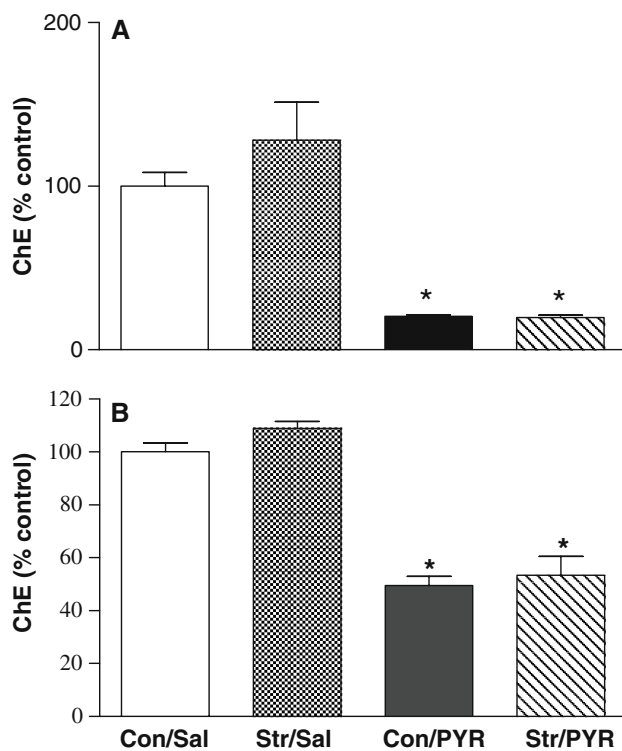
**Table 1** Effects of pyridostigmine, with or without multiple stressors, on core body temperature

Treatment <sup>a</sup>	Pre-exposure <sup>b</sup>	Post-exposure
Con/Sal	38.0 $\pm$ 0.2	38.3 $\pm$ 0.1
Str/Sal	37.9 $\pm$ 0.2	37.9 $\pm$ 0.2
Con/PYR	38.3 $\pm$ 0.1	36.5 $\pm$ 0.2*
Str/PYR	38.3 $\pm$ 0.3	36.9 $\pm$ 0.3*

\*Indicates a significant difference relative to pre-exposure values

<sup>a</sup> Rats ( $n = 4$  per treatment group) were either unstressed (Con) or stressed (Str) by sequential restraint, forced running and forced swimming as described in [Materials and methods](#) and then treated with either saline (Sal) or pyridostigmine (PYR, 30 mg/kg)

<sup>b</sup> Rectal body temperature was measured prior to initiating stress conditions (pre-exposure) and then just prior to sacrifice (post-exposure) using a thermistor probe and tele-thermometer



**Fig. 2** Effects of multiple stressors and pyridostigmine on cholinesterase activity in diaphragm and whole blood. Rats were treated as in Fig. 1 and sacrificed 2 h after treatment for measurement of ChE activity in **a** diaphragm and **b** whole blood. Data are reported as mean percent of control values  $\pm$  SEM. Statistical analyses were conducted on raw data. Control values in diaphragm and blood were  $12.1 \pm 1.0$  nmol/min mg protein and  $1.71 \pm 0.05$  nmol/min  $\mu$ l, respectively. An asterisk indicates a significant difference compared to Con/Sal group

**Table 2** Effects of multiple stressors on pyridostigmine-induced ChE inhibition in selected brain regions

Treatment <sup>a</sup>	Frontal cortex <sup>b</sup>	Cerebellum	Hippocampus
Con/Sal	100.0 $\pm$ 21.0	100.0 $\pm$ 2.0	100.0 $\pm$ 3.5
Str/Sal	113.5 $\pm$ 11.1	104.8 $\pm$ 3.6	100.8 $\pm$ 5.3
Con/PYR	111.7 $\pm$ 8.3	89.6 $\pm$ 3.6*	91.0 $\pm$ 4.1
Str/PYR	110.0 $\pm$ 15.8	85.8 $\pm$ 6.3* <sup>#</sup>	92.6 $\pm$ 5.7

\*Indicates a significant difference relative to Con/Sal

<sup>#</sup>Indicates a significant difference relative to Str/Sal group

<sup>a</sup>Rats ( $n = 8$  per treatment group) were either unstressed (Con) or stressed (Str) by sequential restraint, forced running and forced swimming as described in [Materials and methods](#) and then treated with either saline (Sal) or pyridostigmine (PYR, 30 mg/kg)

<sup>b</sup>Tissues were collected two hours after PYR treatment for measurement of ChE activity. Percent of control was calculated on the basis of values in the Con/Sal group. Control values in frontal cortex, cerebellum and hippocampus were  $95.5 \pm 20.1$ ,  $44.9 \pm 0.9$ ,  $67.7 \pm 2.4$  nmol/min mg protein, respectively

(Tian et al. 2002; Song et al. 2002; Shaikh and Pope 2003). We therefore hypothesized that exposure to combined stressors may be required to increase acute

sensitivity to pyridostigmine. While acute lethality was indeed increased by exposure to all three stressors, there was no increase in the expression of classical signs of cholinergic toxicity between rats stressed or not stressed prior to administration of pyridostigmine. Moreover, while marked reductions in ChE activity were noted in both blood and diaphragm following pyridostigmine exposure, and a slight but significant reduction was seen in cerebellum, stress had no apparent effect on the degree of inhibition in any tissue.

There were significant reductions in core body temperature in both unstressed and stressed rats 2 h after pyridostigmine exposure (Table 1), but no significant differences between those two treatment groups. Rowsey et al. (2001) reported that pyridostigmine (0.1 mg/kg, ip) had relatively little effect on body temperature in nonstressed rats, but that stress-induced (open field) hyperthermia was actually increased by pyridostigmine. We previously reported (Tian et al. 2002) that pyridostigmine (30 mg/kg, po) caused a slight but significant reduction in core body temperature in unstressed rats. Differences in the methods used between these studies (e.g., telemetry vs. thermistor probe, dosage/route of administration of pyridostigmine) may be responsible for the observed differences in changes in body temperature following pyridostigmine. It appears, however, that the peripheral actions of pyridostigmine may contribute to modification of core body temperature.

We conclude that a variety of stressors, either alone or combined, have relatively little effect on acute cholinergic toxicity following oral pyridostigmine exposure in rats. A number of other laboratories have made similar conclusions using mice, rats and guinea pigs exposed to various stressful conditions (Lallement et al. 1998; Grauer et al. 2000; Sinton et al. 2000; Ovadia et al. 2001). Possible increased central cholinergic effects of pyridostigmine under stressful conditions therefore appear of little concern for the continued use of pyridostigmine as a prophylactic agent during wartime conditions. While a number of reports indicate that various physical stressors can increase BBB permeability, many studies have failed to detect an effect of various stress conditions on the ability of pyridostigmine to penetrate the brain following oral administration. In fact, Sinton et al. (2000) reported that stress actually reduced the central effects of pyridostigmine, using brain AChE inhibition as a marker.

Using a variety of stress conditions, we have found little evidence of modulation of pyridostigmine-induced cholinergic toxicity or (based on indirectly brain cholinesterase inhibition) increased entry of pyridostigmine into the brain by stress (Tian et al. 2002;

Song et al. 2002; Shaikh and Pope 2003; data herein). It should be noted, however, that pyridostigmine-induced inhibition of acetylcholinesterase in the peripheral nervous system could indirectly alter central nervous system function. Taysse et al. (2005) reported that pyridostigmine (0.2 mg/kg twice daily for 5 days, sc) significantly increased stress (immobilization and unescapable foot shock)-induced elevations of 5-HIAA, dopamine and c-fos expression in different brain regions of mice. Wang et al. (2005) recently reported that pyridostigmine further increased and prolonged the activation of brain mitogen activated protein-kinase kinase 4 (MKK4) and c-Jun-N-terminal kinase (JNK) in mice elicited by forced swimming stress (three sequential 10-min sessions). Interestingly, pyridostigmine alone elevated brain regional MKK4 and JNK levels in this same study (Wang et al. 2005). Servatius and Beck (2005) reported that peripheral actions of acetylcholinesterase inhibitors may mediate central changes via classical conditioning. It has been known for decades that some stress-related hormones and/or metabolites can efficiently pass the blood-brain barrier and interact with specific receptors to modify neural function (de Kloet 1984; Sapolsky et al. 1986). Corticotropin releasing factor (CRF) may be pivotally involved in stress related changes in nervous system function (Dallman et al. 2004; Bale 2005). Lowry and Moore (2006) reported that CRF was an important mediator of stress-induced neuroendocrine, autonomic and neurobehavioral responses. Stress-induced changes in CRF release may modify central neurotransmission through a variety of mechanisms (Gulpinar and Yegen 2004; Lodge and Grace 2005; de Groote et al. 2005; Keeney et al. 2006). Thus, there remains the possibility that stress and pyridostigmine exposure may interact to alter central nervous system function through indirect mechanisms.

In conclusion, our studies suggest that combined, multiple stressors can increase lethality following acute pyridostigmine exposure in rats. While increased lethality was noted, combined stressors did not enhance signs of cholinergic toxicity or pyridostigmine-induced ChE inhibition. These results, along with a number of studies from other laboratories, suggest that even severe physical stress has relatively little effect on pyridostigmine-induced cholinergic toxicity.

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