The Effects Of Pyridostigmine Bromide On The Hypothalamic-Pituitary-Adrenal Axis And The Acoustic Startle Response

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The Effects of Pyridostigmine Bromide on the Hypothalamic-Pituitary-Adrenal Axis and the Acoustic Startle Response

By

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Abstract

Pyridostigmine bromide (PB), a peripheral cholinesterase inhibitor, was investigated to determine if administration under non-stress conditions alters the circadian rhythm of plasma corticosterone (CORT). Research has suggested that PB may elevate plasma CORT concentrations, but the effect it may have on the circadian cycle is unknown. If PB elevates plasma CORT, a state of anxiety may be induced. In both experiments, rats were administered PB (5mg/kg) in their water bottles for seven consecutive days. In experiment 1, blood was sampled at the circadian trough and peak of plasma CORT to determine shifts in the circadian as well as to determine cholinesterase inhibition. In experiment 2, a three-intensity (82, 92, 102 dB) acoustic startle test was administered at the circadian trough and peak, mimicking blood sampling of experiment 1. In experiment 1, a steady-state cholinesterase inhibition of plasma cholinesterase levels at approximately 30% was produced. Trough levels of plasma CORT were unaffected while a slight overall elevation in plasma CORT was detected at the peak of the circadian. PB did not affect the ASR in experiment 2. It can be concluded that while PB does not alter the circadian rhythm of CORT, it does induce a slight rise in peak CORT without affecting the ASR.
Introduction

In the last decade, concerns have been raised regarding the veterans of the Persian Gulf War and their prophylactic use of pyridostigmine bromide (PB) during combat situations. After returning from the war, many veterans have indicated a range of unexplained illnesses such as fatigue, headaches, neurocognitive difficulty, myalgia, joint pain, and a multitude of other symptoms (Doebbeling, 2000) that led to classifying the sicknesses as Gulf War Syndrome. Many believe that the drug PB may play a role in the origin of this syndrome.

During the war, soldiers were under the threat of possible exposure to nerve agents that could result in immediate death. Pyridostigmine bromide was originally developed for the treatment of the degenerative muscle disease myasthenia gravis and was administered to patients at a dosing range of 200 to 1400 mg per day (Sharabi et al, 1991; Cook et al, 2002). Research found PB to be an excellent prophylaxis against nerve agent exposure with no adverse effects to the individual (Francesconi et al, 1986; Almog et al, 1991; Keeler et al, 1991). The repeated dose of 30mg three times a day did not produce any incapacitating side effects (Almog et al, 1991; Keeler et al, 1991) thus, the drug was administered to soldiers to protect them against nerve agents during the Persian Gulf War.

Nerve agents utilize the cholinergic pathways in the body to immobilize and kill the exposed individual by allowing acetylcholine (ACh) to build up at neuromuscular junctions and synaptic clefts. This excess ACh causes parasympathic
overstimulation which constricts bronchioles and contracts the muscles around the
lungs restricting breathing thereby killing the individual. Briefly, the cholinergic
pathway involves ACh, a neurotransmitter, which is released into the synaptic
cleft or neuromuscular junction by the presynaptic neuron and binds to the
nicotinic (nAChR) and muscarinic (mAChR) receptors on the postsynaptic neuron
or tissue allowing for propagation of the electrical signal. This cholinergic
discharge is relatively short in duration because of the high concentration of the
enzyme acetylcholinesterase (AChE) which hydrolyzes ACh into choline and
acetate. This mechanism allows for reuptake of choline into the presynaptic
neuron thereby clearing the synapse or junction of the excess unbound ACh,
whereupon repolarization of the neurons can occur (Ganong, 1999).

This cholinergic pathway stems from the body’s peripheral autonomic
(ANS) and somatic nervous systems (SNS) which are the motor (efferent) portion
of the nervous system. Both systems have important afferent (sensory) inputs that
provide sensation and modify motor activity. The SNS deals with consciously
controlled movement, respiration and posture (Katzung, 1998). The ANS is not
under direct conscious control and is divided into two divisions whose neurons all
originate on nuclei within the central nervous system. The two divisions,
sympathetic and parasympathetic, give rise to preganglionic efferent fibers that
exit from the brainstem or spinal cord and terminate in motor ganglia. The
sympathetic preganglionic neurons leave the CNS through thoracic and lumbar
spinal nerves with some terminating in ganglia located paravertebral and
prevertebral ganglionic chains around the spinal column. Other preganglionic neurons run to ganglia, and then into the postganglionic fibers that innervate tissues (Katzung, 1998; Ganong, 1999). This is the pathway that is known for the "fight or flight" mechanisms that arise.

The parasympathetic nervous system is the second division of the autonomic nervous system known as the "rest and digest" system. The preganglionic fibers of this division leave the CNS through cranial and sacral nerves, with some terminating in parasympathetic ganglia located outside the organs they innervate. Most terminate, however, on ganglion cells that are distributed diffusely or in networks in the walls of the innervated organs (Katzung, 1998).

The cholinergic fibers of the ANS and SNS synthesize and release ACh in a complex network of nerve, muscle, glands, and organs. All preganglionic efferent autonomic fibers and the somatic motor fiber that run to skeletal muscle are cholinergic. The ACh that is released into the neuromuscular junction will diffuse and bind to the nicotinic receptors located on the muscle tissue, thus depolarizing it and causing contraction (Ganong, 1999) All parasympathetic postganglionic and a few sympathetic fibers are cholinergic as well (Katzung, 1998; Ganong, 1999). Ach released from parasympathetic fibers bind to the muscarinic receptors on cardiac and smooth muscle, glands, and nerve terminals to induce stimulation. ACh also binds to muscarinic receptors on parasympathetic sweat glands and nicotinic receptors on the adrenal medulla (Katzung, 1998).
Nerve agents are organophosphorous AChE inhibitors. Once exposed, the organophosphates (OP) in the circulating plasma bind and irreversibly phosphorylate the enzyme AChE, preventing hydrolysis of ACh. The ACh accumulates at cholinergic receptor sites (synaptic junctions) causing parasympathetic overstimulation, which consequently results in death. Prophylactic PB, when administered peripherally, binds reversibly to AChE inhibiting about 30-40% of its activity (Abdel-Rahman et al 2002, Chaney, et al 1997). Subsequent exposure to OP’s results in competitive binding with PB for AChE, with PB blocking OP’s from irreversibly binding to the AChE molecule. Upon nerve agent exposure, atropine citrate is then administered intramuscularly with an autoinjector, increasing the likelihood that the individual will spontaneously breathe (Keeler et al, 1991). Atropine is a tertiary amine drug that is used to control muscarinic effects of the peripheral and central nervous system during cholinergic crisis (Katzung, 1998). After the threat subsides and the dosing regiment discontinues, PB will dissociate from AChE allowing the free AChE to resume normal cholinergic function. Hence death of the individual is avoided.

Pyridostigmine bromide is a dimethylcarbamate that contains a quaternary amine that does not readily cross the blood brain barrier (BBB). Because of its structure, the drug should remain peripheral and damage to the CNS can be avoided. The dosing regimen was 30mg/po every 8 hours for 3 to 7 days when there was high threat of exposure to nerve agents (Keeler, 1990). After the regimen stopped, AChE levels returned to normal 12 hours later and the side
effects of this drug were shown to be minimal. Overstimulation by PB caused transient, peripheral side effects in some troops such as nausea, diarrhea, flatus, frequent urination, headaches and bradycardia, but these symptoms did not alter military performance (Keeler, 1990; Keeler et al, 1991).

Recent research has shown that the side effects of PB may not be so transient. Studies involving the neuromuscular junction, skeletal muscle, and diaphragm have demonstrated that PB may affect acetylcholinesterase activity after the treatment has stopped. Mice that were treated acutely with PB (4.0 or 2.0 μmoles/kg) showed an increase in skeletal muscle AChE activity that was higher than control levels days after treatment had ended (Lintern et al, 1997a) and variations in enzyme activity were seen in the diaphragms of chronically treated guinea pigs that had 40-50% inhibition for days after treatment ended (Lintern et al, 2001). Pretreatment for three weeks with PB (0.4 μmoles/kg twice daily) showed a sensitization of the muscle (after normal AChE activity returned) to a later exposure to the drug (Lintern et al, 1997b). Neuromuscular junctions from diaphragm and skeletal muscle of rats were found to have morphological changes after exposure to an acute (0.36 mg/kg s.c.) dose of PB and a subacute 2-day (10 mg/ml by osmotic pump) dose that inhibited AChE by 60-70% (Hudson et al, 1985). These studies suggest that prolonged use of PB or PB overstimulation can damage peripheral tissues causing impaired neuromuscular transmission.

During the Gulf War the troops were exposed to a combination of biological, psychological, chemical, and stressful environments (Abdel-Rahman et
that may have altered the effect of PB on the peripheral and central nervous systems. Much research focuses on PB administration and exposure to a combination of other variables that may induce the negative effects of PB by allowing PB to cross the blood brain barrier (BBB). One of the variables is stress, which has been cited as a potential factor that increases BBB permeability (Friedman et al, 1996; Abdel-Rahmen et al, 2002; Beck et al, 2003). In 1996, Friedman et al. found that exposure to swim stress inhibits brain cholinesterase in mice treated with PB (Friedman et al, 1996). Coexposure of PB and other chemicals such as DEET, permethrin, and sarin (a nerve agent) cause significant levels of brain cholinesterase inhibition in rats (Chaney et al, 2000; Abou-Donia et al, 2001, 2002), but PB or DEET alone did not cause brain inhibition (Chaney et al, 2000).

In contrast, other research has suggested that PB does not cross the blood brain barrier when exposed to stress. These studies show that even in high doses that cause blood cholinesterase inhibition, brain AChE activity was not affected (Kant et al, 2001). Moreover, Scremin et al (2003) found that PB either alone or in conjunction with sarin did not affect brain AChE. Heat stress was also utilized in guinea pigs to test the permeability of the BBB to PB, but no brain AChE inhibition was found (Lallement et al, 1998). Furthermore, forced running, forced swimming, and restraint stress did not alter brain regional cholinesterase inhibition in rats (Song, et al, 2002; Tian et al, 2002) a finding that contradicts the earlier Friedman et al (1996) mouse study.
During stressful situations, be it chemical, physiological, or psychological, the body undergoes a hormonal response that allows the individual to cope. Briefly, stress activates the hypothalamic-pituitary-adrenal axis (HPAA), by releasing corticotrophin-releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus (fig. 1). CRH travels to the pituitary via the portal hypophysial vessels where it triggers the pituitary into releasing adrenocorticotropic hormone (ACTH). ACTH travels through the circulation to the adrenal cortex where cortisol in humans, or corticosterone (CORT) in rats, is released into the circulating plasma (Ganong, 1999). The cortisol released from the adrenal allows the individual to survive a stressful event as well as return the body back to its homeostatic state once the stress subsides.

Figure 1: The hypothalomo-pituitary-adrenal axis activated by an arbitrary stressor
The HPAA also has a circadian rhythm, which is generally marked by the 24 hour neural and endocrine fluctuations in hormones, for example, CORT in rats. The biological rhythms of rodents are synchronized to the cycling of light and dark environments, with the dark cycle being the peak activity, feeding and drinking period. Plasma glucocorticoids, such as CORT, peak near the onset of darkness which is the major activity period of the day (Moldow and Fischman, 1984). From morning to afternoon, rat CRH mRNA expression and CRH concentrations rise, then fall rapidly near the dark phase (Moldow and Fishman, 1984; Retana-Marquez et al, 2003), a phenomenon directly related to the circadian fluctuations of corticosterone.

The circadian rhythm is controlled by the suprachiasmatic nucleus (SCN), or the biological clock, of the hypothalamus. This pacemaker is programmed by the light/dark (L:D) cycles when the SCN receives input from the retino-hypothalamic fibers that pass directly from the optic chiasm. Neurons of the hypothalamus discharge rhythmically in response to the L:D cycle secreting ACTH in bursts causing plasma CORT to fluctuate (Ganong, 1999). In rats, these bursts occur more frequently in the evening and less frequent during the day, opposite of that seen in man.

Stress can alter the circadian rhythm of rats by affecting the circulating levels of CORT. Restraint stress, footshock, inescapable tailshock, cold water immersion, restricted feeding or watering, and light/dark alterations all cause elevations in basal CORT or alterations of peak CORT (Ottenweller et al, 1987;
Moldow and Fischman, 1987; Fischman et al, 1988; Pitman et al, 1988; Retana-Marquez et al, 2003). Stress such as inescapable tailshock has been shown to increase basal trough levels in plasma CORT 3-5 days post stress, thus altering the normal AM circadian fluctuation of CORT (Ottenweller et al, 1994; Servatius et al, 1994). Repeated timed stress has been shown to advance the acrophase of the peak CORT levels by 1.7 hours, while restricted feeding and watering produces anticipatory peaks in corticosterone that alter the circadian rhythm (Ottenweller et al, 1987).

The cholinergic system has been investigated with respect to its stimulatory effect on the HPAA. In early studies, Naumenko (1967) found that administering anticholinesterases in guinea pigs caused an increase in glucocorticoid levels. Phystostigmine, another cholinesterase inhibitor with nicotinic and muscarinic cholinomimetic properties, was found to increase plasma cortisol and corticosterone levels in both humans and rats respectively, as well as increase the levels of β-endorphin immunoreactivity (Risch et al, 1980). This effect was later found to be a result of co-release of ACTH and β-endorphin from the anterior pituitary (Risch et al, 1983). Another research group confirmed that acetylcholine stimulates hypothalamic CRH via muscarinic receptors by demonstrating that the mAChR agonist, arecoline, was found to stimulate the HPAA through muscarinic receptor mechanisms, producing a rise in corticosterone in rats (Calogero et al, 1988, 1989).
Much research focuses on the cholinergic interactions of the hypothalamus.

Cholinergic stimulation of nAChR and mAChR located in the PVN of the hypothalamus has been investigated as to how activation of the HPAA might occur. ACh has been shown to activate the HPAA by stimulating the release of CRH in vitro studies (Hillhouse et al., 1975) while hypothalamic CRH mRNA levels and plasma ACTH levels increased after ACh was microinjected into the PVN of rats (Ohmori et al., 1995). Choline, a precursor to acetylcholine known to enhance ACh release in vitro and in vivo, was administered intracerebroventricularly (i.c.v.) and found to increase plasma ACTH along with β-endorphin. This effect was blocked when choline was administered after a pretreatment of mecamylamine (nAChR antagonist) thereby suggesting that activation occurs through central nAChRs (Savci et al., 1996).

Immunoreactive CRH in the hypothalamus increased after exposure to ACh (Calogero et al., 1988) while arecoline (mAChR agonist) increased the release of CRH in vitro (Calogero et al., 1989; Wei et al., 2002). Arecoline, however, did not induce the secretion of CORT in vitro, but in vivo an increase in CORT was elicited (Calogero et al., 1989). This was probably due to stimulation of the PVN which subsequently activated the HPAA (see Fig. 2).

The direct effects of cholinergic agents on the pituitary gland in mammals have not been studied in great detail. Most research in vivo involves multiple pathways or indirect stimulation of the pituitary that is very complex and often conflicting. However, research has been performed using arecoline, nicotine, and
PB. Arecoline was found to have a stimulatory effect in rats by increasing ACTH release, an effect that was blocked with atropine (mAChR antagonist) administration (Calogero et al, 1989). Conversely, the same study found that arecoline could not elicit ACTH release from pituitary cell cultures which suggests that the pituitary secretion of ACTH was actually triggered by CRH release from the hypothalamus.

Nicotine, which acts on nAChR much like ACh, has also been shown to activate the HPAA. Nicotine delivered i.c.v. (200 and 500 nmol/animal) increased plasma CORT in a dose-dependant manner (Okada et al, 2003). Nicotine (2.5-50 μg/kg) was also found to stimulate ACTH release in vivo when delivered IV. Conversely, the nAChR agonist cytosine (35-100 μg/kg IV), which does not cross the BBB, failed to elicit an ACTH response (Romano et al, 1981). Nicotine was researched to determine if anterior pituitary cell cultures could be stimulated. The co-secreted pro-opiomelanocortin (POMC), β-endorphin, was measured because ACTH is unstable under the experimental conditions (Matta et al, 1997). Nicotine was found to have no effect on the corticotropes, thus suggesting that nicotine must act within the brain to stimulate ACTH release.

PB, under normal circumstances, does not cross the BBB, so cholinergic studies involving the pituitary may be performed (see Fig. 2). PB was found to stimulate the secretion of ACTH and cortisol as well as potentiating some of the normal secretory functions of the human pituitary gland. Murialdo et al (1993) demonstrated this in humans who received PB (120 mg) alone and in combination
with CRH. They found that PB alone elicited higher ACTH and cortisol responses than control and that PB potentiated the effects of CRH (Murialdo et al, 1993). The same effect was found on GH when PB in combination with growth hormone-releasing hormone (GHRH) was administered to patients (Corsello et al, 1992; Murialdo et al, 1993).

At the adrenal level, ACh stimulates the adrenal medulla into secreting catecholamines (norepinephrine and epinephrine). Epinephrine is a vasoconstrictor and cardiac stimulant that also activates β2 receptors in some vessels causing dilation. Norepinephrine has similar functions as epinephrine without the β2 interaction and increases both diastolic and systolic blood pressure (Katzung, 1998). Catecholamine secretion is controlled by the CNS through the sympathetic splanchnic nerves that make synaptic connections with nACHR in the chromaffin cells of the adrenal medulla (Akiyama et al, 2003). mACHRs are located extra-synaptically of the chromaffin cells. AChE located at the synapses quickly terminates the actions of ACh before reaching the mACHRs, thus if an anticholinesterase is administered, ACh accumulation spills over to extra-synaptic regions thus stimulating mACHR into releasing norepinephrine and epinephrine in excess (Akiyama et al, 2003) resulting in adverse effects.

Epinephrine, which does not cross the BBB, also has a stimulatory effect on the rat pituitary gland (See Fig. 2). Researchers found that rats injected IP with epinephrine (1 mg/kg IP) had elevated levels of plasma ACTH and β-endorphin as compared to control or saline injected rats (Mougey et al, 1986). Other studies
have also shown that epinephrine (100-10000 ng/kg) and the β-adrenergic agonist, isoproterenol (100-1000 ng/kg) causes a dose-depandant increase of plasma ACTH and CORT in vivo and vitro (Tilders et al, 1981; Berkenbosh et al, 1983).

![Figure 2: The cholinergic interactions of hypothalamo-pituitary-adrenal axis](image)

The effect of PB on the circadian rhythm of the HPA axis remains controversial. Murialdo et al (1993) reported that human subjects showed elevated AM levels of ACTH and cortisol in response to pyridostigmine bromide (120 mg) coupled with CRH administered in the AM. Llorente et al (1996) researched the effects of PB alone (120 mg) and in combination with pirenzipine (PZP, a muscarinic antagonist) on the human circadian rhythm. They found that PB by itself when administered either at night or in the morning, induced a significant
reduction in AM and PM ACTH levels, a finding that contradicts the Murialdo et al (1993) study, while PB + PZP only reduced evening levels of ACTH and cortisol (Llorente et al, 1996). PB alone did not produce any changes in cortisol levels. They concluded that the cholinergic pathway plays an inhibitory role in human ACTH secretion without altering the normal circadian rhythm.

The effect of PB on circadian fluctuations in corticosterone in rats is also unclear. Basal levels of CORT remained unaltered in unstressed rats treated with PB for 7 consecutive days (Servatius, et al, 1998, 2000). Beck et al, (2003) found that non-stressed rats had a slightly elevated CORT response when given a single, i.p. injection of PB (2.0 mg/kg), although their results upon analysis did not prove to be significant. Kant et al (2001) found that stressed and nonstressed rats actually had a slight decrease in plasma corticosterone levels when a lower dose of PB (1.5mg/kg) was delivered through an osmotic pump for 7 days. It is not known whether PB administered orally for 7 consecutive days alters the circadian peak or shifts the cycle in anyway.

The acoustic startle response (ASR) is a useful behavioral measurement developed for investigation of sensorimotor information processing and modulation in mammals (Fendt and Koch, 1999). This response is brought on by a sudden, intense, acoustic stimulus and is composed of a spasm of facial, neck, and limb muscles (Lee et al, 1994; Fendt and Koch, 1999). The response stems from the ventral cochlear nucleus, lateral lamniscus nuclei, caudal pontine reticular nucleus (PnC), amygdala, various tegmental nuclei, and spinal motor neurons
(Davis, 1989; Lee et al., 1994; Servatius et al., 1998; Fendt and Koch, 1999). Figure 3 represents a hypothetical ASR pathway.

![Diagram of hypothetical ASR pathway]

Figure 3: Hypothetical pathways of the ASR. Purple areas are the primary ASR circuit and grey shaded boxes are involved in stress-induced enhancement of the ASR (Koch, 1998)

The ASR has a daily rhythmicity in which the amplitude of the response can differ depending on the time of day (Chabot and Taylor, 1992). Differences in the amplitude of the ASR have been observed due to the photic conditions during ASR measurement. Amplitude was twofold higher during the dark testing than during the light testing at 110dB and 120dB white noise levels (Chabot and Taylor, 1992). The ASR also has a sensitivity to light called the light enhanced startle. The percent responses tend to be greater when the rats are exposed to light conditions (Walker and Davis, 1997).
Activation of the HPA through stress, such as footshock and tailshock, can affect the ASR (Davis 1989; Servatius et al., 1995; Koch, 1998). Rats that had been exposed to tailshock (2hr, 40 2.0 mA shocks) for three consecutive days in the AM had elevated basal CORT for four days post-stress, which resulted in an exaggerated AM ASR on Day 7 and 10 post-stress (Servatius et al., 1995). Footshock (10 0.6 mA), which elevates plasma CORT as well, was administered to rats producing an increase in the amplitude of the ASR 1-40 minutes post shock (Davis, 1989), but did produce an elevated response when tested again 24 hrs later. This may be due to the differences in amplitude and duration of the shock administered.

Rats treated chronically with corticosterone for five days in the PM shortly before lights out did not produce an increase in startle amplitude when tested during the beginning of the rats' dark phase (Lee et al, 1994). However, when given an i.c.v. microinjection of CRH prior to a 120 minute startle testing, there was a potentiation of the startle amplitude. Adrenalectomized and sham rats were also exposed to an i.c.v. infusion of CRH (no CORT pretreatment) then startled to evaluate the role of the HPAA on the CRH enhanced startle (Lee et al, 1994). Both groups showed enhancement of the ASR following the infusion suggesting that CRH potentiated by CORT is regulated through other brain areas such as the amygdala, one of the brain regions known to enhance startle, or the bed nucleus of stria terminalis (BNST) (Lee et al, 1994).
The role of the cholinergic system on ASR has also been investigated, but there are no consistent effects (see Fig. 4). Scopolamine (SCO), a mAChR antagonist, has been shown to slightly increase (0.5-1.0 mg/kg), decrease (0.25-2.0 mg/kg), or have no effect (1.5 mg/kg) on the ASR of rats while methylscopolamine, which does not cross the BBB, also had no effect on ASR (Davis, 1980). ACh increased the ASR in rats (Overstreet, 1977), while the muscarinic agonist, pilocarpine (1-10 mg/kg), depressed ASR. Physostigmine (PHY), a centrally acting reversible AChE inhibitor, did not alter baseline startle at doses of 0.1-4 mg/kg (Overstreet, 1977). PHY was also studied in guinea pigs. PHY, along with SCO, caused enhancement of the ASR (Philippens et al, 1996, 1997). This increase in ASR was probably due to the agonistic action of PHY on nAChR, because PHY, when in combination with mecamylamine (nAChR antagonist), had no effect on the ASR (Philippens et al, 1997). Conversely, another reversible AChE inhibitor, ethyl-p-nitrophenylphosphoramidate (PNF), was found to have no effect on the ASR with and without the presence of SCO (Philippens et al, 1996). It is suggested that the enhancing effects of PHY may not be due to AChE inhibition, but rather through other transmitter systems (Philippens et al, 1996).

Administration of the peripherally acting drug, PB, for seven consecutive days was found to have a delayed-onset, persistently enhanced startle response in Wistar-Kyoto rats fifteen days after the end of treatment (Servatius et al, 1998), but Sprague-Dawley rats did not exhibit this response. Sprague-Dawley rats were
shown to have an enhanced ASR later in a subsequent experiment that investigated PB and stress on startle response (Servatius et al, 2000). Rats treated orally with PB for seven days that were not exposed to stress had an enhanced startle response on Day 7 and Day 8 (Servatius et al, 2000). Exposure to the startle testing occurred at the rats’ circadian trough for both of these experiments.

Figure 4: Cholinergic effects on the primary ASR circuit. Purple areas are the primary ASR circuit and grey shaded boxes are involved in CRH enhancement of the ASR (Koch, 1998)

The present study focuses on the possible effects of pyridostigmine bromide on plasma circadian corticosterone concentrations and the ASR of the Sprague Dawley rat. Recent research has shown that anticholinesterases may activate the HPA axis, thereby possibly altering the natural circadian hormonal
fluctuations (Murialdo et al, 1993; Llorente et al, 1996). Studies have shown that PB elevates basal cortisol in humans, so the effects of PB on circadian trough and peak corticosterone in rats are investigated.

If PB causes an alteration in plasma corticosterone concentrations, a change in the ASR might also occur. Research has shown that acetylcholine may stimulate hypothalamic CRH secretion while anticholinesterases seem to be able to stimulate the pituitary. If the anticholinesterase PB is administered chronically, some of the AChE existing in peripheral synaptic clefts and neuromuscular junctions should be inhibited, causing an increase in ACh that may be able to stimulate the pituitary-adrenal axis causing a rise in CORT. Studies have shown that PB given orally may induce an exaggerated startle response in the circadian trough of rats (Servatius et al, 2000), but it is not known whether this response can also occur at the circadian peak. Here, the effect of PB on the circadian trough and peak of corticosterone was investigated along with the effect PB has on the ASR during the trough and peak of the circadian rhythm.

In experiment 1, Sprague-Dawley rats were administered either water or pyridostigmine bromide in their water bottles for seven days. PB was administered at a dose that would produce approximately 20-30% inhibition of plasma acetylcholinesterase. Blood was sampled in the AM and PM each day according to the circadian trough and peak. Plasma was analyzed for cholinesterase activity and corticosterone. In Experiment 2, the acoustic startle response was used to determine the behavioral differences between rats treated with PB vs. rats that
only had water. Being that the first experiment yielded an elevated level of peak CORT in the rats treated with PB, an acoustic startle response (ASR) was employed to determine if there were response differences between groups due to drug treatment. The startle sessions occurred at the circadian trough and peak to mimic blood sampling in the first experiment. Blood samples were not obtained due to the artifact that the procedure would have on the ASR.
Materials & Methods

Experiment 1: The Effects of PB on Circadian Physiology

A. Animals

Ten adult male Sprague-Dawley rats (225-250g) were obtained from Charles River (Kingston, NY). Rats were individually housed in shoe-box style cages with free access to food and water. Cages were kept in chambers designed to hold up to 16 individually housed rats in a sound attenuated environment. The chambers also provide controlled 12hr light cycles (lights on, 0700h), air filtration and humidity. All rats were allowed a one-week acclimation period prior to the start of the experiment.

B. Experimental design and procedure

Animal water intake was tracked for a period of one week for accurate pyridostigmine bromide (PB) dosing. Animals (n=5 per group) were randomized into 2 groups (PB and vehicle) according to body weight. The average body weight and amount of fluid intake was used in calculating an accurate PB concentration. Baseline blood was taken 0800h the morning prior and morning of the start of the experiment. Blood samples were collected via tailclip at 0800h and 2000h for 7 consecutive days. Blood sampling at 2000h occurred under minimal red light to prevent disruption of the circadian rhythm. Blood was collected at 0800h only on the eighth day, thus concluding the experiment. Blood was
collected in heparinized hematocrit tubes and immediately spun for plasma separation. Plasma was then transferred to fresh hematocrit tubes and stored at -80°C until assay.

C. Drug Treatment

Pyridostigmine Bromide (Sigma, MO) was dissolved in tap water (5mg/kg) and dispensed in water bottles in 250ml amounts. The amount of fluid intake was recorded daily throughout the experiment by measuring the amount of fluid in the water bottle with a graduated cylinder and subtracting from 250ml. Fresh batches of PB were made every two days and refrigerated. The concentration of PB solution was determined by tracking fluid intake for one week prior to the start of the experiment. Animals were weighed the day before starting and the concentration calculated using dose, average body weight and average fluid intake in milliliters.

D. Assay

Plasma CORT levels were determined using a double antibody RIA kit obtained from ICN Biomedicals Inc., Carson, CA. (RSL 125I corticosterone kit #07-120102). Samples were diluted 1:200 as per kit directions. Cholinesterase activity was determined using a radiometric assay based on the liquid-extraction technique procedures of Johnson and Russell (Johnson and Russell, 1975). Briefly, [3H] acetyl labeled ACh (Perkin-Elmer Life Science, MA) and unlabeled ACh are
used as substrate for hydrolysis by BuChE and AChE found in the samples. The reaction is carried out at room temperature in scintillation vials containing a mixture of KPO₄ (pH 7.0), labeled and unlabeled substrate, enzyme in samples, and dH₂O. The reaction is stopped with a strongly acidic buffer (pH 2.5) that protonates the ³H-labeled acetate produced. The acetate is then extracted in a scintillation cocktail of Scintisafe Econo-F (Fisher Scientific, MA) and 10% isoamyl alcohol (ICN, OH) and counted within the scintillation vial using a beta counter (Beckman Coulter LS6500, CA). Plasma samples were diluted 1:5 in dH₂O. Cholinesterase activity was determined by calculating the number of micromoles of hydrolyzed acetate per min then correcting for unit volume of plasma. The final activity is presented in nmol/min/ml plasma.

E. Data Analysis:

Corticosterone levels, cholinesterase activity, and fluid intake data were analyzed using a repeated measure ANOVA for during treatment differences with group (PB or Vehicle) as a between animal measure. Measurement Day and Time (AM\PM) were analyzed within each group. Bonferroni Multiple Comparison tests determined a priori post-hoc differences with p < 0.05.
Experiment 2: The Effects of PB on Circadian Behavior

A. Animals

Sixteen adult male Sprague-Dawley rats (225-250g) were obtained from Charles River (Kingston, NY). Rats were individually housed in shoe-box style cages with free access to food and water. Cages were kept in chambers designed to hold up to 16 individually housed rats in a sound attenuated environment. The chambers also provide controlled 12hr light cycles (lights on, 0700), air filtration and humidity. All rats were allowed a one-week acclimation period prior to the start of the experiment.

B. Experimental design and procedure

Animal water intake was tracked for a period of one week for accurate PB dosing. Animals (n=8 per group) were randomized into 2 groups (PB and vehicle) according to body weight. The average body weight and amount of fluid intake was used in calculating an accurate PB concentration. Animals were startled at 0800 and 2000 hours for eight consecutive days. Startle sessions occurring at 2000h were performed under minimal red light to prevent the circadian rhythm from being disrupted. Blood samples were not obtained due to the artifact the sampling would have on the ASR.
C. Drug Treatment

Pyridostigmine Bromide (PB, Sigma, MO) was dissolved in tap water (5mg/1kg) and dispensed in water bottles in 250ml amounts. Amount of fluid intake was recorded daily by measuring amount of fluid in water bottle with a graduated cylinder and subtracting from 250ml. Fresh batches of PB was made every two days and refrigerated. Concentration of PB solution was determined by tracking fluid intake for one week prior to the start of the experiment. Animals were weighed the day before starting and concentration calculated using dose, average body weight and average fluid intake in milliliters.

D. Startle Testing

The acoustic startle apparatus (white noise generators, rat holders, startle platforms and interface) was obtained from Coulbourn Instruments (Langhorn, PA). Rats were loosely restrained in holders on top of platforms that detect movement. The movement was transduced into an analog signal that runs through an interface where a PC running Labview recorded the A/D signal from a DAS 1600 A/D board. The program controlled the stimulus timing and intensities, as well as signal recording. The acoustic stimuli were white noise bursts (100ms, 5ms rise/fall time) produced from the white noise generators. The rats were exposed to eight trials containing one of three acoustic intensities 82, 92, and 102dB (24 total trials) with an interstimulus interval of 25-35 seconds. To control for circadian
variations in startle responding, rats were run in pairs with a representative from
the PB treatment and control groups run at the same time each startle session.

E. Data Analysis

Data was analyzed on a trial-by-trial basis and a response was scored if the
activity after the stimulus onset exceeded a response threshold. The threshold was
determined as peak baseline activity plus 4 times the standard deviation of the
baseline activity (Servatius et al, 2000). No activity was scored for the trial if the
activity post-stimulus did not exceed the response threshold. Obtaining mean
values for each rat at each stimulus intensity level reduced the startle data for each
session.

Data was analyzed with split-plot ANOVA for repeated measures.
Sensitivity was measured as the percent response for each stimulus intensity.
Responsitivity was measured as the magnitude of responses at the highest stimulus
intensity. Significance levels were set with a p < 0.05 for all post-hoc
comparisons.

Fluid intake was analyzed by a repeated measure ANOVA as described
above.
Results

Experiment 1:

PB Treatment

Baseline water consumption between the two groups was relatively equivalent with the PB group drinking 47.6 (± 1.78) milliliters of water and the vehicle group drinking 51.4 (± 1.78) milliliters of water the day before the start of the experiment. There was an overall difference in fluid consumption with the PB treated group drinking more than the vehicle group throughout the 7-day treatment regimen (Fig 5). The PB group consumed an average of 60.9 (± 1.87) milliliters of fluid and the vehicle group consumed 48.1 (± 1.87) milliliters of fluid over the course of the experiment. This was confirmed by a 2 x 7 (Drug x Measurement Day) repeated measures ANOVA. Significant main effects of Drug $F(1,8) = 23.7$, $p < 0.001$, and Measurement Day $F(7,56) = 14.0$, $p < 0.0001$. This was also confirmed in the Drug x Measurement Day interaction $F(7,56) = 16.6$, $p < 0.0001$.

Cholinesterase Activity

Baseline blood samples were obtained the morning of the start of PB treatment. Cholinesterase activity was measured in nmol/min/ml. The baseline cholinesterase activity in the PB group (143 ± 7.82) was slightly higher than the vehicle group (117 ± 6.99). Treatment with pyridostigmine bromide (0.038 mg/ml) in the drinking water of Sprague-Dawley rats resulted in cholinesterase inhibition of about 30% over the 7-day treatment regimen. The cholinesterase data
for the 7-Day treatment regimen in Figures 6 and 7 is represented as means per
group per day. Cholinesterase activity in the PB treated group was reduced overall
to 99.2 (± 5.36) from baseline levels throughout the experiment. PB treated
animals had an inhibited ChE activity within each sample day as compared to
vehicles, but time of day did not play a role in cholinesterase activity. No
difference in activity was seen within each group for the AM and PM sampling
times. This was confirmed by a 2 x 7 x 2 (Drug x Measurement Day x Time)
repeated measures ANOVA. Significant main effects of Drug, $F(1,8) = 11.84$, $p <
0.01$, Measurement Day, $F(6,101) = 12.04$, $p < 0.0001$ were qualified by
significant Drug x Measurement Day interaction, $F(6,101) = 5.3$, $p < 0.0001$. No
significant difference was observed for the main effect of Time, $F(1,101) = 0.19$, $p
< 0.6$, confirmed by the Drug x Time interaction, $F(1,101) = 0.88$, $p < 0.3$.

Pearson’s Coefficient of correlation revealed an overall correlation between
cholinesterase activity and PB consumption $R(77) = -0.47$, $p < 0.00001$.

Cholinesterase activity was reduced as PB consumption increased.

Corticosterone Levels

Baseline plasma corticosterone levels were obtained the morning of the
start of the experiment yielding equivalent CORT levels between the PB group
(0.91 ± 3.14 ) and vehicle group (1.1 ± 3.14 ). As expected, differences in CORT
levels were seen between AM and PM sampling times within each measurement
day resulting from normal circadian fluctuations of these hormones. Overall, AM CORT levels (0.91 ± 0.84) were significantly lower than PM levels (33.8 ± 5.36) modeling the circadian trough and peak respectively. No difference was observed between groups at the basal AM trough of CORT throughout the 7-day treatment regimen (fig. 8). There were slightly elevated PM levels of CORT were observed in the PB treated rats as compared to the Vehicle rats overall (Fig. 9). This was confirmed by a 2 x 7 x 2 (Drug X Measurement Day x Time) repeated measures ANOVA. Significant main effect of Time $F(1,104) = 764$, $p < 0.0001$, and the Drug x Time interaction, $F(1,104) = 14.2$, $p < 0.0002$. 

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Figure 5: Mean (± SEM) fluid consumption for the PB group and the Vehicle group over the 7-Day treatment regimen. The asterisk (*) represents a significant difference overall for the length of treatment from control group, p < 0.05.
Figure 6: Mean (± SEM) AM differences in cholinesterase activity for the PB group and the Vehicle group over the 7-Day treatment regimen. The yellow bar at the base of the graph represents the time frame of PB treatment. The PB group had a significant difference overall for the length of treatment, p < 0.05.
Experiment 1: PM Cholinesterase Levels

![Bar graph showing cholinesterase levels over 7 days with error bars for group mean (±SEM). There are two groups: Control (Vehicle) and Pyridostigmine Bromide.](image)

Figure 7: Mean (± SEM) PM differences in cholinesterase activity for the PB group and the Vehicle group over the 7-Day treatment regimen. The yellow bar at the base of the graph represents the time frame of PB treatment. The PB group had a significant difference overall for the length of treatment, \( p < 0.05 \).
Experiment 1: AM Corticosterone Levels

Figure 8: Mean (± SEM) AM corticosterone levels in PB group and Vehicle group over the 7-Day treatment regimen. No difference between groups was observed, p > 0.05.
Figure 9: Mean (± SEM) PM corticosterone levels in PB group and Vehicle group over the 7-Day treatment regimen. A significant difference (p < 0.05) between groups was observed with the PB group having elevated CORT overall as compared to the Vehicle group.
Experiment 2:

PB Treatment

There was no difference in fluid consumption between the PB treated group and the vehicle group for the 7-day treatment regimen (Fig. 10). The PB group consumed an average of 63.6 (± 2.47) milliliters of fluid and the vehicle group consumed 58.8 (± 2.49) milliliters of fluid over the course of the experiment. This was confirmed by a 2 x 7 (Drug x Measurement Day) repeated measures ANOVA. Significant main effect of Measurement Day $F(7,97) = 11.99, p < 0.0001$. This was also confirmed in the Drug x Measurement Day interaction $F(7,97) = 12.57, p < 0.0001$.

Acoustic Startle Response:

The startle responses were assessed in the PB group and the Vehicle group using a multiple-intensity protocol for 8 consecutive days 1 hour after lights on (AM) and 1 hour after lights off (PM) to mimic the blood sampling times of Experiment 1. There was no difference between groups for percent response at any intensity therefore the data was collapsed. The percent responses were analyzed for all three intensity levels, and as expected, the overall response to each intensity level was significant (Table 1). There was also an overall difference in responses due to time. Sensitivity was greater in the AM timepoint than in the PM timepoint. The percent responses were analyzed using a 2 x 8 x 2 x 3 (Drug x Measurement Day x Time x Intensity) repeated measures ANOVA. The main effects of Intensity
$F(2,14) = 1798$, $p < 0.0001$, and Time $F(1, 14) = 26.67$, $p < 0.0001$, were significant. The Intensity x Time interaction $F(2,14) = 4.78$, $p < 0.009$, the Intensity x Measurement Day interaction $F(7,14) = 1.79$, $p < 0.04$, and the Time x Measurement Day interaction $F(1,7) = 2.83$, $p < 0.007$ are also significant.

PB did not induce an exaggerated startle response at any intensity level in either AM or PM sessions throughout the experiment. There were many no responses scored for the 82 and 92 decibel intensities so the data was not included in the statistical analysis. The amplitudes of the startle response at 102dB was measured by a $2 \times 8 \times 2$ (Drug x Measurement Day x Time). There was a main effect of Measurement Day $F(1,7) = 4.37$, $p < 0.0002$. There were no significant group differences in amplitude throughout the experiment (Table 2).
**Experiment 2: Fluid Consumption**

![Bar chart showing fluid consumption](image)

Figure 10: Mean (± SEM) fluid consumption for the PB group and the Vehicle group over the 7-Day treatment regimen. There was no significance in drinking for this experiment, p > 0.05.
## Experiment 2: Percent Response

<table>
<thead>
<tr>
<th>INTENSITY</th>
<th>AM</th>
<th>PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>82dB</td>
<td>27 ± 2%</td>
<td>23 ± 1%</td>
</tr>
<tr>
<td>92dB</td>
<td>82 ± 3%</td>
<td>73 ± 1%</td>
</tr>
<tr>
<td>102dB</td>
<td>99 ± 0.3%</td>
<td>98 ± 0.1%</td>
</tr>
</tbody>
</table>

Table 1: Mean (± SEM) of percent responses obtained over the course of the experiment at three different intensities. Significant main effects of Intensity (p < 0.0001) and Time (p < 0.0001) was observed. Sensitivity was greater in the AM than in the PM. There was no significant difference observed between groups so data was collapsed.
Experiment 2: Amplitude of the ASR at 102dB

<table>
<thead>
<tr>
<th>DAY</th>
<th>MEAN AMPLITUDE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.5 ±0.2</td>
</tr>
<tr>
<td>2</td>
<td>2.0 ±0.2</td>
</tr>
<tr>
<td>3</td>
<td>2.0 ±0.1</td>
</tr>
<tr>
<td>4</td>
<td>2.3 ±0.2</td>
</tr>
<tr>
<td>5</td>
<td>2.7 ±0.2</td>
</tr>
<tr>
<td>6</td>
<td>2.4 ±0.2</td>
</tr>
<tr>
<td>7</td>
<td>2.6 ±0.2</td>
</tr>
<tr>
<td>8</td>
<td>2.6 ±0.2</td>
</tr>
</tbody>
</table>

Table 2: Overall means (± SEM) of the amplitude of the acoustic startle response at 102dB. There was no significant difference (p > 0.05) observed between groups so data was collapsed to show amplitude.
Discussion

For the past decade, pyridostigmine bromide has been under scrutiny for its involvement in the unexplained illnesses of Persian Gulf War veterans. PB, given prophylactically at a dose of 30 mg/kg p.o. every eight hours for 3 to 7 consecutive days, gave soldiers a reversible 20-30% inhibition of plasma cholinesterase activity (Keeler et al, 1991), thereby protecting this enzyme from irreversibly binding with nerve agents. The safety of this drug has been questioned given that soldiers have reported a vast number of symptoms since their return from the Gulf.

Pyridostigmine bromide effects remain, for the most part, peripheral, and its effects on circadian physiology are controversial. PB (120mg) administered in the AM has been shown to stimulate the pituitary by inducing ACTH and cortisol at the human circadian peak (Muraiado et al, 1993), as well as potentiating the effects of GHRH and CRH administration on GH and ACTH (Corsello, 1992). Conversely, PB (120 mg) was found to reduce the human peak and trough levels of ACTH and had no effect on cortisol when at either in the morning or the evening (Llorente et al, 1996).

The purpose of the study was to investigate the effects pyridostigmine bromide has on the circadian rhythm, physiologically and behaviorally. Previous research in humans and rats has suggested that PB may affect ACTH and CORT levels through pituitary stimulation, but how it effects the overall circadian
fluctuation of these hormones is unclear. This study was designed to elucidate whether PB, in a non-stress environment, can alter the circadian fluctuation of plasma corticosterone in rats thereby altering their behavioral response to acoustic startle testing.

The results of experiment 1 indicate that the target range of 20-30% cholinesterase inhibition was met by using a 5mg/kg dose. In the first experiment, there was an overall 30% inhibition of cholinesterase activity. There was no difference between AM and PM levels of cholinesterase activity within each treatment group, meaning that the PB ingested caused a steady-state inhibition that lasted throughout the 24 hour circadian cycle. This steady-state inhibition seemed to last throughout the experiment, without any signs of cholinergic overstimulation, such as diarrhea or excessive lacrimation that is sometimes seen in similar treatment regimens (Servatius et al, 1998).

To determine if rats had a normal circadian fluctuation in corticosteroids, blood was sampled one hour after lights on and one hour after lights off to capture trough and peak corticosterone levels. The results showed normal circadian fluctuations of CORT in the AM trough levels of Vehicle animals and the PB treated animals. The PB animals however, showed an elevation of peak CORT. Previous research has suggested that anticholinesterases may stimulate the pituitary causing a rise in either ACTH or CORT or both (Murialdo et al, 1993). The results of this experiment suggest that PB does in fact cause a rise in CORT levels, but only in the peak phase of the circadian cycle.
The PM elevation of CORT in the PB treated animals may possibly be a direct reflection of the rats’ drinking during their active phase. Since rodent activity and CORT tend to peak around the onset of lights out, the exaggerated rise in PM CORT may be a direct result of the immediate ingestion of the PB that occurs during this period. For this experiment, the PB group consumed a little more fluid overall when treated with PB than they did when given water alone, an effect that was not seen in similar experiments (Servatius et al, 1998, 2000). It is known that approximately 90% of the drinking activity of rats occurs during the lights out phase (Stephan and Zucker, 1974), so the effect of PB on CORT appears to be acute and does not affect the overall circadian rhythm.

In experiment 2, the effect of PB on circadian ASR was investigated. The ASR is a sensitive method used by many investigators to determine how different neurotransmitter systems modulate sensori-motor activity (Phillipens et al, 1997). The effect that the cholinergic system has on the ASR remains unclear. Davis (1980) had found that ACh plays a minor, if not, indirect role in startle modulation while PB was found to have an exaggerated startle response on the ASR (Servatius et al, 2000). This exaggerated ASR was elicited from non-stressed, PB treated rats, but this effect was only seen on Day 7 and Day 8 of a 7-Day treatment regimen. Conversely, other researchers found that PB did not produce an exaggerated startle response, even after a 21-Day treatment regimen (Scremin et al, 2003). Nonetheless, a sensitivity difference in the AM versus the PM measurements was
observed, reinforcing previous data that the ASR is influenced by light (Walker and Davis, 1997).

In Experiment 1, rats that were treated orally with PB for 7 days had elevated levels of plasma CORT overall. The ASR was utilized to determine if PB created a behavioral change by elevated plasma CORT. This did not happen. The response of the PB treated animals and the vehicle animals were the same. There were no exaggerations in the ASR. Blood was not sampled during this experiment because of the artifact the sampling itself would have on the ASR. The ASR was tested at AM and PM timepoints, thereby pre-startle blood sampling for corticosterone would have interfered with accurate ASR responses. Sampling blood post-startle would have yielded elevated levels of CORT resulting from startle exposure.

Blood was not sampled for cholinesterase inhibition at either timepoint for similar reasons. The ASR can be a mild stressor in itself and stress had been shown to produce inhibition in cholinesterase activity (Servatius et al, 2000) thereby accurate cholinesterase activity may have been difficult to obtain. With this in mind, if employing the ASR at two timepoints in one sampling day while ingesting PB may cause a mild stress state, an exaggerated ASR should have been seen.

Drinking differences between the two experiments may have played a minor role in the outcome of the results. In the first experiment, rats that received PB in their drinking water drank approximately 18 mls more than control rats
overall. This means they ingested more PB creating the 30% inhibition during the experiment. The dose per rat was 2.01 mg per day as calculated at the start of the experiment. The average dose for the PB treated animals over the course of the experiment was 2.58 mg. The rats ingested a slightly higher amount of PB, yet the targeted range of inhibition was still met.

In the second experiment, PB treated rats drank almost the same amount of fluid as the Vehicle rats over the course of the experiment. PB treated animals drank about 5 mls more fluid than Vehicle rats over the course of the experiment. PB rats were calculated to receive 1.83 mg per day. These animals actually received 2.23 mg of PB per day. Previous research has shown that the dosing regimen used in this experiment has produced a 20-30% inhibition of cholinesterase in rats, while the fluid consumption between groups remained the same (Servatius et al, 1998, 2000; Scremin et al, 2003). It is possible however, that because the rats in the first experiment drank more fluid overall causing a 30% inhibition of AChE, the rats in the second experiment may have only ingested enough PB to reach the lower end of the targeted range (i.e. 20%). If there was only a 20% inhibition of AChE overall, a rise in CORT would not be found, and this would have no effect on ASR.

Elevations on CORT may or may not be a direct effect of ACh on the pituitary. Research seems insufficient when it comes to cholinergic stimulation on the pituitary in vitro. One in vitro study found that arecoline (mAChR agonist) failed to elicit ACTH release from pituitary cell cultures (Calogero et al, 1989).
Conversely, PB was found to trigger the pituitary-adrenal axis in humans (Murielado, et al 1993). The permeability of the BBB in certain regions of the hypothalamus may be questioned, such as the arcute nucleus because of the cholinergic neurons located here, but some research has suggested that the BBB is in fact intact at this site (Peruzzo et al, 2000). Further research is needed to explore this possibility.

Research where elevations in human plasma cortisol were seen had high doses given to the subjects. The normal dosing regimen is 30 mg orally three times a day (Keeler, 1991) yielding 90 mg per day per soldier. In the Murielado et al. study (1993), elevated plasma cortisol was observed after a single AM 120 mg dose of PB was given to the subjects. This high dose administered at one time may cause adverse side effects similar to those reported by Keeler (1990; Keeler et al 1991), thereby elevating plasma cortisol. It is not known whether the subjects of the Murielado et al (1993) study experienced any ill effects from PB administration.

Side effects of PB may induce a minor stress state thus activating the HPAA. Side effects like diarrhea, headaches, flatus, nausea, and the occasional slight bradycardia (Keeler et al, 1991) have been reported from soldiers who were under the normal dosing regimen. It is not known whether the animals used in these experiments had experienced these side effects. There were no signs of cholinergic over-stimulation as seen in similar experiments (Sevavius et al, 1998) however, the animals that received PB could have experienced mild ill effects that
were unnoticeable to visual observations. Rats do not have the ability to vomit; therefore it is difficult to determine if the rats had experienced nausea.

PB may have induced a state of nausea in the rats, thereby triggering a hormonal response. Vasopressin and oxytocin have been cited as hormonal indicators of visceral illness in rats (Verbalis et al, 1986; Carter and Lightman, 1987) Vasopressin (VP) and oxytocin (OT) are peptides that are synthesized in the parvocellular neurons of the PVN. OT has been found to trigger the release of ACTH from the pituitary through VP receptors type V1b (Schlosser et al, 1994). CRH is also found in the parvocellular neurons of the PVN and is simultaneously released with VP (Kandel et al, 1991) which is known to potentiate the effect of CRH on ACTH. Nausea caused by agents such as lithium chloride has been shown in rats to cause a rise in plasma levels of VP (Verbalis et al, 1986; Carter and Lightman, 1987).

VP neurons are spontaneously active and maintain a basal level of VP in the blood. Changes in VP levels results from changes in an animals body i.e., a decrease in water intake causes an increase in VP neuronal firing thereby increasing VP blood concentrations. Stress, pain, and anxiety have also been found to cause increases in VP levels (Kandel et al, 1991).

In this study, plasma CORT levels were elevated in PB treated rats. It is possible that the PB in their water bottles induced a mild state of emesis that resulted in the parvocellular neurons of the hypothalamus releasing VP simultaneously with CRH. The HPAA would then be activated and plasma CORT
would rise. This may explain why elevated levels of CORT were only seen during PM sampling. Rats consuming PB at night may have a mild state of nausea that may cease once the rats stopped drinking. CORT levels were also not significantly elevated each night, suggesting that the rats may not have felt ill. Further research would be needed to elucidate vasopressin's role in the effects of PB on CORT.

PB's inhibition of AChE may have also indirectly stimulated the pituitary-adrenal axis through epinephrine release. Previous in vitro studies have shown that the β-adrenergic agonist stimulates the pituitary intermediate lobe into releasing ACTH (Tilders et al, 1981). The increase in ACh at synapses and junctions may have stimulated the adrenal medulla or other peripheral adrenergic neurons into releasing catecholamines. These catecholamines could have stimulated the pituitary into releasing ACTH thereby elevating plasma CORT concentrations.

Pyridostigmine bromide did not alter the ASR, yet other behavioral measures might have been altered. The ASR is only one behavioral measure that utilizes a complex central and peripheral pathway. Many other behavioral measures involve different pathways that may have been affected by PB. PB has affected the acquisition of the lever pressing response in rats by delaying the onset of their responses (Van Haaren et al, 1998). Learning and behavioral tasks such as open field, two-way shuttle box avoidance, and rat coordination tests have also been affected (Van Haaren et al, 1998).

In conclusion, the results of this study indicated that PB does not alter or disrupt the circadian rhythm of corticosterone concentrations; however CORT is
elevated during peak concentrations suggesting an acute effect directly related to PB ingestion. PB did not produce a state of anxiety as measured by the acoustic startle response at either circadian trough or peak. Further research is needed to elucidate how PB is activating the HPAA, whether it is through visceral illness or stimulation of the pituitary through cholinergic or adrenergic mechanisms.
Summary

Pyridostigmine bromide (PB) has been implicated as a cause of Gulf War syndrome for the immediate and delayed side effects experienced by veterans. In this study, the effects of PB on circadian plasma corticosterone (CORT) and the acoustic startle response were investigated in non-stressed rats. PB was administered in water bottles at a dose and route similar to that of the soldiers.

The results of this study suggest that PB does not affect the circadian fluctuations of plasma CORT concentrations. It does however, suggest that the immediate ingestion of PB during the rats’ active phase can cause acute effects that diminish when the rat ceases drinking. Peak plasma CORT concentrations were found to be slightly elevated during the experiment which would imply that the PB may cause gastric upset leading to a mild activation of the HPAA. This activation of the hypothalamic-pituitary-adrenal axis (HPAA) was not evident in the acoustic startle response. This would further imply that the effects of PB are subtle and transient.

This research has provided insight into the effects of PB on rat circadian plasma CORT concentrations and anxiety behavior. With further research, the underlying causes that affect Gulf War veterans may be elucidated.
References


Murialdo G, Fonzi S, Torre F. (1993) Effects of pyridostigmine, corticotrophin-releasing hormone and growth hormone-releasing hormone on the pituitary-


