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The combined effects of pyridostigmine and chronic stress on brain cortical and blood acetylcholinesterase, corticosterone, prolactin and alternation performance in rats☆

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Abstract

Thousands of soldiers who served in the Gulf War have symptoms that have been collectively termed Gulf War Illness (GWI). It has been suggested that a combination of operational stress and pyridostigmine, a drug given as a pretreatment to protect soldiers against the effects of exposure to nerve agents, might have had unexpected adverse health effects causing these symptoms. Our laboratory has previously modeled operational stress in rats using a paradigm of around-the-clock intermittent signalled footshock. In the present studies, this model was used to investigate the potential synergistic effects of chronic stress and pyridostigmine on physiology and behavior. Seventy-two rats were trained to perform an alternation leverpressing task to earn their entire daily food intake. The rats were then implanted with osmotic minipumps containing vehicle, pyridostigmine (25 mg/ml pyridostigmine bromide) or physostigmine (20 mg/ml eserine hemisulfate). The pumps delivered 1 μl/h, which resulted in a cumulative dosing of approximately 1.5 mg/kg/day of pyridostigmine or 1.2 mg/kg/day of physostigmine, equimolar doses of the two drugs. The rats were then returned to their home cages where performance continued to be measured 24 h/day. After 4 days, 24 of the 72 rats were trained to escape signalled footshock (avoidance-escape group) and 24 other rats (yoked-stressed group) were each paired to a rat in the avoidance-escape group. The remaining 24 rats were not subjected to footshock (unstressed group). Shock trials were intermittently presented in the home cage 24 h/day for 3 days, while alternation performance continued to be measured. Since only 12 test cages were available, each condition was repeated to achieve a final *n* of six rats per group. Pyridostigmine and physostigmine each decreased blood acetylcholinesterase levels by approximately 50%. Physostigmine also decreased brain cortical acetylcholinesterase levels by approximately 50%, while pyridostigmine had no effect on cortical acetylcholinesterase activity. Alternation performance was impaired on the first day of stress and then recovered. Neither pyridostigmine nor physostigmine affected performance in the absence of stress or increased the effects of stress alone. Corticosterone was significantly increased in the yoked stress group compared to unstressed controls. These data suggest that pyridostigmine does not exacerbate the effects of stress on performance or levels of stress hormones. Furthermore, these data do not suggest that stress enables pyridostigmine to cross the blood brain barrier.

Keywords: Pyridostigmine; Physostigmine; Chronic stress; Gulf War Illness; Blood brain barrier; Corticosterone; Prolactin

1. Introduction

Following the Persian Gulf War and continuing to the present time, soldiers and veterans have complained of ailments that are collectively termed Gulf War Illness (GWI) (Institute of Medicine and Health Consequences of Service During the Persian Gulf War, 1995; Landrigan, 1997; Nisenbaum et al., 2000). Among these symptoms are unexplained fatigue, memory loss, joint pain and reproductive difficulties. It is currently unknown whether these symptoms can be ascribed to deployment and service in the

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Persian Gulf War and, if so, what factor or combination of factors present during the war might be causative. Soldiers were exposed to operational stress, the drug pyridostigmine taken prophylactically to protect against potential nerve agent attacks, insecticides, smoke from burning oil fires and possibly small amounts of chemical threat agents. Any of these factors alone or in combination might be related to GWI (Abou-Donia and Wilmarth, 1996).

Nerve agents *irreversibly* inhibit acetylcholinesterase, resulting in an overabundance of acetylcholine at synapses in the brain and periphery thereby causing convulsions and respiratory failure. Pyridostigmine, a carbamate with a charged quaternary nitrogen that does not cross the blood brain barrier, is a peripherally acting *reversible* cholinesterase inhibitor that has been shown to afford significant protection against lethality caused by exposure to chemical organophosphate nerve agents in animals (Dirnhuber et al., 1979). Since carbamates have been used clinically for many years to treat myasthenia gravis, and because pyridostigmine has been shown to offer significant protection to animals exposed to chemical nerve agents, soldiers were given a moderate dose regimen of pyridostigmine to protect them against exposure to chemical threat agents. However, the question has been raised of whether pyridostigmine administered in a combat environment may have adverse side effects not seen in a civilian patient population. Specifically, the question is whether the operational stress that is part of a soldier's environment may have altered the blood brain barrier to allow the entry of pyridostigmine into the brain.

A study published by Friedman et al. (1996) reported that pyridostigmine appeared to enter mouse brains after 10 min of forced swimming as judged by inhibition of brain cortical acetylcholinesterase activity and this report generated considerable comment and publicity as a possible mechanism for GWI, i.e., the combination of stress and pyridostigmine leading to altered brain biochemistry of an important neurotransmitter known to be involved in numerous physiological functions. It was with this background that the research reported herein was initiated.

Our laboratory has been characterizing a chronic model of stress in rats (Anderson et al., 1993, 1996; Bauman and Kant, 1992; Bauman et al., 1998; Dave et al., 2000; Kant et al., 1987, 1991, 1992, 1995, 1997). Unlike the short swim stress reported by Friedman et al., this model exposes rats to sustained stress over a period of days. The stress, intermittent footshock, is signalled and can be avoided or escaped by one of each of two paired rats. The second rat in the pair must rely on its partner to escape the shock for both of them. We have found that rats tolerate this paradigm well, avoiding or escaping over 99% of the shock trials presented. Food intake is initially reduced but generally returns to prestress levels even though the stress paradigm continues. Hormones, sleep and circadian rhythms are disrupted but the paradigm is not debilitating as judged by numerous indices. This type of sustained stress was designed to model the environmental conditions that soldiers might face when deployed, i.e., a background of underlying psychological stress engendered by constantly being in a place associated with the occurrence of intermittent aversive events. Loss of control and disrupted sleep are also part of the model. In the current research, rats living in this stressful environment were also chronically treated with pyridostigmine. The purpose of these experiments was to determine whether pyridostigmine under conditions of chronic stress had deleterious effects on physiology and behavior that would not be seen under nonstressful conditions. Since memory impairment is among the reported symptoms of GWI, we selected a delayed alternation task that depends upon working memory. We have previously shown that chronic stress impairs performance on this task (Bauman et al., 1998).

In addition to the experiments with pyridostigmine, we performed similar experiments with the carbamate physostigmine as a positive control. Lacking a quaternary charged nitrogen, physostigmine readily traverses the blood brain barrier and inhibits brain acetylcholinesterase.

2. Methods

2.1. Subjects

The subjects were male Sprague Dawley rats from Charles River. Upon arrival, rats were singly housed in the Institute vivarium in hanging cages with food and water freely available. All procedures were approved by the IACUC of the Walter Reed Army Institute of Research and adhered to all federal guidelines with respect to animal care and use. Rats weighed 388 ± 57 g (mean ± S.D.) at the time of pump implantation (see below).

2.2. Chemicals

Pyridostigmine bromide (3-dimethylaminocarbon pyridinium bromide) and eserine hemisulfate (physostigmine) were dissolved in saline immediately prior to being used in the osmotic minipumps. Saline was used as the vehicle. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.3. Experimental procedures

Preliminary experiments were conducted to determine the appropriate concentration of pyridostigmine (to achieve approximately 50% inhibition of blood acetylcholinesterase) to place in the Alzet osmotic minipumps (model 2001, delivers 1 μl/h from a 200-μl reservoir for 7 days). Several rats were implanted as described below and the animals were sacrificed after 7 days to determine blood acetylcholinesterase levels (data not shown). Based on these studies, a concentration of 25 mg/ml pyridostigmine bromide was selected. In the first three runs, pyridostigmine or vehicle was administered via minipumps. A second set of three runs

was conducted with physostigmine (see below). Physostigmine is known to cross into the brain through the blood brain barrier. To achieve equimolar concentrations of physostigmine to the dose of pyridostigmine used in the first experiment, 20 mg/ml of eserine hemisulfate (physostigmine) was used.

Two experiments, one with pyridostigmine and one with physostigmine, were conducted. The pyridostigmine experiment was performed first. Twelve operant boxes were available for these experiments. To achieve a final *n* of six for each stress and drug treatment group, three runs with 12 rats were performed for each of the drug experiments.

For each run of 12 rats, as shown in Table 1, four rats were assigned to the unstressed control group, four rats were assigned to the avoidance-escape stress group and the remaining four rats were each yoked to a rat in the avoidance-escape stress group, such that the rat in the escapable group terminated shock for both itself and its yoked partner rat by pulling the chain (details below). Thus, each paired set of rats received an equal amount of shock simultaneously, which was terminated whenever the controlling rat of the pair pulled the ceiling chain. In previous work, we have found that the yoked rat is generally more "stressed" as judged by reduction in food intake and elevation of plasma corticosterone levels (Kant et al., 1992).

As described in more specific detail below, each run began with the 12 rats being placed in standard operant boxes learning to leverpress to obtain food pellets. Houselights were on from 07:00 to 19:00 h daily. Then, rats were required to alternate lever presses to obtain food pellets. When alternate lever pressing was learned, as judged by approximately an 80% correct criterion, rats were anesthetized with halothane and implanted with the Alzet osmotic minipumps. In each run, half of each experimental group received the saline pump and half the carbamate pump (Table 1). With 12 rats in each run and a design with two drugs and three stress conditions, each run yielded an *n* of two rats treated similarly, e.g., escapable stress with pyridostigmine and each experiment (consisting of three runs) consisted of six rats in each cell.

Table 1
Experimental design

	Runs 1, 2 and 3[a]	Runs 4, 5 and 6
Stressor-none	Pump-saline	Pump-saline
Stressor-none	Pump-pyridostigmine	Pump-physostigmine
Stressor-avoidance/escape	Pump-saline	Pump-saline
Stressor-avoidance/escape	Pump-pyridostigmine	Pump-physostigmine
Stressor-yoked	Pump-saline	Pump-saline
Stressor-yoked	Pump-pyridostigmine	Pump-physostigmine

[a] There were two rats in each cell in each run. Rats were trained to leverpress alternately for food pellets prior to pump implantation. Then, 12 rats were implanted with the minipumps for each run. Four days after pump implantation, four rats were trained to avoid/escape signalled footshock, four rats were paired to the avoidance-escape group and four rats were not stressed. Three days after stress initiation, the rats were sacrificed.

After pump implantation, lever pressing performance continued to be measured around the clock for 4 days and percent correct leverpresses were calculated. This 4-day period was used to assess the effect of drug alone on performance. After 4 days, the escapable stress rats were trained to pull a ceiling chain to avoid or escape signalled footshock. This training required approximately 30 min. The rats continued to live in the operant chambers for another 3 days during which they alternately lever pressed for food and, depending upon stress group assignment were exposed around-the-clock (approximately one presentation per 5 min) to no shock, escapable or inescapable (yoked) intermittent footshock. This 3-day period was used to assess the effects of stress alone (in the saline-implanted rats) and the effects of the combination of stress and drug on performance. Nonstressed rats were evaluated over the entire 7-day period for the effect of drug alone. Specific methodological details are described below.

2.4. Alternation

As a measure of working memory, we chose a procedure that required rats to alternate leverpresses for the delivery of food pellets. A press on any one lever was immediately followed by the delivery of a food pellet if and only if the immediately preceding press had been executed on the alternate lever. Since accurate alternation requires that the rat maintain a representation of the lever last pressed, this procedure constituted a simple test of working memory.

Rats were placed in standard operant boxes. The boxes were equipped with house lights, levers and a ceiling chain. Lever presses resulted in the delivery of a 45-mg food pellet (Noyes, Formula A) delivered to a central trough. Initially, the levers were baited with peanut butter to facilitate learning to leverpress; each press on either of the two levers (one on each side of the trough) resulted in a pellet delivery. After rats had learned to earn their entire daily food ration through lever presses, rats were then required to alternately press the levers to receive consecutive pellets. A 1-s delay was imposed between activation of the alternate levers and, during this delay, the cue lights above each lever were illuminated. Consecutive presses on the same lever or presses made prior to the 1-s delay did not result in a pellet delivery. Correct and incorrect presses were recorded.

2.5. Minipump implantation

After rats had learned to leverpress on the alternation task, they were anesthetized with halothane and an Alzet minipump filled with 200 μl of 25 mg/ml pyridostigmine bromide, 20 mg/ml physostigmine hemisulfate or saline was inserted in a small subcutaneous pocket between the shoulder blades. Complete filling of the pump reservoir was confirmed by weighing the pumps before and after filling. Wound clips were used to close the incision and antibiotic ointment was applied to the incision. Rats were typically

awake and moving about within minutes after this surgery. Rats were placed back into the operant cage and continued to leverpress for their entire daily food intake. Water was available ad lib. Four days after pump implantation, the stress portion of the experiment began.

2.6. Stress

The rats assigned to the avoidance-escape stress group were shaped to pull a ceiling chain to escape scrambled footshock delivered by the experimenter. Footshock was delivered from a programmable shocker (Coulbourn Instruments, model E13-10). A ceiling chain was suspended from the center of the cage and a 5-cm diameter metal ring was attached to the end of the chain, which hung approximately 10 cm off the floor. During escape training, a strip of towel was attached to the chain ring to make it more visible and to facilitate grabbing the chain. Rats in the uncontrollable stress (yoked) group were paired to a rat in the avoidance-escape stress group during training and thereafter such that the yoked stress group rat received shocks whenever its paired partner did. After each controlling rat was trained, a procedure which generally required no more than 30 min of intermittent shock, shock delivery was subsequently controlled by a PDP11 computer programmed in SKED (Snapper and Ingliss, 1985). Shock presentation trials began with a 5-s sonalert auditory warning tone, and then 5-s each of 0.16, 0.32, 0.65, 1.3 and finally 2.6 mA of footshock. Trials could be avoided or escaped at any point in the trial sequence by the controlling rat pulling the ceiling chain. The rats in the yoked stress group had no control over shock delivery. Rats responding during the sound warning (first 5 s) did not receive any shock (avoided shock). Rats responding during the 25-s of shock did receive footshock as described above until the point in the sequence at which the ceiling chain was pulled (escaped from shock). Thus, the duration of footshock to which a rat was exposed in any specific trial could vary from 0 (if the chain was pulled during the warning) to 25 s if the rat failed to pull the chain during any of the five shock intensities. Typically, rats' responses cluster at shock levels 2, 3 and 4 (0.32, 0.65 and 1.3 mA) with only a few avoidance responses (Bauman and Kant, 1992; Kant et al., 1991). Very few rats wait until the highest shock level to pull the ceiling chain and less than 1% of trials are not avoided or escaped. Rats acquire the avoidance/escape procedure very rapidly (within hours) and performance does not change over days although there is a circadian factor in that rats respond earlier in the trial sequence during the dark hours.

Shock trials were initially presented at average intertrial intervals of 1 min. Following 35 successful escapes, the average intertrial interval was increased to 5 min. A safeguard contingency stopped shock delivery if 20 consecutive escape failures from shock trials occurred; however, this condition was never met. Leverpress and chain pull data were collected and stored by the PDP11 for daily analysis.

2.7. Tissue and blood collection

Rats were sacrificed during a trough in the rhythm of plasma corticosterone between 09:00 and 12:00 h, approximately 2–5 h after lights "on." Since shock trial frequency was approximately 1 trial/5 min and since rats were sacrificed after removal from the operant cages, transport to an adjacent room and anesthesia induction, the approximate length of time between the last shock and sacrifice was approximately 4 min (2.5 min average time since last shock trial and 1.5 min from cage to sacrifice). Rats were removed from their home cage, transported to a nearby room and placed in a chamber with halothane until they lost consciousness. Duplicate 13-μl samples of whole blood were collected from the tail of each Sprague Dawley rat using a positive displacement pipette. The blood was transferred to a 200-μl microcentrifuge tube containing 1000 mU of heparin followed by thorough mixing. The sample was frozen on dry ice and stored at −80°C until batch analyzed for cholinesterase content using the method of Feaster and Doctor (2000) and Feaster et al. (2000). The rats were then decapitated and trunk blood was collected in a beaker containing heparin (0.3 ml) and aprotinin (0.1 ml or 3 TIU) a peptidase inhibitor, and then centrifuged at 4°C for 20 min at 3000 rpm. Aliquots of plasma were stored at −40°C until assayed for corticosterone and prolactin. Brains were removed and chilled in cold saline and then cortical tissue was dissected and frozen on dry ice and stored at −70°C until assayed for acetylcholinesterase.

2.8. Blood acetylcholinesterase assay

Stock reagents ATC, PTC, DTP and buffer were prepared and stored at −20°C until needed, or stored at 4°C when in use: ATC = 30 mM acetylthiocholine prepared in 18.2 MΩ water, PTC = 30 mM propionylthiocholine prepared in 18.2 MΩ water, DTNB = 30 mM 5,5′-dithiobis(2-nitrobenzoic acid) in 50 mM sodium phosphate buffer, pH 8.00, buffer = 50 mM sodium phosphate buffer, pH 8.00. Working reagents A, P and D were prepared in buffer and stored at room temperature: A = 1.0 mM acetylthiocholine and 500 μM 5,5′-dithiobis(2-nitrobenzoic acid), P = 1.0 mM propionylthiocholine and 500 μM 5,5′-dithiobis(2-nitrobenzoic acid), D = 500 μM 5,5′-dithiobis(2-nitrobenzoic acid).

Briefly, the 13-μl blood samples were thawed and 187 μl of room temperature water was added to each sample and rapidly mixed. Ten microliters were then dispensed into each column of a 96-well microtiter plate (i.e., eight test samples were dispensed into 12 columns = 96 wells). A total of 290 μl aliquots of working reagent D was added to columns 1–3, 290 μl aliquots of working reagent A were added to columns 4–6, 290 μl aliquots of working reagent P were added to columns 7–9, and 290 μl aliquots of 18.2 MΩ water were added to columns 10–12. Next, a kinetic assay was used to extract the acetylcholinesterase activity, while, in a second experiment, an endpoint assay was used for

sample normalization. The kinetic assay consisted of monitoring the increase in the absorbance at 412 nm for columns 1–9 using a Molecular Devices SpectrMax Plus microtiter plate spectrophotometer set for a 60-s preread shaking, a 3-s shaking between reads, a 5-min collection time and a linear least squares data analysis. The endpoint assay was conducted on columns 10–12 using the following parameters (1) two wavelengths, 415 and 445 nm and (2) 5-s preread shaking. The concentration of AChE was calculated as previously described (Feaster and Doctor, 2000; Feaster et al., 2000).

2.9. Brain cortical acetylcholinesterase assay

At the time of assay for acetylcholinesterase activity, the cortex was thawed, placed in 2 vols. of ice cold 100 mM sodium phosphate buffer (pH 8.0) and disrupted via a Brinkmann Polytron at medium power for 5 s. The tissue homogenate was diluted further (1:6) and homogenized (four passes) in a Potter-Elvehjem tissue grinder using a glass vessel and a teflon pestle attached to a rotary motor. Final dilution of the tissue homogenate was made immediately prior to the initiation of the reaction. Assays for acetylcholinesterase were conducted according to a variation of the spectrophotometric methods suggested by Ellman et al. (1961) as adapted for 96-well microplates (Ved et al., 1991) with modification of tissue preparation and experimental procedures suggested by Norstrandt et al. (1993) for assay of tissue containing reversible inhibitors. The total assay volume was 200 μl and consisted of 100 mM sodium phosphate buffer (pH 8.0), with 1 mM 5,5′-dithiobis(2-nitrobenzoic acid), DTNB, used as the chromogen, the substrate, 1 mM acetylthiocholine iodide (ATCI) and homogenate prepared from 0.75 mg of cortex. The temperature of the buffer was adjusted prior to addition of the tissue so that the reaction temperature in the wells was 24 ± 1°C. The assay solution was thoroughly mixed by shaking for 1 min before commencement of absorbance readings. Absorbance at 410 nm was measured in a BIOTEK EL310 automated microplate reader at 40-s intervals for 5 min. The slope of the best fitting straight line for change in optical density versus time was considered reflective of initial enzyme activity. Assays were conducted in triplicate with a parallel set of wells containing 1 μM 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW 284c51). Acetylcholinesterase activity was defined as the amount of 1 mM ATCI hydrolyzed that was inhibited by 1 μM BW 284c51 (Marks et al., 1981). Background absorbance at 410 nm produced by tissue thiols and degradation of substrate were subtracted to determine net absorbance due to enzymatic hydrolysis of ATCI. Protein content of samples was determined by a variation of the Lowry method (Lowry et al., 1951) using bovine serum albumin as the comparative standard (study mean ± S.E.M. = 74 ± 0.9 μg/assay well).

2.10. Hormone assays

Materials for the prolactin assay were provided by the National Institutes of Health through the National Hormone and Pituitary program (NHPP). Intraassay variation was < 8% and interassay variation < 12%. Corticosterone was measured using antibody-coated tube radioimmunoassay kits (COAT-A-COUNT, Diagnostic Products, Los Angeles, CA). The procedure described by the manufacturer was modified slightly to include an overnight incubation at 4°C. Assay sensitivity was 0.2 μg/dl and coefficients of variation were < 5.7% for intraassay and < 10.8% for interassay.

3. Results

3.1. Blood acetylcholinesterase

As shown in Fig. 1, the minipumps loaded with 25 mg/kg pyridostigmine bromide reliably inhibited blood acetylcholinesterase levels by approximately 50% (top panel). The cumulative dosing is approximately 1.5 mg/kg/day pyridostigmine bromide. In the physostigmine experiment (bottom

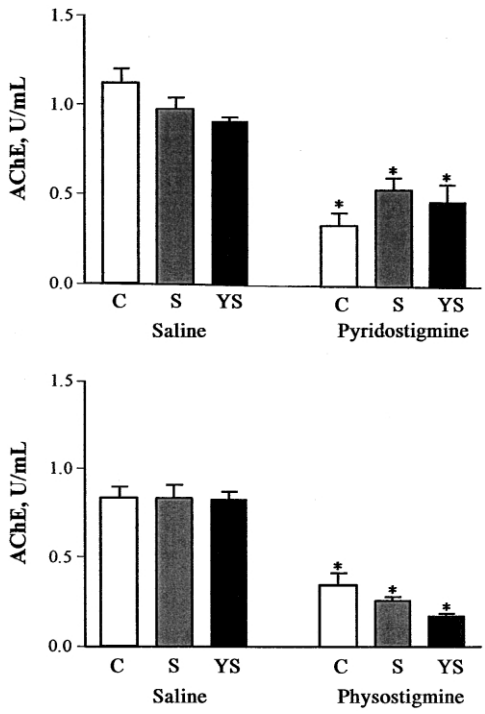

Fig 1. Effects of pyridostigmine (top) and physostigmine (bottom) on blood cholinesterase levels. Each bar represents five to seven rats (mean ±S.E.M.). The bar labeled "C" represents unstressed (control) group. The bar labeled "S" represents the avoidance-escape stressed group. The bar labeled "YS" represents the yoked-stressed group. * Significantly different from same stress group given saline. Tukey's multiple comparison test, $P < .05$. A unit of activity equals 1 μmol of 1 mM acetylthiocholine metabolized per minute. In the physostigmine study, the apparent difference between the C group mean and the Y group mean is not significant, Tukey's multiple comparison test, $P = .053$.

panel), we found similar inhibition using 20 mg/kg physostigmine in the pumps (1.2 mg/kg/day of physostigmine hemisulfate). There were no significant differences in blood acetylcholinesterase among stress treatment groups (unstressed, stressed, yoked-stressed).

3.2. Brain cortical acetylcholinesterase

Cortical acetylcholinesterase was inhibited approximately 50% by physostigmine (Table 2) but was unaffected by pyridostigmine administration. Results of a two-way ANOVA indicated a significant drug effect for physostigmine [$F(1,29) = 22$, $P < .001$] but not pyridostigmine [$F(1,28) = 0.092$, $P = .076$]. There were no significant differences in cortical acetylcholinesterase activity among stress treatment groups (unstressed, avoidance-escape, yoked-stressed) or any significant interactions among drug and stress treatment groups [pyridostigmine study $F(2,28) = 1.98$, $P = .16$ for stress treatment and $F(2,28) = 0.20$, $P = .82$ for interactions; physostigmine study $F(2,29) = 0.20$, $P = .82$ for stress treatment and $F(2,29) = 0.18$, $P = .84$ for interaction].

3.3. Plasma hormones

As shown in Table 3, corticosterone levels were markedly increased in the yoked-stress groups but not the avoidance-escape groups compared to unstressed controls. We have previously reported that, after 3 days of stress in this paradigm, corticosterone levels were highest in the yoked-stress group (Kant et al., 1992). However, corticosterone levels have been generally found to be elevated in the avoidance-escape groups compared to controls as well, albeit not as elevated as the yoked-stressed groups. Control (unstressed) levels were relatively high in the current experiments compared to previous published reports from our laboratory and may have been a factor in the lack of measurable effects in the avoidance escape group. This may have been the result of the greater time taken between removing the rat from the cage and collection of trunk blood for the hormone assays in these experiments due to the extra time required for the halothane anaesthesia and taking the tail vein samples for the blood acetylcholinesterase assays. Corticosterone levels were lower in five of the six carbamate-treated groups as compared to the saline-treated groups. This difference was significant for the pyridostigmine-treated groups [two-way ANOVA, $F(1,28) = 5.9$, $P = .021$].

As shown in Table 4, plasma prolactin, which is also a stress marker, appeared to be increased in all eight stressed groups as compared to controls. Two-way ANOVA showed no significant effects of drug treatment, but prolactin levels were significantly higher in both the pyridostigmine and physostigmine experiments for the yoked-stress groups as compared to the unstressed control groups.

3.4. Alternation performance

In the alternation procedure, a lever response was recorded as "correct" if it was made on the different lever than the previous response and "incorrect" if it was the second consecutive response on the same lever. The percent correct responses were determined as the number of correct responses divided by the total number of responses per day for each rat.

Table 2
Brain cortical acetylcholinesterase activity in saline, pyridostigmine and physostigmine-treated rats

Group	Saline	Pyridostigmine
Unstressed	100	100.8 ± 4.7
Avoidance-escape stress	95.4 ± 3.6	92.0 ± 3.6
Yoked-stress	95.7 ± 3.5	95.6 ± 3.9
	Saline	**Physostigmine[a]**
Unstressed	100	52.0 ± 13.2
Avoidance-escape stress	89.9 ± 10.3	50.7 ± 8.3
Yoked-stress	88.2 ± 16.2	52.4 ± 7.8

Values are the mean ± S.E.M. of five to six samples (cortex) expressed as percent control of the saline control animal within the same biochemical assay.

[a] Significantly different from saline treatment. The mean hydrolysis of acetylthiocholine iodide was 39.5 ± 0.9 nmol/min/mg protein for the saline animals in the pyridostigmine study and 39.8 ± 2.2 nmol/min/mg protein for the saline animals in the physostigmine study.

Table 3
Plasma corticosterone, μg/dl

Group	Saline	Pyridostigmine**
Unstressed	9.8 ± 2.8	6.2 ± 3.6
Avoidance-escape stress	8.6 ± 3.5	2.9 ± 1.1
Yoked-stress	47.6 ± 2.3*	35.0 ± 7.0*
	Saline	**Physostigmine**
Unstressed	2.3 ± 0.8	2.7 ± 0.9
Avoidance-escape stress	5.6 ± 0.5	3.7 ± 1.5
Yoked-stress	45.1 ± 4.6*	34.0 ± 6.7*

Values represent the mean ± S.E.M. of five to seven rats.

* Significantly greater than unstressed in same drug group, Dunnett's *t* test (one-sided).

** Pyridostigmine treatment significantly lower than saline (two-way ANOVA, $P < .021$).

Table 4
Plasma prolactin, ng/ml

Group	Saline	Pyridostigmine
Unstressed	5.9 ± 2.8	9.6 ± 6.2
Avoidance-escape stress	9.7 ± 2.8	11.6 ± 4.2
Yoked-stress*	18.8 ± 6.7	21.3 ± 6.5
	Saline	**Physostigmine**
Unstressed	9.0 ± 2.8	5.9 ± 3.1
Avoidance-escape stress	16.5 ± 6.7	7.6 ± 2.3
Yoked-stress*	39.7 ± 12.1	22.8 ± 10.3

Values represent the mean ± S.E.M. of five to seven rats.

* Significantly greater than unstressed group mean, Dunnett's test (one-sided).

Separate ANOVAs for percent correct were performed for the pyridostigmine and physostigmine experiments. In each analysis, data from the day before shock was introduced, and first day of shock exposure were used. Thus, each ANOVA included a repeated measure analysis for the main effect of Day as well as analyses for the main effect of Group and the interaction of Group × Day. If an F score for the Group × Day interaction was significant in either the pyridostigmine or physostigmine analyses, Tukey's test was used to evaluate all pairwise comparisons between the six groups on the day before shock and the first day of shock. Alpha was .05 for all main effects, interactions and pairwise comparisons.

For both the pyridostigmine and physostigmine analyses, the main effects of Group and Day and the Group × Day interaction were significant [Pyridostigmine, Group, $F(5,28)=2.81$, $P=.0355$; Days, $F(1,28)=30.02$, $P=.0001$; Days × Group, $F(5,28)=4.74$, $P=.0029$] [Physostigmine, Group, $F(5,28)=4.58$, $P=.0032$; Days, $F(1,28)=32.9$, $P=.0001$; Days × Group, $F(5,28)=6.16$, $P=.001$]. On the day before the introduction of footshock, there were no group differences in either the pyridostigmine or physostigmine experiments. Shock caused a decrease in percent correct performance in all stressed groups but only some decreases were statistically significant. In the pyridostigmine study, on the first day of shock, the accuracy of the yoked-stress saline group was significantly lower than that of both the unstressed saline and unstressed drug groups. No other differences were significant. In the physostigmine study, on the first day of shock, the performance of the yoked-stress drug group was significantly decreased compared to the unstressed drug groups and significantly better than the performance of the yoked-stress saline group. The accuracy of the avoidance-escape stress group in the physostigmine experiment was impaired compared to both the unstressed control and unstressed drug groups. No other comparisons were statistically significant.

The data from the unstressed and yoked-stress groups are shown in Fig. 2A–F. Graphs from the avoidance-escape groups (not shown) were similar to that of the yoked stress groups. As can be seen, surgery to implant the pumps sometimes caused a small and transient decrease in performance on the delayed alternation task. Performance was decreased in the stressed groups (Fig. 2D–F) on the first day of stress compared to nonstressed controls. However, neither pyridostigmine nor physostigmine affected performance prior to stress initiation nor did they modify the effects of stress alone on performance.

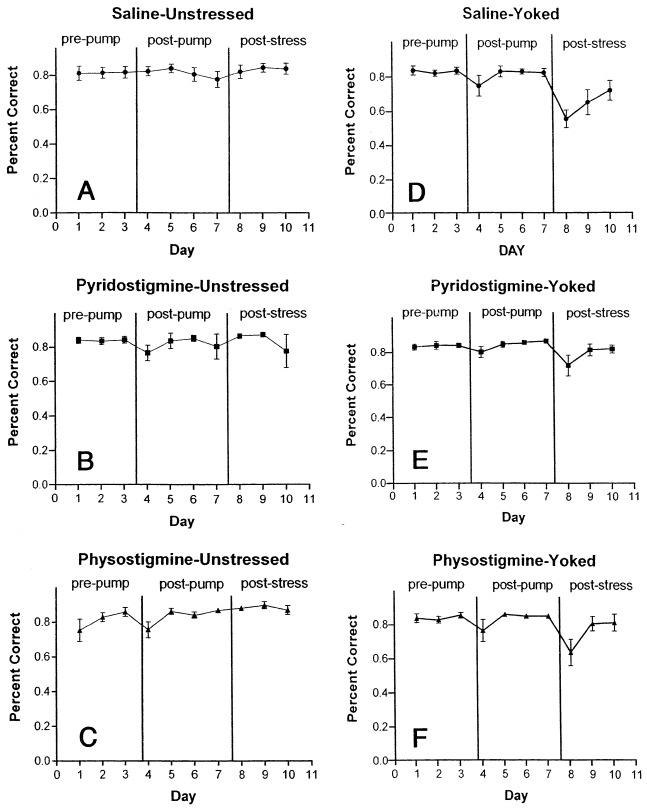

Fig 2. Effects of stress, pyridostigmine or physostigmine on alternation performance. Each graph depicts 3 days of baseline performance, followed by 4 days of alternation following pump implantation and finally, 3 days after stress initiation. The points are the mean of six rats ± S.E.M. in all graphs. (A) Saline unstressed animals, (B) pyridostigmine unstressed rats, (C) physostigmine unstressed rats, (D) saline yoked-stressed rats, (E) pyridostigmine yoked-stressed rats, (F) physostigmine yoked-stressed rats.

4. Discussion

Soldiers perform in an uncertain and dangerous environment. Military operations, especially in times of war, are inherently stressful. In addition to stress, soldiers are exposed to many specific battlefield threats. One of the dangers of modern warfare is the use of chemical weapons including organophosphates such as soman and sarin which act by irreversibly inhibiting the enzyme acetylcholinesterase. Carbamate compounds such as pyridostigmine and physostigmine offer protection against these chemical agents. They protect by reversibly binding to acetylcholinesterase and "masking" a portion of the enzyme during exposure to nerve agents. After the nerve agent has been cleared from the body, some of the protected cholinesterase is then available to maintain function. Both pyridostigmine and physostigmine pretreatment have been shown to afford considerable protection against organophosphate exposure in animals when followed by postexposure treatment with atropine and oximes (Dirnhuber et al., 1979; Harris et al., 1989; Philippens et al., 1998), the standard treatment in the field for chemical exposures. Pyridostigmine does not cross readily into the brain while physostigmine, which does not have a charged quaternary nitrogen does cross the blood brain barrier. To avoid potential performance effects of pretreatment with a compound that enters the brain, pyridostigmine was chosen as the pretreatment drug of choice. Thus, soldiers in the Gulf War were administered three 30-mg tablets of pyridostigmine daily as a pretreatment to pro-

tect against chemical attack. Since pyridostigmine and similar drugs are widely used in clinical medicine (Breyer-Pfaff et al., 1985; Furey et al., 2000; Molloy and Cape, 1989), their use was not expected to cause significant adverse side effects.

Given to soldiers as a protectant, there is now some controversy as to whether pyridostigmine may have contributed to the symptoms of GWI. It has been postulated that under the stressful conditions of war, pyridostigmine might have been able to penetrate a stress-compromised blood brain barrier (Hanin, 1996). This hypothesis has been driven primarily by a report that found that in mice forced to swim for 10 min, pyridostigmine appeared to inhibit brain cortical acetylcholinesterase activity, facilitate penetration of Evan's blue dye into mouse brain and increase brain levels of c-*fos* oncogene and acetylcholinesterase messenger RNAs (Friedman et al., 1996). The present study was initiated to model a chronic condition of both stress and pyridostigmine administration that was more similar to the conditions experienced by soldiers than a 10-min swim and acute single injection of pyridostigmine. In our study, the stress was sustained over 3 days and the pyridostigmine was administered for 7 days.

We have previously reported that rats exposed to this stress paradigm initially decrease food intake, have decreased and disrupted sleep patterning, have elevated levels of the stress hormones corticosterone and prolactin, have increased levels of mRNA for prolactin in the anterior pituitary gland, demonstrate impaired performance on delayed alternation and fixed interval tasks, have decreased thymus weight, and disrupted rhythms of body temperature (Bauman and Kant, 1992; Bauman et al., 1998; Dave et al., 2000; Kant et al., 1987, 1991, 1992, 1995, 1997). All these measures return to baseline over a 2-week period even though the stress paradigm continues. The continuous nature of the paradigm appears to facilitate habituation and adaptation, although the habituation rate varies tremendously among the parameters studied. For example, corticosterone levels remain elevated for a longer period than prolactin. Performance on the alternation and fixed interval tasks returns to baseline within a matter of 1 or 2 days (as we saw in the current experiments). Sleep and circadian rhythm patterning generally return to normal within a few days even while the stress exposure continues. Thus, the paradigm is moderately stressful but not debilitating.

In addition to differences in adaptation rate among measured parameters, we also generally see a difference between rats that have control over stressor termination (the avoidance-escape group) and rats that have no control (the yoked group). For most measures, the yoked-stress group demonstrate greater disruptions with stress as has also been reported by others using different stress paradigms (Weiss, 1972, 1988). In the current experiments, we again see greater increases in plasma corticosterone in the yoked groups, suggesting that this group is more stressed.

Both pyridostigmine and physostigmine have been reported to affect performance in animals (Bartus, 1979; Blick et al., 1994; Doty et al., 1999; Frederick et al., 1995; Liu, 1991; Servatius et al., 2000; Shurtleff et al., 1992; Van Haaren et al., 2000; Wolthuis et al., 1995), although behavioral effects have been reported more often and at lower doses with physostigmine, a drug that crosses the blood brain barrier, than with pyridostigmine that does not cross the blood brain barrier. For example, Wolthius et al. reported that 25 times more pyridostigmine than physostigmine were required to induce a significant behavioral decrement in hand–eye performance in marmosets (Wolthuis et al., 1995). Pyridostigmine, administered by gavage at a single dose of 1.5 mg/kg shortly before the behavioral testing, delayed response acquisition of a lever pressing task for food in rats (Van Haaren et al., 2000). Orally administered pyridostigmine (3–12 mg/kg) has also been reported to produce dose-dependent decreases in the rate of responding for water reinforcement under a simple light intensity discrimination task (Liu, 1991). However, not all behavioral effects are negative. Doty et al. (1999) reported that physostigmine enhanced odor discrimination performance in a dose-related manner in rats given 0.05–0.2 mg/kg physostigmine. Frederick et al. (1995) report both task improvements and decrements following physostigmine administration in Rhesus monkeys depending upon the task selected and the dose of physostigmine administered. They concluded that physostigmine at lower doses may facilitate acquisition and performance of simple tasks but high doses may impair performance due to cholinomimetic side effects such as tremor or hypothermia.

Tolerance to the behavioral effects of physostigmine and pyridostigmine despite continued blood acetylcholinesterase inhibition have been reported following chronic or repeated administration (Francesconi et al., 1986; Galbicka et al., 1989; Genovese et al., 1988). Acute doses of pyridostigmine (2 mg/kg) were found to decrease exercise performance of rats in the heat; however, oral consumption of pyridostigmine administered over a period of 7–14 days in the drinking water (20 mg/kg/day) did not significantly affect clinical chemical indices of heat/exercise injury (Francesconi et al., 1986). Galbicka et al. (1989) report tolerance to the behavioral effects of physostigmine in rats under interval schedules of positive and negative reinforcement, with tolerance developing more rapidly to some aspects of the performance than to others.

In the present experiments, we did not see performance effects of either pyridostigmine or physostigmine, although the doses administered were sufficient to inhibit blood acetylcholinesterase by approximately 50%. By administering the drugs via osmotic minipump, we administered cumulative doses of approximately 1.2–1.5 mg/kg/day. The failure to see effects of pyridostigmine or physostigmine in our experiments compared to other behaviorally reported effects may be due to the task we selected lacking sufficient sensitivity to detect a change in performance, an effectively lower dose of drug at any one point in time in our experiments compared to others because the dose was

delivered over 24 h, tolerance to the effects of these drugs due to the continuous delivery method or motivated adaptation to the drug effects because rats earned their entire food ration by performing the task. Another possibility is that the direction of performance alteration caused by carbamates on this task might not be negative. Physostigmine and other centrally active cholinesterase inhibitors at appropriate doses have been shown to improve performance in some animal models and have been considered for use in humans with memory deficits such as Alzheimer's patients (Furey et al., 2000; Molloy and Cape, 1989). The baseline performance of our task was fairly high as it was selected to detect impairments, not improvements in performance. The drugs, however, were not able to prevent the stress-induced impairment that was seen on the first day of the stress exposure.

The dose of pyridostigmine administered was sufficient to inhibit blood acetylcholinesterase by 50%. Yet, we found no additional effects of pyridostigmine beyond that which were caused by the stress itself on performance. In addition, we found no significant inhibition of brain cortical acetylcholinesterase although there was a marked decreased in blood acetylcholinesterase levels. Thus, we see no evidence that a combination of stress and pyridostigmine acts synergistically. Since we initiated our studies, three other reports have appeared using various acute stressors in mice, rats and guinea pigs (Grauer et al., 2000; Lallement et al., 1998; Sinton et al., 2000) that also fail to show that stress enables pyridostigmine to enter the brain. Sinton et al. (2000) reported no increased blood brain barrier permeability in rats subjected to forced swimming for two 10-min periods, restrained for 30 min or exposed to a combination of swimming and restraint. Lallement et al. (1998) found no penetration of pyridostigmine into the brain of guinea pigs subjected to heat stress for 2 h. Finally, Grauer et al. (2000) examined the stress, pyridostigmine, blood brain barrier interface in mice using a similar stress protocol to that utilized in the original Friedman report (short swim stress) but measuring blood brain barrier penetration not only by cholinesterase activity but also by [^{11}C]pyridostigmine penetration into brain and by Evans blue visualization. They found no stress-induced penetration of mouse brain using any of these techniques (Grauer et al., 2000).

In addition to these three reports that focused on acute stress and pyridostigmine administration, Servatius et al. (2000) tested the effects of repeated stress (1 h restraint/day) in combination with chronic pyridostigmine administration via drinking water on plasma butyrylcholinesterase and the acoustic startle response in rats. They report decreases in butyrylcholinesterase activity and an exaggerated startle response in both the stressed and the pyridostigmine-treated groups but no additive effects on these measures by a combination of both stress and pyridostigmine.

The present report extends the negative findings of the reports cited above. We found no stress-induced inhibition of brain acetylcholinesterase activity in cortex in pyridostigmine-treated animals, although blood acetylcholinesterase was inhibited by 50%. The preponderance of evidence by a number of laboratories now points to some unknown experimental artifact associated with the original positive report.

In summary, chronic pyridostigmine administration at doses that inhibit blood acetylcholinesterase by 50% does not penetrate the blood brain barrier in chronically stressed rats or affect a test of working memory in rats.

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