PART I: INTRODUCTION

A STATEMENT OF HYPOTHESIS

The hypothesis was that repeated stress would modulate the toxicity of cholinesterase inhibition on cholinergic and glutamatergic systems of the central nervous system. This hypothesis was based on studies that indicated stress altered the glutamatergic and cholinergic responses in the hippocampus (McEwen 1999, Kaufer et al. 1999, Sunanda et al 2000). It was expected that these stress-induced responses would have additive or synergistic effects on the responses to cholinesterase inhibitors. Cholinesterase inhibitors increase acetylcholine in synapses with excess acetylcholine desensitizing cholinergic receptors. Cholinesterase inhibitors also alter concentrations of catecholamines and glutamate (Lallement et al. 1991, El-Etri et al. 1992, Giacobini et al.1996, Ali et al. 1980).

An interaction between concurrent repeated stress and exposure to a cholinesterase inhibitor on the cholinergic and glutamatergic systems has not been reported. These two systems are mainly distributed in the hippocampus, an important brain region for learning and memory (Bear et al. 2001), and the cerebral cortex, a center of sensory and motor systems and cognition (Purves et al. 2001). Stress has been reported to cause dendritic atrophy of CA3 cells in the hippocampus (Magarinos et al. 1997). The research for this dissertation examined differences in the concentrations of neurotransmitters (glutamate and monoamines), enzyme activities associated with acetylcholine synthesis and metabolism, and densities of related glutamatergic and cholinergic receptors with a focus on the hippocampus and cerebral cortex of rats under conditions of concurrent exposure to repeated stress and chlorpyrifos.

JUSTIFICATION FOR THE STUDY

Sustained stress causes secretion of corticosteroids, which induces negative feedback of the hypothalamic-pituitary-adrenal axis (HPA). Stress and excess glucocorticoids promote neurodegeneration in the hippocampus. For example, chronic stress appears to cause atrophy of apical dendrites of neurons in the hippocampus (Magarinos et al. 1997, McEwen 1999).

Mechanisms contributing to hippocampal atrophy have not been well defined but may involve the effects of stress on neurotransmitter systems, especially the glutamatergic and cholinergic systems (Kaufer et al. 1998, McEwen 1999). The effects of stress-induced modulation of glutamatergic and cholinergic systems in the presence of cholinesterase inhibition are of concern and may contribute to illness seen in veterans of the 1991 Gulf War (Sapolsky 1998). Gulf War veterans were exposed to chronic stress and cholinesterase inhibitors (such as organophosphate insecticides); however, these were among several risk factors that troops experienced (Sapolsky 1998). Furthermore, veterans may have been exposed to cholinesterase inhibitors (organophosphate insecticides) after they returned home. We hypothesized that concurrent exposure to repeated stress and cholinesterase inhibitors may have caused additive or synergistic effects on the glutamatergic and cholinergic systems of the central nervous system. The study was justified because stress induces release of glutamate and modulates expression of *N*-methyl-D-aspartate (NMDA) receptors in the hippocampus (McEwen 1999). Restraint and swim stress of rats increases extracellular concentrations of glutamate and aspartate in the prefrontal cortex, hippocampus, striatum, and nucleus accumbens

(Moghaddam 1993). Increases of glutamate and aspartate are largely associated with neuronal release, because concentrations were significantly attenuated by tetradotoxin, a sodium channel blocker (Moghaddam 1993). Restraint of rats for 6 hours daily over a period of 21 days increases concentration of glutamate in the hippocampus (Sunanda et al. 2000). Acute restraint stress of rats for 1 hour increases extracellular concentrations of glutamate in the hippocampus, but the increase is attenuated by adrenalectomy (Lowy et al. 1993). Stress not only affects glutamate concentration but also is reported to modulate glutamatergic receptor responses (Kim et al. 1996, Armanini et al. 1990, Watanabe et al. 1992). For example, restraint plus tailshock of rats reduces long-term potentiation (LTP) but enhances long-term depression (LTD) in the CA1 region of the hippocampus (Kim et al. 1996). DL-(E)-2-amino-4-methyl-5-phosphono-3-pentanoic acid, a competitive NMDA receptor antagonist, prevents the stress-induced effect on LTP and LTD (Kim et al. 1996). Stress also causes a bi-directional response of genes that regulate acetylcholine availability (Kaufer et al. 1999). Chronic stress modulates muscarinic receptors (González and Pazos 1992, Gilad et al. 1983, Finkelstein et al. 1985). For example, repeated immobilization stress of rats for 2 hours daily over 3 or 7 days increases the maximal number of muscarinic receptors (Bmax) in the cerebral cortex, CA1 region of the hippocampus, and the caudate-putamen (González and Pazos 1992). However, repeated immobilization for 10 minutes over 3 or 7 days did not affect Bmax of the muscarinic receptors as determined by autoradiographic technique (González and Pazos 1992). Cholinesterase inhibitors directly inhibit acetylcholinesterase activity, resulting in an excess of acetylcholine and desensitization of cholinergic responses. Some cholinesterase inhibitors increase extracellular glutamate levels in the hippocampus

during seizures (Lallement et al. 1991). Excess acetylcholine is proposed to mediate glutamate release (Savolianen 2001).

Proposed mechanisms for the observations reported above were that stress affected the hypothalamic-pituitary-adrenal axis (HPA), causing release of adrenocorticotropin hormone and glucocorticoids. The excess of glucocorticoids causes negative feedback to the HPA axis (Sapolsky 1998). The hypothalamus receives, sums, and integrates neuronal information from other brain regions such as brain stem, midbrain, limbic structure including hippocampus, and hypothalamus itself (Akil et al 1999). Repeated restraint stress leads to release of glutamate (Moghaddam 1993, Sunanda et al. 2000) and modulates NMDA responses in the hippocampus (McEwen et al. 1995, McEwen 1999). NMDA receptors are widely distributed in the brain and spinal cord, with particularly high densities in the hippocampus and cerebral cortex (Cooper et al. 1996). Modulation of glutamatergic transmission in the hippocampus may alter the function of hypothalamus. Cholinesterase inhibitors increase concentration of acetylcholine in the synapses in both peripheral and central nervous systems via an inhibition of esterase enzyme activities (Ecobichon 2001). Cholinergic pathways in the basal forebrain project to the entire telencephalon, including the cerebral cortex and hippocampus (Cooper et al. 1996) and muscarinic receptors (M_1 receptors) predominate in the hippocampus and cerebral cortex (Taylor and Brown 1999). Both glutamatergic and cholinergic systems are distributed in the hippocampus and cerebral cortex; therefore, an interaction of concurrent exposure to stress and cholinesterase inhibitors on the two systems is likely. Furthermore, excess glutamate (an excitatory neurotransmitter) can

endanger and/or cause cell death of neurons (McEwen et al. 1995); it could modulate the toxicity of cholinesterase inhibition.

A proposed model for neurochemical effects of concurrent exposure to repeated stress and cholinesterase inhibition was proposed (Figure 1).

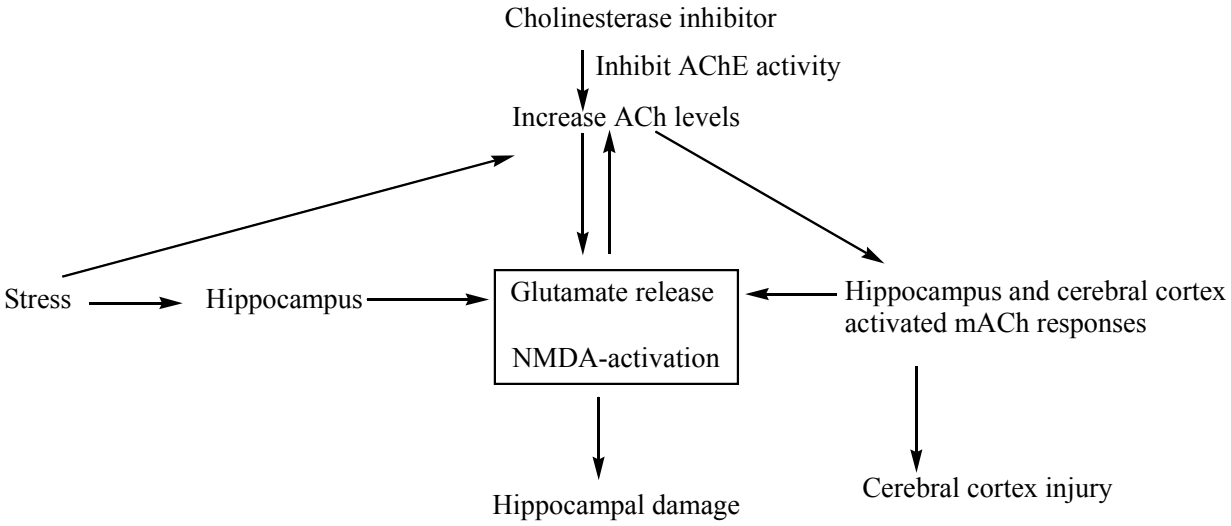


Figure 1. A proposed model for neurochemical effects of concurrent exposure to repeated stress and cholinesterase inhibition

The model was justified because stress and cholinesterase inhibitors each affect the glutamatergic and cholinergic systems and both systems are mainly located in the hippocampus and cerebral cortex. Some literature supporting the proposed mechanism and model is presented as follows: Stress is reported to modulate glutamatergic responses; for instance, stress induces the release of glutamate in the hippocampus and other brain regions (Magarinos et al. 1996). Restraint stress for one hour increased extracellular glutamate levels in the hippocampus with peaks at 20 minutes following the

initiation of stress and also immediately after the termination of stress (Lowy et al. 1993). Blockade of the glutamatergic *N*-methyl-D-aspartate (NMDA)-receptor is also effective in preventing stress-induced dendritic atrophy in neurons of the hippocampus (McEwen et al. 1995). Neurodegeneration and neuronal death following apoptosis of granule neurons in the hippocampus are regulated by stress, by an enriched environment, and by seizure-like activity (McEwen 1999). Glucocorticoid treatment causes dendritic atrophy, and stress-induced atrophy is prevented by treatment with a blocker of adrenal steroid synthesis (McEwen and Sapolsky 1995); therefore, endogenous glucocorticoids are involved in stress-induced dendritic atrophy. Glucocorticoids affect the excitatory amino acid system by regulating the release of glutamate (Lowy et al. 1993) and modulating expression of NMDA receptors in the hippocampus (Bartanusz et al. 1995).

Stress modulates cholinergic responses. For example, restraint stress 6 hours daily for 21 days decreases acetylcholinesterase activity in the hippocampus (Sunanda et al. 2000). Acute stress transiently increases the release of acetylcholine and enhances neuronal excitability (Imperato et al. 1991). Swim stress modulates genes that regulate the synthesis and metabolism of acetylcholine in the brain (Kaufer et al. 1998, Kaufer et al. 1999). In swim-stressed mice, the blood-brain-barrier is disrupted and that leads to the penetration of anticholinesterase agents (Kaufer et al. 1999). However, the disruption of the blood brain barrier by stressors is still controversial (Kant et al. 2001, Tian et al. 2002).

Cholinesterase inhibitors such as organophosphates and carbamates cause excess acetylcholine levels in the synapses of both peripheral and central nervous systems. Some organophosphates induce seizures via the excess of acetylcholine (Lallement et al. 1992).

These seizures involve glutamatergic NMDA-receptors (Raveh et al. 1999). Convulsions induced by cholinesterase inhibitors in rats increase glutamate concentrations in the hippocampus (Lallement et al. 1991). Effects of non-convulsive doses of cholinesterase inhibitors on the glutamatergic system (neurotransmitter and receptor levels) especially in presence of stress have not been studied.

Glutamate and glutamatergic receptors have been studied for their involvement in learning and memory (Francis 2003). NMDA agonists impair (Francis et al. 1993), whereas NMDA antagonists facilitate learning and memory (Lynch 1998). Francis (2003) delineated the pathways of glutamatergic and cholinergic systems in the hippocampus and neocortex. The cholinergic innervation of neocortex and hippocampus arise from the nucleus basalis of Meynert and the medial septum (Francis 2003). Both muscarinic and nicotinic cholinergic receptors activate glutamatergic pyramidal neurons and increase glutamate release (Chessell and Humphrey 1995, Dijk et al. 1995). Glutamatergic transmission in the hippocampus is from the entorhinal cortex to the dentate gyrus, CA3, CA1 and subiculum. Glutamatergic pathways also innervate from the entorhinal cortex to the neocortex (Francis 2003).

In summary, we hypothesized glutamatergic and cholinergic systems in the hippocampus and cerebral cortex were affected by stress and chlorpyrifos. Stress activates the glutamatergic system by stimulation of glutamate release and activation of NMDA receptors in the hippocampus. Overactivation of glutamatergic transmission may cause hippocampal injury. Stress also modulates cholinergic system by inhibition of acetylcholinesterase, release of acetylcholine and enhancement of neuronal excitability. When exposure to stress was concurrent with exposure to chlorpyrifos, an

acetylcholinesterase inhibitor, an addition, synergism or antagonism of glutamatergic and cholinergic responses could occur. Furthermore, chronic stress and chlorpyrifos also affected concentrations of monoamines and their metabolites (Campmany et al. 1996, Helleriegel and D'Mello 1997, Dam et al. 1999, Karen et al. 2001).

Neurochemical mechanisms associated with the interaction between repeated restraint stress, swim, unpredictable stress and toxicant-induced cholinesterase inhibition were studied. Stress models were repeated restraint, swim, and restraint with occasional swim. Repeated restraint stress represented psychological stress (Magarinos et al. 1997) that soldiers may have experienced. Swim represented acute stress (Kaufer et al 1998) (physical and probable psychological stress) and soldiers certainly experience acute stress in battle. Unpredictable stress (restraint and occasional swim stress) represented psychological and physical stress that the troops experienced during the war. Unpredictable stress could be a logical model for psychological and physical stress because rats could not predict what would happen. Chlorpyrifos was selected as a cholinesterase inhibitor because it is widely used for agriculture in the USA and chlorpyrifos was on the list of chemicals that were shipped to the Gulf War.

Effects of concurrent exposure to repeated stress and chlorpyrifos on glutamatergic and cholinergic systems in the hippocampus, cerebral cortex, and hypothalamus were studied. The hippocampus and cerebral cortex have an important role in learning, memory, and cognition and both NMDA and muscarinic receptors locate in these regions. The hypothalamus is the key region of the hypothalamic-pituitary-adrenal axis that is the major response to stress. Glutamatergic and cholinergic systems and these brain regions are involved with stress-mediated reactions. Endpoints included

determinations of glutamate and aspartate concentrations, enzyme activities associated with acetylcholine synthesis and metabolism, densities of related receptors (NMDA and total muscarinic receptors) in the hippocampus and cerebral cortex, and plasma corticosterone concentrations. Aspartate was included because it is an excitatory amino acid and reported to activate NMDA receptors. Concentrations of monoamines and their metabolites in the hippocampus, cerebral cortex, and hypothalamus were determined as well because the concentrations were affected by stress and chlorpyrifos.

SPECIFIC OBJECTIVES

These studies were designed to examine the interaction between concurrent repeated stress (restraint, swim, or restraint with occasional swim stress) and exposure to a cholinesterase inhibitor on cholinergic and glutamatergic neurotransmitter systems in a rat model. Study I was to determine a dose of chlorpyrifos that would be useful for further experiments, which included repeated stress and chlorpyrifos exposure. Study II was to determine the effects of concurrent exposure to repeated stress and chlorpyrifos on activities of acetylcholinesterase, carboxylesterase and choline acetyltransferase, concentrations of glutamate and aspartate, in the hippocampus and cerebral cortex, and concentrations of monoamines and their metabolites in the hippocampus, cerebral cortex and hypothalamus. Study III was to determine the effects of concurrent exposure to repeated stress and chlorpyrifos on maximum binding density (Bmax) and equilibrium dissociation rate constant (Kd) of NMDA and total muscarinic receptors.

Study I: Chlorpyrifos dose finding experiment

Chlorpyrifos was used to induce cholinesterase inhibition. A subcutaneous dose that decreased blood acetylcholinesterase activity 50% or more with no noticeable outward signs of poisoning except for an alteration in levels of neurotransmitter glutamate and/or norepinephrine in the hippocampus, cerebral cortex or hypothalamus or 60% of the maximum tolerated dose was suggested as a dose to examine the interaction between stress and exposure to anticholinesterase agents because this dose may not be too high with concurrent exposure to repeated stress and it may not be too low to cause

the effects on both cholinergic and glutamatergic systems. The hippocampus and cerebral cortex were chosen as brain regions of interest because they contain high densities of NMDA and muscarinic cholinergic receptors. The hypothalamus was chosen because it is the "master region" of neuroendocrine regulation (Akil et al. 1999), including the regulation of stress hormones (Akil et al 1999).

Study II: Effects of concurrent exposure to repeated stress and chlorpyrifos on concentrations of neurotransmitters in the hippocampus and cerebral cortex

The effects of three different stressors were examined: repeated restraint stress, swim stress, and repeated unpredictable stress (restraint stress plus occasional swim). Handled rats were included as control. The interaction between repeated restraint stress, swim stress, or restraint stress plus occasional swim and exposure to chlorpyrifos was examined. Control rats and treated rats exposed to the various stressors were subcutaneously injected with corn oil or chlorpyrifos on day 24 of stress treatment. The rats were continued on their stress model until day 28. The time between chlorpyrifos dosing and sacrifice was based on Lewis (2003) and Pope et al. (1991).

Specific objectives were to:

1. Determine effects on acetylcholine (ACh) activities by measuring activities of cholinergic enzymes. Activities of acetylcholinesterase (AChE), choline acetyltransferase (ChAT), and carboxylesterase (Cbxy) in the hippocampus and cerebral cortex were determined. AChE specifically hydrolyzes ACh in

the synapses to degrade ACh after neuronal transmission. Inhibition of AChE specifically increases the concentrations of ACh in the synapses, and this can result in cholinergic poisoning (Taylor and Brown 1999). Cbxy hydrolyzes a variety of esters and amides, including ACh. Cbxy detoxifies foreign compounds and protects AChE in some organophosphate poisoning. Choline acetyltransferase is an acetylcholine synthesis enzyme located in the axon terminals. High activities of ChAT can correlate with high levels of ACh synthesis (Cooper et al 1996).

2. Determine the concentrations of glutamate and aspartate in the hippocampus and cerebral cortex. Glutamate and aspartate are excitatory amino acids distributed in the brain for both protein structural and neurotransmission functions (Dingledine and McBain 1999). Several types of stress affect concentrations of excitatory amino acids (McEwen 1999, Sunanda et al 2000). The hippocampus and cerebral cortex function in learning and memory (Bear et al. 2001) and are affected by stress (especially the hippocampus) (Magarinos et al. 1997, McEwen 1999).
3. Evaluate plasma concentrations of corticosterone. Plasma corticosterone in rats is one of the stress indicators. High concentrations of plasma corticosterone indicate immediate stress response. Elevated corticosterone causes negative feedback of the HPA responses (Akil et al. 1999).
4. Assess concentrations of monoamines and their metabolites in the hippocampus, cerebral cortex, and hypothalamus. Repeated stress alters concentrations of monoamines (Campmany et al. 1996, Hellriegel and

D'Mello 1997, Adell et al. 1997). Some cholinesterase inhibitors alter concentrations of monoamines (Karen et al. 2001, Bloomquist et al. 2002).

Study III: Effects of concurrent exposure to repeated stress and chlorpyrifos on NMDA and total muscarinic receptors

Effects of concurrent exposure to repeated stress and chlorpyrifos on total muscarinic and NMDA receptors were determined. The experimental design was similar to Study II. Specific objectives were to determine maximal binding (Bmax) and equilibrium dissociation rate constant (Kd) of cholinergic (total muscarinic) and glutamatergic receptors (NMDA receptors) by radioligand binding (Motulsky and Neubig 1997, McKinney 1998, Zheng et al. 2000, Bellinger et al. 2002). Organophosphate insecticides not only inhibit cholinesterase but also down-regulate cholinergic receptors (Liu et al. 2002, Ward and Mundy 1996). Glutamate receptors (NMDA receptors) distribute mainly in the hippocampus and cerebral cortex (Cooper et al. 1996) and have been proposed to be involved in stress responses (McEwen 2000a). Plasma concentrations of corticosterone, norepinephrine and epinephrine were examined as well to assess stress hormones.

Evaluation followed these guidelines:

1. If dose-related effects of the cholinesterase inhibitor are seen in blood and brain actylcholinesterase activities and/or brain glutamate levels, the effects of

cholinesterase inhibitor on the cholinergic and/or glutamatergic systems will be considered to exist.

2. If repeated unpredictable stress (restraint and occasional swim stress) affects the cholinergic, and/or glutamatergic systems but repeated restraint stress or swim stress do not, repeated unpredictable stress (psychological and physical stressor) will be considered to be a more severe effect. In contrast, if repeated restraint stress affects the neurotransmitter systems more than swim stress or repeated unpredictable stress, the restraint stress (psychological stress) may have more involvement with the neurotransmitter systems. If swim stress affects the neurotransmitter systems more than the other stressors, swim stress (physical and probable psychological stress) will be acknowledged as more severe.
3. If the repeated stress rats concurrently treated with the cholinesterase inhibitor show different effects from individual stress or cholinesterase inhibition on cholinergic and glutamatergic systems in the hippocampus, cerebral cortex, and hypothalamus, the repeated stress will be considered to contribute to the cholinesterase inhibitor effects on the cholinergic and/or glutamatergic systems that are altered. We expected that repeated stress would cause an additive or synergistic effect with cholinesterase inhibition on the cholinergic and/or glutamatergic responses in the hippocampus and cerebral cortex.

PART II: LITERATURE REVIEW

CHAPTER 1

LITERATURE REVIEW

I. STRESS RESPONSES IN GENERAL

Stress is usually defined as a threat to homeostasis and it often refers to negative events. Stress responses depend on the past experiences (McEwen 2000a). The brain, immune system, endocrine system, and cardiovascular system are targets of stress (McEwen 2000a). Physiological systems that respond to stress are the hypothalamic-pituitary adrenal axis and the autonomic nervous system, especially the sympathetic nervous system (McEwen 2000a).

Stress hormones (corticosteroids) mediate both adaptive and maladaptive responses. Stress hormones activate protective mechanisms in a short term (flight or fight); however, the excess of these hormones can be deleterious (McEwen 2000a). Glucocorticoids (cortisol in humans and corticosterone in rodents) play many functions during both normal and stressful situations. During normal activity, the steroids manifest circadian rhythm. The diurnal cycle is regulated by activity in the hippocampus, acting through neuronal pathways to the hypothalamus, which results in a secretion of corticotropin releasing hormone. Negative feedback pathways also regulate the release of stress hormones. Cortisol potentiates activities of the sympathetic nervous system, increases the utilization of glucose and mobilization of fat, and suppresses the immune functions (Lovallo 1997).

The brain is an important organ in control of stress responses through physiological responses and behavior. The brain may respond to repeated stress over weeks by adaptation of its plasticity, which alters the neurotransmitters and the hormones. Acute stress enhances the memory of situations that cause threats to life

whereas chronic stress causes adaptive plasticity in the brain where the neurotransmitters and systemic hormones interact to produce structural and functional alterations (McEwen 2000a).

The sympathetic nervous system is responsible for the sensation most immediately associated with stress. It speeds up the heart, constricts the blood vessels, dilates the pupils, and slows the digestive system. Norepinephrine and epinephrine are the major neurotransmitters. Visceral sensation signals are sent to the brain by a visceral afferent system. The parasympathetic nervous system turns off the immediate responses and reestablishes physiology to homeostasis. When the stressors are overwhelming, the parasympathetic nervous system does not fully function to serve internal needs of the body (McEwen and Lasley 2002).

The importance of memory in stress suggests that it can be both protective and damaging to the brain. The hippocampus is thought to have a declarative memory function (ability to help remember facts) and a role in learning. The hippocampus is rich in corticosteroid receptors (McEwen and Lasley 2002). Cortisol works in the hippocampus to consolidate some kinds of memory; however, this summation still needs more confirmation (McEwen and Lasley 2002). Excitatory amino acids (especially glutamate) are abundant in the hippocampus and are essential to many kinds of learning and memory. Neurons change structure and function in response to what is learned. However, high concentrations of glutamate cause toxic effects to the brain and spinal cord (McEwen and Lasley 2002).

The hippocampus also contributes to the regulation of the hormonal stress response by sending inhibitory signals to the hypothalamus. During stress, the hippocampus takes more time to shut off the stress hormone; therefore, corticosterone can rise to high levels for longer periods of time (McEwen and Lasley 2002).

Stress affects two forms of structural plasticity in the hippocampus. Repeated stress causes atrophy of dendrites in the CA3 in the hippocampus and both acute and chronic stress suppresses neurogenesis of granule neurons in dentate gyrus (McEwen 1999). Glucocorticoids, excitatory amino acids, and *N*-methyl-D-aspartate (NMDA) receptors are involved in both atrophy of the CA3 and suppression of neurogenesis and also in neuronal death that is caused by seizures and ischemia. The hippocampal plasticity is relevant to the hippocampal atrophy in some brain disorders such as the deficits in visual and contextual memory (McEwen 1999).

II. THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS (HPA)

A) General HPA and negative feedback

The negative feedback regulation of glucocorticoid secretion is important for life because either a deficiency or excess of glucocorticoids can cause diseases. The brain-pituitary-adrenal axis is thought to function via the negative feedback of glucocorticoids on the pituitary, hypothalamus, and other brain regions that contain glucocorticoid receptors, especially in the hippocampus (Akil et al. 1999).

Brain-pituitary-organ axes have a hierarchical organization. The brain receives input information from spinal cord and cranial nerves and controls the secretion of releasing and inhibitory hormones. The pituitary gland relays the response and controls the secretion of stimulating hormones. Visceral organs produce hormones that respond to the stimulation (Akil et al. 1999). The releasing and inhibitory hormones are secreted from axons of the paraventricular nucleus in the hypothalamic neurons. The axons terminate in median eminence. Hormones and neurotransmitters are released to anterior pituitary via hypothalamic-hypophysial portal vessels. Two neuronal hormones, vasopressin and oxytocin, are secreted from large neurons that originate in the supraoptic and paraventricular nucleus in the hypothalamus, and terminate in the posterior pituitary gland (Akil et al. 1999, Guyton and Hall 1996).

The anterior pituitary gland secretes six important hormones: growth hormone, adrenocorticotropin hormone (ACTH), thyroid-stimulating hormone, follicle-stimulating hormone, luteinizing hormone, and prolactin. The posterior pituitary secretes antidiuretic

hormone and oxytocin. ACTH diffuses to blood circulation and reaches the adrenal gland (Guyton and Hall 1996).

ACTH and catecholamines exert intracellular actions in adrenal cortex using cAMP as the secondary messenger. The steroid hormones secreted by adrenal cortex, ovaries, and testes bind with their specific receptors in the cytoplasm. The combined molecules of hormone-receptor diffuse or are transported into the nucleus, and stimulate the transcription of specific genes to produce messenger RNA. The mRNA diffuses into the cytoplasm and is translated to form new proteins (Guyton and Hall 1996).

The cortex of the adrenal gland secretes corticosteroids, including mineralocorticoids, glucocorticoids, and androgens. Androgens are secreted in small amount and function like the sex hormone, testosterone (Guyton and Hall 1996). The adrenal medulla secretes norepinephrine and epinephrine.

Glucocorticoids are involved in the metabolism of carbohydrates, proteins and fat. They stimulate gluconeogenesis, decrease glucose utilization of cells, decrease the quantity of protein in most tissues, but increase proteins in the liver and plasma, increase concentrations of amino acid acids in the plasma, and mobilize fatty acids (Guyton and Hall 1996).

Glucocorticoid feedback is divided into three types depending on time and characteristics of glucocorticoid administration: fast, intermediate and delayed feedback (Young 1995). Fast feedback is the immediate response to stress or exogenous glucocorticoids resulting in increase plasma ACTH and glucocorticoids within seconds to minutes. Intermediate feedback is active in 30-90 minutes after stress or steroid exposure. Both fast and intermediate feedbacks inhibit corticotropin releasing factor (CRF), which

controls ACTH secretion (Young 1995). Glucocorticoid receptor antagonists in the hippocampus modulated the negative feedback of the hypothalamic-pituitary adrenal axis following photic (dark area) and acoustic stimuli (Feldman and Weidenfeld 1999). The regulation of negative feedback may rely on glucocorticoid-receptor function. Delayed feedbacks are thought to be mediated by inhibition of transcription of genes that code for CRF and ACTH (Akil et al. 1999). Different types of feedback respond to stress differently in time and mechanisms. It may cause an excess of glucocorticoids if the feedback does not work well. Chronic glucocorticoid from the administration of corticosteroids (Magarinos et al. 1998) or repeated stress (Magarinos et al. 1997) can cause morphological changes in the hippocampus.

B) Glucocorticoid receptor and mineralocorticoid receptor and their balance during stress

There is negative feedback control when the corticosteroid levels are very high. This feedback inhibits the secretion of CRF of the paraventicular nucleus in the hypothalamus, ACTH in the anterior pituitary gland and corticosteroids in the adrenal cortex. Corticosteroids exert the feedback in two ways: a proactive mineralocorticoid receptor (MR) mode and a reactive glucocorticoid receptor (GR) mode. The mineralocorticoid receptor mode maintains the basal HPA activity, whereas the glucocorticoid receptor mode facilitates the recovery from stress-induced activation. Mineralocorticoid receptors are located at hypothalamic sites that control salt appetite and the autonomic nervous system. The highest MR expression is found outside the

hypothalamus, especially in the hippocampus. The MRs bind to corticosterone with an affinity about 10 times higher than co-localized GRs. The GRs are found throughout the brain in both neuronal and glial cells, especially in the hypothalamic CRF neurons and anterior pituitary cells. Feedback to the GRs terminates stress-induced HPA activation. The balance of MR and GR expression is postulated to be important in the stress response (De Kloet et al. 1998). Therefore, the amount of corticosterone that reaches the brain is critical to function as a modulator or regulator.

MR and GR in the hippocampus are thought to be crucial sites in the regulation of stress response and in aging (Sapolsky et al. 1986). Repeated stress or chronic glucocorticoid exposure down-regulates hippocampal MR and GR, but not hypothalamic or pituitary receptors. Sapolsky et al. (1986) suggested that periods of stress and of excessive corticosterone secretion down-regulate the number of MR receptors per hippocampal neuron. When the period of corticosterone secretion terminates, the receptors recover. However, at some point the down-regulation of these receptors is sufficient to damage hippocampal feedback to HPA axis and consequently hypersecretion of corticosteroids occurs. The further down-regulation and the further hypersecretion of corticosteroid can cause permanent loss of the hippocampal neurons. The relationships between down-regulation, hypersecretion and hippocampal damage have not been defined.

C) Effects of stress on the HPA

Stress and glucocorticoids have specific effects on cognitive function in humans and in animal models (McEwen 2000a). Glucocorticoid administration and stressful conditions causes reversible decrease of spatial memory in animal models. The acute effects of these stressors on spatial memory are expressed from a few hours to a day after the last stressor (Lupien and McEwen 1997).

Acute immobilization in non-adrenalectomized rats for 1 hour increased extracellular glutamate in the CA3 of the hippocampus. The peak rose at 20 minutes following the initiation of stress. However, there was a decrease in the basal extracellular concentrations of glutamate and there was no elevation of this neurotransmitter after acute immobilization in the adrenalectomized rats. The results suggest that glucocorticoid is important in the maintenance of basal extracellular concentrations of glutamate and may be involved in the effects of stress on the hippocampus (Lowy et al. 1993).

Chronic stress regulated glucocorticoid negative feedback differently from acute stress (Mizoguchi et al. 2001). Acute stress by water immersion and restraint for 2 hours increased the basal plasma concentrations of corticosterone and these levels remained elevated for 5 hours after the termination of stress. With chronic stress for four weeks plasma corticosterone was increased at similar levels but more quickly decreased within 2 hours. The results suggested that repeated exposure to the same stress could cause partial habituation and adaptation to the stressor (Mizoguchi et al. 2001). Implantation of 50% corticosterone pellet in rats to give constant plasma corticosterone levels of 7-8 μg/dL affected the ACTH response in both the morning and the afternoon (Young 1995). However, this regimen did not affect the fast feedback response to cortisol injection.

Pre-treatment with repeated restraint stress before a second restraint stress in rats reduced the response of plasma corticosterone but did not affect plasma adrenocorticotropin hormone (ACTH) up to the pretreatment interval (De Souza and Van Loon 1982). Acute restraint stress for 2 minutes increased plasma ACTH with the peak in 2 minutes and return to basal level by 30 minutes. The plasma corticosterone levels reached a peak by 15-30 minutes and returned to basal levels by 60-90 minutes. Pre-restraint stresses for 30 and 60 minutes intervals reduced the elevated corticosterone after the second acute restraint stress but had no effect on ACTH. Pre-restraint at 90 minutes intervals did not alter the response of corticosterone or ACTH (De Sousa and Van Loon 1982).

Subcutaneous implantation of 50 mg corticosterone in rats for 10-20 days decreased plasma corticosterone levels and caused atrophy of the adrenal gland (Lin and Singer 1990). The results suggested that exogenous corticosterone implantation impaired the HPA function. In another study, implantation of corticosterone pellets at low doses that elevated plasma corticosterone to 1.2 and 4 μg/dL for 4-6 days slightly increased morning plasma corticosterone levels and provoked the decrease of evening plasma corticosterone; however, the higher dose that elevated plasma corticosterone to 10 μg/dL only increased the morning plasma corticosterone (Akana et al. 1992).

III. THE SYMPATHETIC NERVOUS SYSTEM RESPONSE TO STRESS

A) Effects of stress on the sympathetic nervous system

The sympathetic nervous system initiates physiological changes to prepare the body for the fight and flight responses. Sympathetic activation is mediated by either direct or indirect pathways. The direct pathway is the one in which neurons terminate on target organs and release norepinephrine whereas the indirect pathway is the one which causes the release of norepinephrine and epinephrine from the adrenal medulla into circulation (Toates 1995a).

The immediate responses to stress by the sympathetic nervous system are to accelerate heart rate, constrict the blood vessels, dilate the pupils, and slow the digestive system. Peripheral sensations are sent to the central nervous system and also visceral sensation signals are sent to the brain by the visceral afferent system (McEwen and Lasley 2002). The actions of the sympathetic and parasympathetic nervous systems are antagonistic to each other (Toates 1995a). The parasympathetic nervous system turns off the immediate responses and reestablishes homeostasis. In chronic stress or prolonged stress, the parasympathetic nervous system may be inhibited from maintaining homeostasis (McEwen and Lasley 2002).

The major efferent pathways responding to stress are either through neuronal hormones in the HPA or through nerves terminating on neurons in the medullary parasympathetic ganglion or spinal sympathetic preganglion (Palkovits 1999). Different stressors may mediate through different afferent and efferent pathways, suggesting that

there are specific stress-response pathways (Palkovits 1999). General stress pathways in the neuronal and hormonal systems are proposed (Palkovits 1999). The afferent inputs are carried by either somatosensory or viscerosensory nerve fibers into the dorsal horn of the spinal cord or to the brainstem. The stress signals diffuse to various parts of the spinal cord and the brain. The stress responses are formed in different regions. The neuronal responses include short (spinal reflex) and long circuits (supraspinal integrated responses in the brain). The efferent pathways activate either the hypothalmic-pituitary adrenal axis or medullary parasympathetic or sympathetic preganglionic neurons. The descending pathways may activate spinal cord or brainstem motor neurons or neurons in the hypothalamus (Palkovits 1999).

B) Interactions between sympathetic nervous system and the HPA axis

There are neuronal pathways in the hypothalamus that provide inputs to both the HPA and to the sympathetic nervous system (Toates 1995a). The HPA axis is controlled by various neuronal pathways that relay stressful information to the paraventricular nucleus (PVN) in the hypothalamus (Herman and Cullinan 1997). The activation of PVN may involve adrenergic pathways in the brainstem (Herman et al. 1999). The functions of locus coeruleus (a brain region that contains high density of adrenergic neurons) in the HPA axis are not clear; however, the locus coeruleus is involved in stress response and may be the influence on the release of corticotropin releasing hormone (Valentino et al. 1991). Herman et al. (1999) have reported that lesions in the locus coeruleus (LC) affect the secretion of corticosterone following restraint stress. However, lesions in the LC do

not change corticosterone levels, body weight or adrenal atrophy. Herman et al. (1999) also proposed the different pathways of LC and adrenal medulla norepinephrine. The LC mediated the stress responses through its influence on suprahypothalamic structures including the limbic system and the cerebral cortex. The adrenal medulla norepinephrine system is involved in integration of systemic stimuli mediated through direct pathways to the PVN.

Within the brain, corticotropin releasing factor (CRF) is a crucial neuronal hormone in activation of the sympathetic branch of the autonomic nervous system (Toates 1995a). Corticotropin releasing hormone receptors have been located in the adrenal medulla and the receptors can increase the secretion of catecholamines (Udelsman et al. 1986).

Corticosteroids influence the biosynthesis of catecholamines at the stage of converting norepinephrine to epinephrine (Axelrod and Reisine 1984). After hypophysectomy, the activity of the enzyme that methylates norepinephrine to form epinephrine is decreased. The activity was reversed after administration of ACTH or corticosterone.

Epinephrine released from the adrenal medulla can stimulate corticotropin-releasing neurons in the hypothalamus. Epinephrine and norepinephrine can stimulate the pituitary to release ACTH (Toates 1995b).

IV. CENTRAL NERVOUS SYSTEM

A) Basic cellular structure of nervous tissues

The nervous tissue consists of two distinct cell types: neurons and glial cells (Kandel 1991). The neuron is a special cell that generates and conducts nerve impulses. The transmission of signal is very fast and measured in milliseconds. Glial cells provide insulation, support, and nourishment for functions of neurons. Glial cells are composed of astrocytes, oligodendrocytes, and microglia (Bear et at. 2001). The following information reviews some basic cellular structure of the nervous tissue and its neurochemical aspects.

The typical structures of a neuron are a cell body or soma, a dendrite, and an axon. The soma is a metabolic center for many biochemical processes including protein synthesis. The soma and the dendrite are two major regions that receive synaptic input and the dendrite plays a very important role in increasing the receptive function of the neuron by arborization of the membrane and having specialized protein molecules called receptors. The nerve impulses flow one direction from dendrite to soma and are integrated in the axon hillock. When the potential is higher than a threshold, an action potential will be generated. The action potential is conducted along the axon to the axon terminal. There are two types of axons: unmyelinated and myelinated. The myelinated axon is insulated by a myelin sheath, which is formed by an oligodendrocyte or Schwann cell. Action potentials are transmitted through a myelinated axon faster than through an unmyelinated axon (Hof et al. 1999).

Dendrite spines are specialized dendritic arbors of certain neurons. They are abundant in large pyramidal neurons but deficient in most interneurons. Spines are more numerous on the apical shafts of the pyramidal neurons than on the basal shafts. Spines receive most of excitatory input from other neurons (Hof et al. 1999). The axon terminal contains numerous synaptic vesicles that store small neurotransmitters. The terminal also contains large dense core vesicles, which store large molecule neurotransmitters such as neuropeptides.

B). Synapses

The synapse is a place where neural information is transmitted from one neuron to another. The synapse consists of presynaptic membrane, synaptic cleft, and postsynaptic membrane (Bear et al. 2001). Many types of synapses are present in the brain; however, they are divided into two general classes: electrical and chemical synapses. Electrical synapses are prevalent in the nervous systems of invertebrates and also occur in the central nervous system (CNS) in mammals. Electrical synapses permit direct and passive flow of electrical current from one neuron to another through a gap junction. At chemical synapses, the arrival of the action potential in the presynaptic nerve terminal causes neurotransmitters to be released (Purves et al. 2001). The release of neurotransmitters depends on the intracellular concentrations of calcium that result when action potentials cause calcium channels to open. The neurotransmitters bind to either ionotropic receptors or metabotropic receptors (Nicholls et al. 2001, Purves et al. 2001).

There are two major types of neurotransmitters: small molecule neurotransmitters and neuropeptides. Biogenic amines (norepinephrine, epinephrine, dopamine, serotonin, and histamine), acetylcholine, and amino acids (glutamate and γ-aminobutyric acid, (GABA), glycine and aspartate) are classified as the small molecule neurotransmitters (Purves et al. 2001). These small neurotransmitters are synthesized in the axon terminals, which receive synthetic enzymes from the cell body. The neuropeptides are synthesized in the cell body and transported to the axon terminals. The small neurotransmitters are packaged in small clear-core vesicles, but the neuropeptides are stored in the large dense-core vesicles. The mechanisms of removal of neurotransmitters from the synaptic cleft involve diffusion in combination with reuptake into nerve terminals or surrounding glial cells, degradation by transmitter-specific enzymes, or a combination of both mechanisms (Purves et al. 2001).

C) Cholinergic system

Acetylcholine

Acetylcholine (ACh) is the neurotransmitter at cholinergic neuromuscular junctions, at synapses in the ganglia of the autonomic nervous system, and at a variety of sites of cholinergic synapses within the central nervous system (Purves et al. 2001). Chemical structure of acetylcholine is seen in Figure 1.

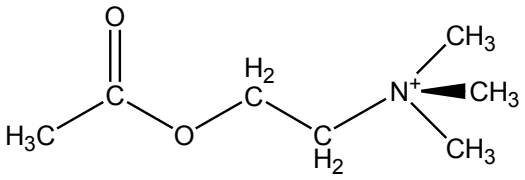


Figure 1. Chemical structure of acetylcholine

Acetylcholine is synthesized in nerve terminals from acetylcoenzyme A and choline, and the reaction is catalyzed by choline acetyltransferase (ChAT). High activities of ChAT indicate an increase of ACh synthesis. ACh is removed from the synaptic cleft by acetylcholinesterase (AChE). Cholinesterases are subdivided into AChE and butyrylcholinesterase (pseudocholinesterase). Although their molecular forms are similar, they are different entities, which are encoded by specific genes (Cooper et al. 1996). AChE hydrolyzes ACh faster than it hydrolyzes butyrylcholine, and other choline derivatives. AChE exhibits Michaelis-Menten enzyme kinetics with saturation at high substrate concentrations. In fact, with very high substrate concentrations, substrate inhibition is noted. AChE predominates in neurons, whereas butyrylcholinesterase usually is found in glial cells and non-neural tissue (Cooper et al. 1996). The active center of AChE has two main subsites. The first is an anionic site that attracts the positive charge of the ACh molecule; the other is an esteratic site that binds the carbonyl carbon atom of ACh. Acetylcholinesterase inhibitors are classified as reversible and irreversible inhibitors. Both inhibitors combine with the enzyme at the esteratic site (Cooper et al. 1996). Some drugs or insecticides inhibit AChE activity causing accumulation of ACh at cholinergic synapses. This causes ACh depolarization of the postsynaptic cells, which

can lead to neuromuscular paralysis (Purves et al. 2001). Carboxylesterase (Cbxy) hydrolyzes a variety of esters and amides, including acetycholine. Cbxy detoxifies foreign compounds to metabolies that cannot bind to AChE, resulting in protection of AChE from cholinesterase inhibition.

Cholinergic receptors

Cholinergic receptors are subgrouped into nicotinic and muscarinic receptors based on pharmacological activities of the two alkaloids nicotine and muscarine. The pharmacological activity of agonist and antagonist also help to determine the subtypes of nicotinic and muscarinic receptors. At present, nicotinic receptors are divided into N_1 receptors (selective for the agonist, phenyltrimethylammonium with location in neuromuscular junctions) and N_2 receptors (selective for the antagonists, trimethaphan and bungarotoxin with location in ganglia of the autonomic nervous system). A large number of distinct neuronal nicotinic receptors are found in the central nervous system. They are closer relatives to the nicotinic receptors in ganglia than to the receptors in muscle. Muscarinic receptors are divided into M_1, M_2, M_3, M_4, and M_5 subtypes (Taylor and Brown 1999). Atropine and l-quinuclidinylphenyl-4-benzylate (QNB), well-known muscarinic antagonists, do not distinguish the subtypes but block them equally (Cooper et al. 1996) and they are used in assays that measure total muscarinic receptor binding. M_1 has high affinity for pirenzepine and is found mainly in neuronal tissue. M_2 has high affinity for methoctramine and AFDX-116 and is mainly located in the mammalian heart. M_3 selectively binds to hexahydrosiladifenidol and is present in smooth muscle and glands. M_4 has high affinity for himbacine and is located predominantly in the striatum.

M_5 is classified due to the cloning by recombinant DNA technique (Taylor and Brown 1999).

Immunohistochemical techniques and autoradiography with muscarinic receptor antagonists provide more information on cholinergic pathways in the CNS. Both nicotinic and muscarinic receptors are widespread in the CNS. M_1 receptors predominate in the hippocampus and cerebral cortex, whereas M_2 receptors are mainly found in the cerebellum and brain stem. M_4 receptors are abundant in the striatum (Taylor and Brown 1999). Nicotinic receptors have been found predominantlt in the hippocampus and cerebral cortex (Webster 2001b). Basal forebrain cholinergic pathways project to the entire telencephalon, including the hippocampus and cerebral cortex (Cooper et al. 1996).

Cholinergic pathway

Cholinergic neurons in the central nervous system consist of in two different types: interneurons within a local circuit and projection neurons that send their axons to other regions (Cooper et al. 1996). The interneurons are found in the caudate-putamen, nucleus accumbens, olfactory tubercle and *Islands of Calleja complex*. Projection neurons are identified in two major cholinergic regions. The basal forebrain cholinergic complex projects to the entire nonstriatal telencephalon (including cerebral cortex and hippocampus (Cooper et al. 1996). The major of source of cholinergic innervation to the hippocampus is the medial septum and vertical limb of the diagonal band (Aigner 1995). The pontomesencephalotegmental cholinergic complex projects to the thalamus and other diencephalic loci and also projects to the pons, cerebellum and cranial nerve nuclei (Cooper et al. 1996, Aiger 1995). Cholinergic pathways have reticular connection and

influence directly and indirectly the cortex and limbic systems. Neuroanatomical findings suggest the connections of the cholinergic nuclei in the basal forebrain and the brainstem influence cortical and limbic system activity directly and indirectly (Aigner 1995).

The modulation of cholinergic neurons in the basal forebrain by other neurotransmitters, especially glutamate, has not been clearly elucidated. The basal forebrain neurons receive glutamatergic afferent input from the brain stem and cerebral cortex (Carnes et al. 1990). The septal nuclei receive a glutamatergic input from the hippocampus (Fonnum and Walaas 1978). The cholinergic pathway in the septo-hippocampus is indirectly modulated by NMDA receptors located on septal GABAergic neurons. Therefore, in the septum, NMDA receptors activate GABAergic neurons, which in turn inhibit the septo-hippocampal cholinergic pathway (Giovannini et al. 1994). Furthermore, Giovannini et al. (1997) demonstrate that the cholinergic system projection in the cortex is indirectly regulated by a glutamatergic input via polysynaptic GABAergic circuitry in the septum.

The relationship of the cholinergic and glutamatergic systems has been studied. Both muscarinic and nicotinic receptors activate glutamatergic pyramidal neurons and increase glutamate release (Chessell and Humphrey 1995, Dijk et al. 1995, Francis 2003). The increase of acetylcholine in the cortex facilitated neurotransmission in the corticostriatal pathway, resulting from depolarization of cortical pyramidal neuron by endogenous excitatory amino acids. The basal forebrain and brainstem cholinergic nuclei indirectly activate cortical areas via a projection to the thalamus that is thought to utilize glutamate (Aigner 1995, Levey et al. 1987). Physostigmine (a carbamate acetylcholinesterase inhibitor) intramuscular injection increases extracellular glutamate in

the striatum (Dijk et al. 1995). Furthermore, acetylcholine potentiates responses that were induced by electrophysiological stimulus to NMDA receptors (Markham and Segal 1990). Moreover, the glutamatergic input indirectly regulates the cholinergic projection in the parietal cortex via a polysynaptic GABAergic circuit in the septum (Giovanninin et al. 1997). Antagonists of both muscarinic cholinergic and NMDA receptors are thought to block the induction of long-term potentiation (LTP) (Bliss and Collingridge 1993).

Stress has been reported to affect on serotonin receptors, NMDA, and dopamine receptors (Adell 1997, McEwen 2000b). Cholinesterase inhibitors cause an accumulation of acetylcholine in the synapses, resulting in stimulation of the neuromuscular junctions, and fasciculation followed by a depolarizing blockage. Prolonged levels of ACh lead to downregulation of the cholinergic receptors (Ecobichon 2001). We anticipated that the effects of combination of stress and cholinesterase inhibitors would alter the levels of neurotransmitters and the density of neurotransmitter receptors.

D) Glutamatergic system

Glutamate

Glutamate and aspartate are thought to be the most important neurotransmitters for excitatory functions and are widely distributed in the brain. Glutamate and aspartate are nonessential amino acids and cannot cross the blood-brain barrier (Dingledine and McBain 1999). Glutamate in the brain is synthesized in the nerve terminal from two sources: from transamination of α-oxoglutaric acid, a metabolite in the Krebs cycle, and

from hydrolysis of glutamine that is synthesized in the glia cells and transported to the neuronal terminal (Cooper et al. 1996, Purves et al. 2001). Newly synthesized glutamate is packaged and stored in synaptic vesicles. Glutamate and aspartate are released through a Ca^{2+}-dependent mechanism during electrical stimulation *in vitro* (Dingledine and McBain 1999). The majority of neurons and glia are likely to be influenced by glutamate because they have glutamate receptors (Francis 2003). The released glutamate is removed from the synaptic cleft rapidly by a reuptake system. The majority of glutamate is taken up into astrocytes and converted into glutamine. Glutamine is then returned to the nerve terminal. The synthesis of glutamate is regulated by end-product inhibition, the accumulation of newly synthesized glutamate and concentrations of glutamine (Cooper et al. 1996).

Glutamate is thought to be the major neurotransmitter in the cerebral cortical and hippocampal pyramidal neurons and contributes to cognitive and memory functions (Francis et al. 2003). For examples, disturbance of glutamate transmission is associated with long-term potentiation (Baudry and Lynch 2001), Alzheimer's disease (Francis et al. 1993), and epilepsy (Meldrum and Chapman 1999). Excess of exogenous and endogenous glutamate could cause excitotoxic cell death (Francis 2003). Excitotoxicity is mediated by an excessive synaptic release of glutamate and followed by overstimulation of glutamate receptors (Sattler and Tymianski 2001).

Glutamate is one major excitatory neurotransmitter to the paraventricular neurons (PVN) in the hypothalamus and the HPA axis (Ziegler and Herman 2000, Van den Pol et al. 1990). The PVN are known to receive glutamatergic projections from upstream HPA-inhibitory regions that are regulated by hippocampus (Herman and Cullinan 1997).

Glutamatergic pathways innervating the PVN and surrounding GABAergic areas may play an important role in both inhibition and activation of the HPA axis (Ziegler and Herman 2000).

The balance of the activity of the glutamatergic and GABAergic systems controls the physiological levels of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) mRNAs in hippocampal neurons. The blockage of the glutamate receptors and/or stimulation of the GABAergic receptors reduce BDNF and NGF mRNAs in the hippocampus and septum (Zafra et al. 1991).

Glutamate receptors

Glutamate receptors mediate synaptic transmission in the nervous system. They are classified into two major groups: ionotropic and metabotropic receptors. Ionotropic receptors are also subdivided to *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA), and kainate receptors due to the pharmacological responses to agonists and antagonists.

NMDA receptors have become a major area of attention because they may be involved in the physiology and pathology of memory and learning. Glutamate and glutamatergic receptors have been considered to be involved in learning and memory (Francis 2003). NMDA agonists may improve memory whereas NMDA antagonists could contribute to deficits in learning and memory (Francis et al. 1993). NMDA receptors appear to be an important mechanism in long-term depression, long-term potentiation and developmental plasticity (Cooper et al. 1996). Studies report that NMDA

receptors may be involved in the effects of stress (McEwen and Sapolsky 1995). The following review focuses mainly on the NMDA receptors.

NMDA receptors are ligand-gated ion channels like nicotinic receptors but appear to have six distinct binding sites (Dingledine and McBain 1999, Cooper et al. 1996), including a binding site for L-glutamate and related agonists. Binding to this site promotes conduction and allows Na^+ and Ca^{2+} to enter the cell. Another binding site of NMDA receptors is the glycine site. Activation of NMDA receptors requires both glutamate and glycine binding to their sites. A site, within the channel, binds phencyclidine and noncompetitive antagonists (MK801 or 5H-dibenzo [a,d] cyclohepten-5,10-imine,10,11-dihydro-5-methyl-, (5S)-, (Z)-2-butenedioate(1:1), ketamine). This binding site acts most effectively when the receptor is activated. A polyamine regulatory binding site is activated by spermine and spermidine. Voltage-dependent Mg^{2+} binding sites and inhibitory binding site of Zn^{2+} are other sites (Cooper et al. 1996).

An excessive release of excitatory amino acids and an overstimulation of glutamatergic receptors are thought to produce neurodegeneration (Sattler and Tymianski 2001, Simon et al. 1984). Molecular mechanisms of glutamate-mediated toxicity are uncertain. However, the influx of Ca^{2+}, Na^+, and Zn^{2+} are proposed (Choi 1988, Sattler and Tymianski 2000, Weiss et al. 2000). Two major classes of Ca^{2+} channels that are activated by glutamate are voltage-sensitive Ca^{2+} channels and ionotropic glutamate receptors (Sattler and Tymianski 2001). NMDA receptors have been demonstrated to mediate excitotoxicity mainly via high Ca^{2+} permeability and individual subunits of receptors contributed to susceptibility to toxicity (Tymianski 1996). Localization of glutamate receptor clusters in spines on the dendrites is critical for function and efficacy

of physiological neuronal synaptic transmission (O'Brien et al. 1998). NMDA receptors trigger excitotoxicity both synaptically and extrasynaptically and the major factor is proposed to be the location of extracellular neurotransmitter accumulation (Sattler and Tymianski 2001). Glutamate is proposed to induce membrane depolarization by the influx of Na^{+}. This will turn on the voltage-sensitive Ca^{2+} channels and allows Ca^{2+} entry through NMDA receptors by releasing the voltage-dependent Mg^{2+} block of the channel (Sattler and Tymianski 2001).

The blockage of glutamate receptors can disrupt memory in animal models. NMDA antagonists can reversibly inhibit long-term depression and this suggests that the NMDA receptor is involved in the induction of long-term depression. The mechanism of action of long-term potentiation (LTP) has been studied in the CA1 in the hippocampus. This study suggested that an induction of LTP required activation of NMDA receptors by synaptically released glutamate during sufficient postsynaptic depolarization (Farrant 2001).

Metabotropic glutamate receptors are linked to G proteins and secondary messenger systems. The widespread distribution of metabotropic receptors in the CNS, coupled with the prevalence of glutamate as a neurotransmitter, indicates that this system is a major modulator of secondary messengers in the mammalian CNS (Cooper et al. 1996).

Glutamatergic location

Glutamate and aspartate are present in high concentrations in the brain and they have powerful stimulatory effects on neuronal activity (Cooper et al 1996). Glutamate is

thought to be the major neurotransmitter in the cerebral cortical and hippocampal pyramidal neurons (Francis et al. 2003). NMDA receptors are widely distributed in mammalian brain and spinal cord, with high density in the hippocampus and cerebral cortex.

Glutamatergic and cholinergic systems interact in learning and memory processes (reviewed by Castellano et al. 2001). For example, combined administration of noncompetitive MK-801 (a NMDA receptor antagonist) and scopolamine (a muscarinic cholinergic antagonist) impaired performance on a delayed nonmatching to sample task in monkeys while MK-801 or scopolamine administration alone had no effect (Aigner 1995). Castellano et al. (1996) demonstrated an interaction of glutamatergic and cholinergic systems in a passive avoidance task in mice. Memory consolidation improved following administration of oxotremorine (a muscarinic cholinergic agonist) but became impaired following atropine (a muscarinic cholinergic antagonist) or MK-801. The effect of MK-801 is attenuated by oxotremorine but enhanced by atropine. Li et al. (1996) demonstrated that NMDA receptors contributed to a regulation of the central cholinergic function for spatial learning in rats.

E) Catecholaminergic system

Catecholamines are biosynthesized from a common precursor, tyrosine. Tyrosine is converted to *L*-dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase. Tyrosine hydroxylase is reported to be the rate-limiting step in the synthesis of catecholamines. DOPA is then decarboxylated by aromatic amino acid decarboxylase or DOPA

decarboxylase to form dopamine. Then, dopamine is hydroxylated by dopamine-β-hydroxylase to norepinephrine. This process occurs in synaptic vesicles whereas other reactions take place in the cytoplasm. Norepinephrine is methylated to epinephrine by phenylethanolamine-*N*-methyl-transferase (PNMT), which requires *S*-adenosylmethionine as a cofactor. PNMT activity is regulated by corticosteroids (Toates 1995b). Specific neurons that contain the synthetic enzymes at each step in the biosynthetic pathway are able to release those neurotransmitters (Purves et al. 2001, Kuhar et al. 1999). Synthesis pathway is shown in Figure 2.

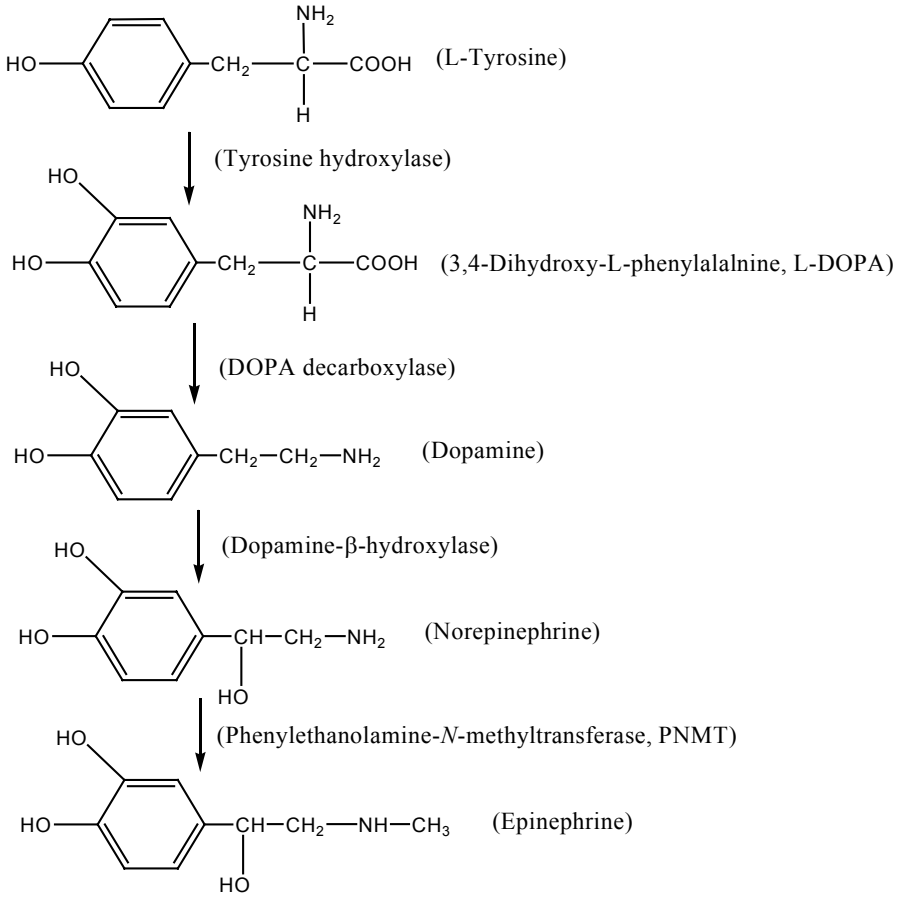


Figure 2. Biosynthesis pathway for catecholamines (Taylor and Brown 1999)

Norepinephrine is synthesized and released by neurons in the locus coeruleus and lateral tegmental of the central nervous system. The locus coeruleus nuclei are present in the lateral region of the fourth ventricle and project their axons to the spinal cord, cerebellum and through the medial forebrain for innervation of the entire cerebral cortex and hippocampus. Neurons in the lateral tegmental system innervate the axons to the brainstem and hypothalamus (Kuhar et al. 1999). The adrenergic system influences sleep and wakefulness, attention, and feeding behavior. Norepinephrine predominantly distributes in sympathetic ganglion cells (Purves et al. 2001). The activity of norepinephrine neurons within the locus coeruleus is governed by two major afferent systems of GABA inhibition input and glutamate excitatory input (Stanford 2001a). Norepinephrine is taken up from the synaptic cleft by membrane-bound norepinephrine transporters, which have hydrophobic transmembrane domains. After reuptake into the cytosol, some norepinephrine molecules are taken up into the synaptic vesicles; however, some molecules are metabolized by monoamine oxidase.

Central adrenergic pathways may contribute to depression and anxiety disorders because symptoms in patients with those two disorders are diminished by drugs that modify adrenergic transmission (Stanford 2001b). An *in vivo* microdialysis study suggested that neither novelty nor an aversive of stimulus is the cause of increased norepinephrine levels. The adrenergic pathway may influence emotion by acting on the limbic system (McQuade and Stanford 2000). Epinephrine-containing neurons in the central nervous system are found in the ventral portion of the pons and medulla. Their fibers terminate in the brainstem and hypothalamus (Kuhar et al 1999).

F) Effects of stress on neurotransmitter concentrations

Sustained stress alters the activity of the nervous system and changes the rate of release of hormones both in the central and the peripheral nervous systems (Toates 1995b). Immobilization stress elevates plasma levels of corticosterone, adrenocortocotropin hormone (ACTH), norepinephrine, and epinephrine. Immobilization stress is considered to be a combination of physical stress and psychological stress. Therefore, immobilization activates input and output of various neuronal pathways. Immobilization stress of rats increased norepinephrine levels in the paraventricular nucleus of the hypothalamus, and in the amygdala (Pacak et al. 1992, Pacak et al. 1993).

Previous chronic exposure to restraint and immobilization for 27 days reduced adrenocorticotropin hormone response of rats to an acute restraint stressor (Campmany et al. 1996). Previous chronic restraint and immobilization induced a few changes in basal levels of norepinephrine, 4-hydroxy-3-methoxyphenyl-glycol (MHPG), and 5-hydroxy indoleacetic acid (5-HIAA) (serotonin metabolite) in the pons plus medulla, midbrain, hypothalamus, hippocampus, and frontal cortex (Campmany et al. 1996). Concentrations of norepinephrine and 5-HIAA increased in the hypothalamus during chronic restraint stress. Acute restraint stress increased MHPG levels of the hypothalamus and 5-HIAA in the pons-medulla, hypothalamus, hippocampus, and frontal cortex. Chronic immobilization increased norepinephrine in the hypothalamus but did not alter MHPG and 5-HIAA levels. Acute immobilization increased 5-HIAA in the pons-medulla, midbrain, and frontal cortex. The authors suggested that the effects of repeated stress on

brain norepinephrine and their metabolites were less notable than the ACTH response for adaptation (Campmany et al. 1996).

Restraint stress to rats 6 hours per day for 21 days decreased the levels of norepinephrine, dopamine, serotonin, and acetylcholinesterase activity in the hippocampus. In contrast, levels of glutamate were increased in the stressed rats. The results suggested that chronic restraint stress (6 hours/day for 21 days) decreased aminergic and cholinergic neurotransmission; however, the stress increased the glutamatergic transmission in the hippocampus (Sunanda et al. 2000).

Stress and glucocorticoid administration have deleterious effects on the hippocampus and it has been proposed that these effects may be mediated by mechanisms involving excitatory amino acids (Lowy et al. 1993). Immobilization for 1 hour in non-adrenalectomized rats increased extracellular glutamate levels in the hippocampus, which peaked at 20 minutes following the initiation of stress. Ten minutes after termination of stress, extracellular glutamate levels were still increased. In adrenalectomized rats, basal levels of extracellular glutamate were reduced compared to those levels in non-adrenalectomized rats and there was no elevation in glutamate levels during and after immobilization in the adrenalectomized rats. The results implied that there was an interaction of the hypothalamic-pituitary-adrenal axis and glutamate neurotransmitter (Lowy et al. 1993). Adrenalectomy (ADX) attenuates the restraint stress-induced extracellular glutamate in the dorsal hippocampus and prefrontal cortex. The corticosterone replacement in ADX abolishes this effect. This confirms that glucocorticoids and glutamate contribute to the stress effects and may control each other (Moghaddam et al. 1994). An anti-epileptic drug (phenytoin) prevented stress-and

corticosterone-induced atrophy in the hypothalamus (McEwen 1999). Phenytoin inhibits glutamate release and antagonizes sodium channels that are activated during glutamate-induced excitation.

Stress causes the release of corticosterone. Sustained stress and corticosterone administration cause the release of excess glutamate in the hippocampus, resulting in damage of the hippocampal neurons (McEwen 1999, McEwen 2000a). However, serotonergic, GABAnergic and adrenergic systems are also participants in stress responses (McEwen 1999, McEwen 2000a). This study proposes that both glutamatergic and cholinergic systems are involved in stress response.

Swim stress of two 4-minute sessions caused deleterious alterations on the synthesis of acetylcholine and increased activities of acetylcholinesterase in mice corticohippocampal slices (Kaufer et al. 1998). Kaufer et al. (1999) also reported that swim stress caused disruption of the blood-brain barrier resulting in the penetration of a non-permeantcholinesterase inhibitor. Kaufer et al. (1998) reported that there was a bi-directional response of genes that regulate acetylcholine availability after swim stress in mice and the inhibition of AChE caused by cholinesterase inhibitors. Both stress and AChE inhibitors initially increased the excitatory cholinergic tone, then they caused a prolonged increase of AChE mRNA levels and a long-lasting reduction of levels of choline acetyltransferase and vesicular acetylcholine transporter. Kaufer et al. (1999) reviewed these data and suggested that acute psychological stress or exposure to cholinesterase inhibitors caused a disruption of the blood brain barrier. The increased transport of cholinesterase inhibitors into the brain in stressed mice caused more accumulation of acetylcholine, resulting in an induction of *c-Fos* gene transcription.

Additional studies have also been conducted to determine the effects of stress on brain cholinesterase and response to cholinesterase inhibitors. In these studies, swim stress did not result in enhanced inhibition of brain cholinesterase when followed by the administration of the cholinesterase inhibitor pyridostigmine (a quaternary carbamate that cannot cross blood brain barrier and was used in Gulf War) in mice. Thus, the effects of stress on access to the central nervous system by normally non-permeant cholinesterase inhibitors remain controversial (Kant et al. 2000, Grauer et al. 2000, Tian et al. 2002).

There are only a few studies demonstrating that stress interrupts the cholinergic system; however, stress affects the glutamatergic system by increasing the levels of glutamate in the hippocampus. Cholinergic pathways also project to the hippocampus. When two neurotransmitters act on common target cells, each neurotransmitter would act at its own pre- or post synaptic transmitter receptor and would interact on that target cells when both neurotransmitters occupy their receptors simultaneously (Cooper 1996). Therefore, the chance that high levels of glutamate would cause an injury to cholinergic neurons is likely. Adrenal steroids are involved in three types of the hippocampal plasticity (McEwen 1999). Glucocorticoids reversibly modulate excitability of hippocampal neurons and influence the long-term potentiation. They along with excitatory amino acids regulate neurogenesis of dentate gyrus granule neurons. Adrenal steroids and excitatory amino acids also participate in a reversible stress-induced atrophy of apical dendrites of CA3 in the hippocampus of rats (Magarinos et al. 1998).

As noted above, stress affects concentrations of glutamate and catecholamines. The glutamatergic system is involved in excitation of nervous system. An increase in

glutamate concentration in the brain may alter long-term potentiation or contribute to long-term depression.

G) Effects of stress on neurotransmitter receptors

Stress effects are associated with neurotransmitter receptors. The glucocorticoid hormones usually exert effects together with neuronal activity. NMDA and serotonin receptors play an important role in functional and structural changes induced by steroid hormones in the hippocampus (McEwen 2000a). Acute psychosocial stress affects a marker of DNA synthesis (thymidine analog bromodeoxyuridine, BrdU-labeled cell) by decreasing the number of BrdU-labeled cells in the dentate gyrus of adult tree shrew (Gould et al. 1997). Blockage of NMDA receptors with MK801 (channel blocker) increases the number of BrdU-labeled cells. Psychosocial stress and NMDA activation could regulate adult neurogenesis in the tree shrews (Gould 1997). Adrenal steroids and NMDA activation regulate neurogenesis in the dentate gyrus of developing and adult rats (Cameron et at. 1998). High concentration of corticosterone and activation of NMDA receptors reduce proliferation of granule neurons whereas adrenalectomy and blockage of NMDA receptors increased production of granule neurons. The NMDA receptor antagonist (MK801) prevents the corticosterone-induced decrease of the proliferation of granule neurons in the dentate gyrus of adult rats (Cameron et al. 1998).

The hippocampus is a plastic (reversible or flexible) and vulnerable region of the brain and a target of stress. The effects of stress hormones depend on the degree of plasticity of the hippocampus (McEwen 2000b). Repeated stress causes shortening and

debranching of dendrites in the CA3 region of the hippocampus and suppresses neurogenesis of granule neurons in the dentate gyrus. These two effects appear to be reversible and are mediated by glucocorticoid hormones working together with excitatory amino acid (EAA) (such as glutamate) and NMDA receptors, along with transmitters such as serotonin and GABA. Glucocorticoids, EAA, and NMDA receptors are involved in neuronal damage and death in pyramidal neurons by seizures, ischemia, and in severe and prolonged psychosocial stress. The relationship of dendritic remodeling to permanent hippocampal damage is complex and still is not well defined (McEwen 2000b).

The atrophy of the hippocampus caused by stress and corticosterone was prevented by phenytoin, an antiepileptic drug (McEwen 1999). Because epileptic seizures are associated with elevated glutamate levels, this implied that the mechanism of atrophy could involve the release and action of an excitatory amino acid (glutamate). Stress also induced release of glutamate in the hippocampus and other brain regions, especially through the NMDA receptor (McEwen 1999, McEwen and Sapolsky 1995).

McEwen (1999) proposed a model of the cellular and neurochemical interactions in the hippocampus influenced by stress response (Figure 3). Granule neurons in dentate gyrus proliferate in adult life. Stress and an enriched environment as well as seizures regulate neurogenesis and apoptosis of these neurons. Granule neurons send fibers to the CA3 pyramidal neurons and interneurons in the hilus, which, in turn, send inhibitory input to the CA3. The balance of the excitatory and inhibitory inputs is important for the function of CA3 neurons. Excitatory amino acids accompany glucocorticoid release during repeated stress cause reversible atrophy of apical dendrites of the CA3 (McEwen

1999). The balance of NMDA and GABAergic receptors in CA3 was proposed to play an important role in the atrophy of dendrites of neurons in this region.

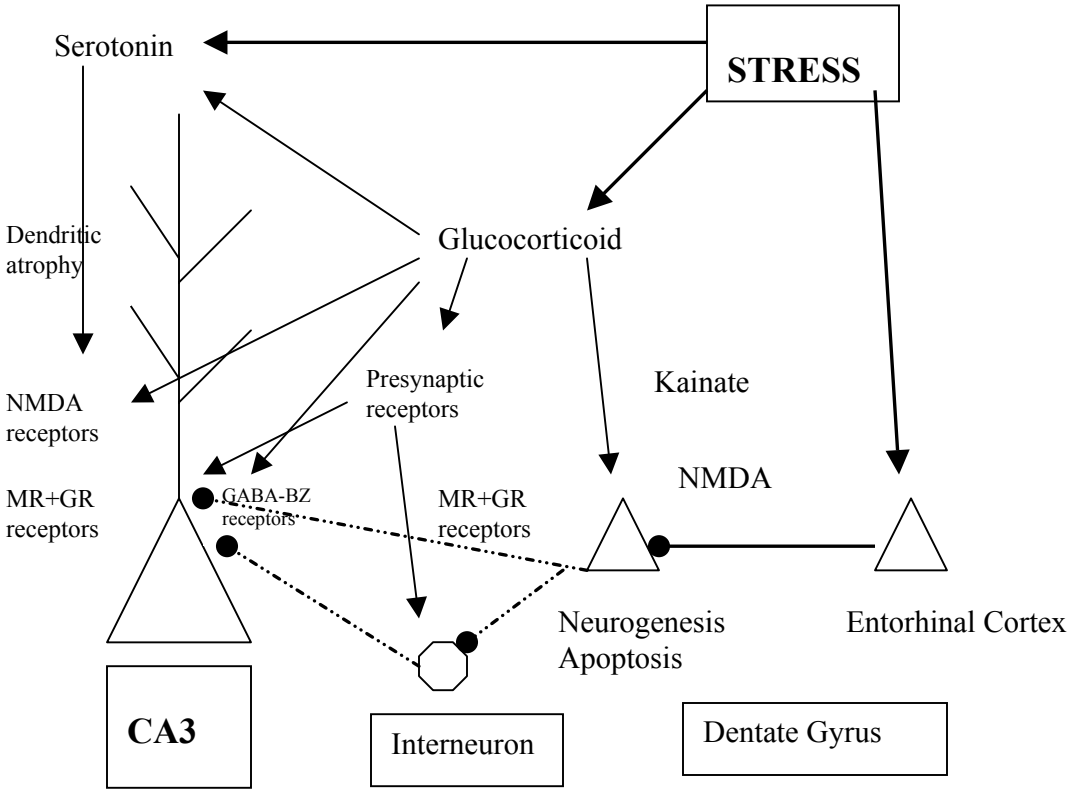


Figure 3. McEwen's proposed model of cellular and neurochemical effects in the hippocampus after exposure to stress

In conclusion, NMDA receptors in the hippocampus are involved in the stress response. The cholinergic receptors may also be involved in stress responses. Excess acetylcholine is proposed to mediate glutamate release (Savolainen 2001). The

interaction between stress and cholinesterase inhibition is likely because the glutamatergic and cholinegic systems are located in the same brain regions.

Table 1 summarizes the effects of stress on the glutamatergic and cholinergic systems.

Table 1. Summary of effects of stress on glutamatergic and cholinergic systems

Type of stress	Glutamatergic system	Cholinergic system
Chronic restraint stress 6 hour per day for 21 days	Increased glutamate levels in the hippocampus (Sunanda et al. 2000)	Decreased AChE activity in the hippocampus (Sunanda et al. 2000).
Immobilization stress	Increased glutamate levels in the hippocampus but did not alter glutamate levels in the adrenoectomized rats (Lowy et al. 1993).	
Restraint Stress and corticosterone in drinking water	Caused atrophy of dendrites of neurons in the hypothalamus. The atrophy could be prevented by phenytoin, an antiepileptic drug (McEwen et al. 1995, McEwen 1999, McEwen 2000b)	

Swim stress		Caused a bi-directional response of genes that regulate acetylcholine availability (Kaufer et al. 1998, Kaufer et al. 1999)

V. CHOLINESTERASE INHIBITOR (CHLORPYRIFOS)

A) Effects of cholinesterase inhibitors on the central and the peripheral nervous systems

There are two major classes of cholinesterase inhibitors: reversible and irreversible agents. Most of the reversible agents that are used as insecticides and therapeutic drugs are carbamates. The irreversible agents are primarily used as insecticides and chemical warfare agents, and most of them are organophosphates (Brown 1995). The irreversible organophosphates form covalent bonds with the esteratic binding sites of the acetylcholinesterase enzyme. These covalent bonds are hydrolyzed very slowly; therefore, recovery occurs only after synthesis of new enzyme (Brown 1995).

Organophosphorus insecticides are effective in protecting crops from pests, improving production of grains, vegetables, and fruits. They also improve animal wellness (Ecobichon 2001). The use of these chemicals can be safe with proper procedures. However, accidental and intentional poisoning of domestic, wildlife, and humans can occur. Since hundreds of tons of organophosphates are produced and used in many countries every year, the problems of individuals exposed to these chemicals in agriculture, at home, and in chemical plants continue. Nerve gas (some are organophosphate types) exposure is also a consideration, especially after the war in the Persian Gulf (Brown 1995).

Mechanisms of action of cholinesterase inhibitors are related to the inhibition of acetylcholinesterase, the enzyme responsible the destruction and termination of the bioactivity of acetylcholine (Ecobichon 2001). The toxic effects of organophosphates are primarily an exaggeration of the muscarinic effects and nicotinic effects of acetylcholine, and include salivation, lacrimation, urination, defecation, respiratory secretions, tremor, and paralysis (Brown 1995). Muscarinic receptors are found in the central nervous system, blood vessel walls, and endocrine and exocrine glands. Nicotinic receptors are found in autonomic nervous ganglia, central nervous system, adrenal and neuromuscular junctions (Savolainen 2001). Cholinergic muscarinic receptor activation facilitates brain metabolism and induces electrophysiological effects that may be associated with convulsions. Nicotinic receptors mediate the effects in autonomic ganglia and at neuromuscular junctions in the peripheral nervous system.

B) Toxicity and mechanisms of chlorpyrifos

Chlorpyrifos is an organophosphate insecticide that is widely used in domestic and agricultural setting. Chlorpyrifos is *O,O*-diethyl-*O*-(3,5,6-trichlor-pyridyl) phosphorothioate (a synthetic organophosphate insecticide). Trade names of chlorpyrifos are Dursban and Lorsban (Pope 1998).

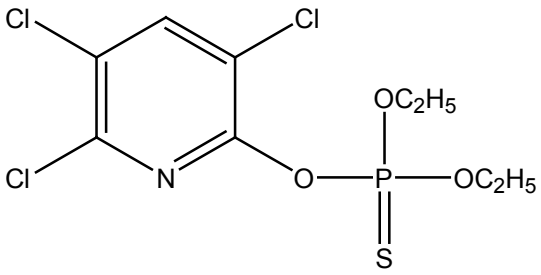


Figure 4. Chemical structure of chlorpyrifos

The solubility of chlorpyrifos in water at 25 °C is 2 mg/L and in methanol is 43 % w/w (O'Neil et al. 2001). It is usually mixed with an oily liquid before it is applied to crops or animals. Chlorpyrifos is unstable in the environment. Chlorpyrifos acts on pests basically as a contact poison, with some systemic actions (Agency for Toxic Substances and Disease Registry (ATSDR), 1996).

Chlorpyrifos enters the body through oral, dermal or inhalation routes. Chlorpyrifos is metabolized mainly in the liver by oxidative desulfuration to chlorpyrifos-oxon, which produces a neurotoxicosis following inhibition of target esterases in the peripheral and central nervous systems (Richardson 1995, Pope 1998). Detoxification is mainly via hydrolysis of chlorpyrifos-oxon to trichloropyridinol and diethylphosphate (ATSDR 1996, Sultatos and Murphy 1983). Organophosphate insecticides interact with carboxylesterase and A-esterase (Fonnum and Sterri 1981), leading to detoxicification of organophosphates. Carboxylesterases stoichiometrically bind to oxons, resulting in a decrease of molecules available for inhibiting AChE (Fonnum et al. 1985) whereas A-esterases including chlorpyrifos-oxonase protect from anticholinesterase toxicity by catalytically inactivating oxons (Li et al.1995). Reactive

chlorpyrifos-oxon can be inactivated by carboxylesterases in tissues and plasma (Chambers and Carr 1993, Karanth and Pope 2000). Carboxylesterases and A-esterases are present mainly in liver and plasma, but carboxylesterases also are located in the brain tissues. Chlorpyrifos exhibits only moderate acute toxicity in many mammalian species. This may be because the active chlorpyrifos-oxon is rapidly hydrolyzed by A-esterases (Richardson 1995, Pope 1998). Large doses of chlorpyrifos given to rats especially by subcutaneous injection can cause prolonged inhibition of brain AChE, probably because chlorpyrifos is slowly released from its deposit sites (Richardson 1995).

The toxic effects of chlorpyrifos are due to inhibition of acetylcholinesterase in the cholinergic synapses in both peripheral and central nervous systems. Accumulation of acetylcholine causes an overstimulation of the postsynaptic neurons, producing excessive activation of both muscarinic and nicotinic receptors (Pope 1998, Ecobichon 2001). Signs and symptoms of toxicosis include autonomic dysfunction (salivation, lacrimation, urination, and defecation), involuntary movements (tremors and muscle fasciculations), hyperthermia, convulsion and even death (Ecobichon 2001). Median lethal dose (LD_{50}) by the oral route is 145 mg/kg in rat. The maximum tolerated dose is 279 mg/kg sc in adult rats (O'Neil et al. 2001, Pope et al 1992). Chlorpyrifos can cause organophosphate induced delayed neuropathy (OPIDN) following chemical modification of neurotoxic esterase (NTE) (ATSDR 1996), but this only occurs at doses greater than that would cause lethality by acute cholinergic poisoning (Richardson 1995). It can also cause "intermediate syndrome", which is muscle weakness that appears after exposure and which is not associated with concurrent autonomic dysfunction (Ecobichon 2001).

C) Specific effects of cholinesterase inhibitors on the cholinergic and glutamatergic systems

The primary mechanism of action of organophosphates is inhibition of acetylcholinesterase resulting in an accumulation of acetylcholine in the synapse. However, some organophosphates and their metabolites may have additional effects besides the inhibition of acetylcholinesterase enzyme. For example, there is a direct interaction of organophosphates with muscarinic receptors (Liu et al. 2002). Some organophosphates also modulate muscarinic agonist binding, muscarinic cholinergic receptor (mAChR) expression and associated signal transduction (Ward et al. 1993, Huff and Abou-Donia 1994). Chlorpyrifos, paraoxon and malaoxon interact with M2 and/or M4 mAChRs in the rat frontal cortex (Ward and Mundy 1996, Ward et al. 1993). Chlorpyrifos-oxon also binds to M2 muscarinic receptors in rat cardiac tissue and modulates their function (Bomser and Casida 2001).

Chlorpyrifos and methyl parathion inhibit cholinesterase activity, causing down-regulation of muscarinic receptors (QNB and AFDX binding) in cortex and striatum in neonate and adult rats (Liu et al. 1999). Eight days after the last administration of chlorpyrifos or methyl parathion, AChE in neonate rats recovers relatively faster than in adult rats. With repeated chlorpyrifos subcutaneously injected, cholinesterase activity was correlated with both QNB and AFDX binding in cortex and striatum.

A single maximal tolerated dose of chlorpyrifos (279 mg/kg sc) caused inhibition of cortical and striatal cholinesterase activity and reductions in muscarinic receptor binding in both cortex and striatum in rats at 2, 4, and 6 weeks after treatment (Pope et al.

1992). However, cholinesterase activity and muscarinic receptor binding at 12 weeks were not different from control. The results suggest that muscarinic receptors are modified to down-regulate, which could be result of direct (chlorpyrifos binds to the receptor) or indirect (chlorpyrifos inhibits AChE, causing an excess of acetylcholine in the synapse) action. Paraoxon, the active oxon metabolite of parathion, binds directly with M2 receptors by displacing the binding of agonist [^{3}H] *cis*-dioxolane from the receptors (Silveira et al. 1988).

The down-regulation of cholinergic receptors and a tolerance to organophosphate inhibition can increase sensitivity to cholinergic antagonists. Pope et al. (1992) challenged chlorpyrifos treated rats with scopolamine to induce hyperactivity and expected high locomotor activity in 6 weeks as the muscarinic receptor binding was reduced. Surprisingly, hyperactivity of locomotor continued to 12 weeks even though muscarinic receptors were recovered within 6 weeks. The authors proposed that other regions than cortex and striatum might be involved in regulation of locomotor activity or chlorpyrifos did not mediate effects only through a cholinergic mechanism. In contrast, chlorpyrifos may affect motor activity through an interaction of dopaminergic and cholinergic systems. This interaction has been known to play a crucial role in mediation of some motor behaviors.

It has been speculated that excess acetylcholine induces effects on other modulatory neurotransmitters such as glutamate, norepinephrine, dopamine, and serotonin. These may be involved in the toxicity of organophosphates including contributing to the induction and/or maintenance of convulsions (Hoskins et al. 1986, Lallement et al. 1991).

An accumulation of acetylcholine in synapses is thought to have a major role in convulsions induced by the nerve gas soman, a potent acetylcholinesterase inhibitor. Lallement et al. (1992) reported that subcutaneous injection of soman inhibited acetylcholinesterase activity in the hippocampus and also increased the levels of acetylcholine in the CA1, CA3 and dentate gyrus of the hippocampus and amygdala as well. The levels of acetylcholine in the CA1, CA3 and dentate gyrus increased in two phases. The author suggested that soman inhibited the activity of acetylcholine in the first phase and that soman may open the blood brain barrier in the hippocampus and septum, resulting in the high levels of acetylcholine in the second phase (Lallement et al. 1992).

Glutamate is thought to be involved in soman-induced seizures (Lallement et al. 1991). Subcutaneous injection of soman at dose of 0.9 LD_{50} transiently increased the extracellular glutamate levels in the CA3 of the hippocampus within 30 minutes of seizures. The levels of glutamate in the CA1 were increased in two phases. There was an immediate increase of glutamate that lasted 20 minutes after the initiation of seizures. Glutamate levels then declined. This was followed by a second increase of glutamate that was persistent throughout a 90-minute period of microdialysis (Lallement et al. 1991). The author reported that the levels of glutamate in soman-treated animals both without seizures and during the preseizure period were not different from the baseline. Moreover, the uptake of glutamate was also related to the levels of extracellular glutamate. The early excess of acetylcholine and, subsequently, the high levels of glutamate suggest that acetylcholine elicits the seizure activity and glutamate may maintain the seizure. Glutamate has been involved in seizures induced by a cholinergic agonist (McDonough and Shih 1997). Glutamate acts through a number of excitatory amino acid receptors

including NMDA receptors. Savolainen (2001) reviewed and proposed that glutamatergic receptors were involved in organophosphate-induced convulsions.

Organophosphates not only alter cholinergic and glutamatergic systems but they also alter adrenergic, dopaminergic, and serotonergic systems. For example, an intramuscular injection of a single dose of 78 μg/kg soman decreased norepinephrine levels in the forebrain in convulsive rats but dopamine and serotonin metabolites were increased slowly and progressively (El-Etri et al. 1992). Soman at a convulsive dose of 31 μg/kg inhibited guinea pig brain AChE by 90%, and high levels of ACh were prolonged in most parts of the brain. In addition, soman also reduced levels of norepinephrine, but did not alter the levels of dopamine and serotonin. However, levels of metabolites of dopamine and serotonin were increased. An alteration of amino acid neurotransmitters also correlates well with the change of ACh in the brain (Fosbraey et al. 1990). The authors suggested that the changes in several neurotransmitters were secondary to the initiation of the excess of ACh, including the release of norepinephrine, the increase of dopamine and serotonin turnover, and the release of amino acid neurotransmitters (including glutamate).

In summary, cholinesterase inhibitors inhibit AChE activity resulting in an accumulation of ACh in the peripheral nervous system (PNS) and CNS, including hippocampus and cerebral cortex. Concurrent exposure of chronic stress and cholinesterase inhibitors may cause an additive or synergistic effect of ACh accumulation in the hippocampus and/or cerebral cortex. Chlorpyrifos and parathion also reduce mAChR activity in the cortex (Liu et al. 1999, Pope et al. 1992), whereas swim stress causes rapid and delayed phases of mediation of mAChR (Kaufer et al. 1999). When

cholinesterase inhibition concurrently occurs with chronic stress, it may modulate mAChR responses. Some organophosphates, such as soman, increase extracellular glutamate levels in the hippocampus during seizures (Lallement et al. 1991, Lallement et al. 1992). Excess acetylcholine is proposed to mediate glutamate release (Savolianen 2001). Stress has been discussed previously; it causes the release of glutamate and alters NMDA-response in the hippocampus. Therefore, concurrent exposure to stress and cholinesterase inhibition is proposed to have additive or synergistic effects on the glutamatergic system in the hippocampus.

VI. THE INTERACTION OF STRESS AND CHOLINESTERASE INHIBITORS

Stress has been reported to modulate the glutamatergic system; for example, stress induces the release of glutamate in the hippocampus and other brain regions (Magarinos et al. 1996). Chronic restraint stress 6 hours daily for 21 days increases glutamate levels in the hippocampus (Sunanda et al. 2000). Immobilization stress increases glutamate levels in the hippocampus (Lowy et al. 1993). Stress and corticosterone administration cause atrophy in the hippocampus; however, this atrophy could be prevented by an antiepileptic drug, phenytoin (McEwen et al. 1995).

Stress also affects the cholinergic system. Stress decreases acetylcholinesterase (AChE) activity in the hippocampus (Sunanda et al. 2000). There is controversy about the hypothesis that stress may alter blood-brain barrier permeability and let some cholinesterase inhibitors penetrate into the brain (Kaufer et al. 1998, Kaufer et al. 1999, Kant et al. 2001, Tian et al. 2002, Zhu et al 2001). Kaufer et al. (1998) reported that there was a bi-directional response of genes that regulate acetylcholine availability after swim stress in mice and the inhibition of AChE caused by cholinesterase inhibitors. The rapid and delayed phases are mediated by muscarinic cholinergic receptors. Both stress and AChE inhibitors initially increased the excitatory cholinergic tone that resulted in a prolonged increase of AChE mRNA levels and a long-lasting reduction of levels of choline acetyltransferase and vesicular acetylcholine transporter. Kaufer et al. (1999) reviewed these data and suggested that acute psychological stress or exposure to cholinesterase inhibitors caused a disruption of blood brain barrier. Zhu et al. (2001) reported that microinjection of neostigmine (a cholinesterase inhibitor) into the

hippocampus in rats increased plasma ACTH and expression of *c-Fos* in the hypothalamic paraventricular nucleus, which is widely used as a marker of neuronal activation. The authors concluded that the microinjection of neostigmine into the hippocampus could be a laboratory model of acute stress because stress also elevates ACTH and increases *c-Fos* expression. These data suggest that stress and cholinesterase inhibition may mediate responses in similar way.

Some reports show that stress may not enhance cholinergic responses to cholinesterase inhibition. For example, avoidance escape stress from footshock and yoked-stress performed in either pyridostigmine or physostigmine implanted rats was examined for the stress effects on esterase activities (Kant et al. 2001). Both pyridostigmine and physostigmine decreased blood acetylcholinesterase. Physostigmine reduced brain cortical acetylcholinesterase but pyridostigmine did not alter brain acetylcholinesterase activity. The data suggest that stress may not induce pyridostigmine to cross the blood brain barrier (Kant et al. 2001). Neither forced running nor forced swimming affected acute pyridostigmine toxicity, the inhibition cholinesterase in frontal cortex, cerebellum and hippocampus, and the entry of pyridostigmine into the brain (Tian et al. 2002). Song et al. (2002) reported that acute restraint stress neither enhanced pyridostigmine-induced toxicity including salivation, lacrimation, urination, and defecation or brain cholinesterase inhibition. The stress also did not alter horseradish peroxidase accumulation seen in cases when the blood brain barrier is disrupted.

Cholinesterase inhibitors inhibit acetylcholinesterase activity in the cholinergic synapses resulting in an accumulation of acetylcholine (Ecobichon 2001). Excess acetylcholine mediates glutamate release (Sovalainen 2001). Some cholinesterase

inhibitors also modulate muscarinic agonist binding, muscarinic receptor (mAChR) expression and association signal transduction (Ward et al. 1993, Huff and Abou-Donia 1994). Chlorpyrifos causes down-regulation of muscarinic receptors in cortex and striatum (Liu et al. 1999). Some cholinesterase inhibitors induce convulsions by increase glutamate levels and modulating NMDA receptors (Lallement et al. 1991, Raveh et al. 1999).

The glutamatergic system modulates cortical acetylcholine release in rats (Giovannini et al. 1997). Microdialysis, retrograde transport, and immunohistochemical techniques indicate that the glutamatergic input indirectly regulates the cholinergic projection in the parietal cortex via a polysynaptic GABAergic circuit in the septum. The glutamatergic system is involved in long-term potentiation, which is thought to be a fundamental process of learning and memory (Francis 2003). In case of Alzheimer's disease, glutamatergic and cholinergic system dysfunctions are highly correlated. Francis (2003) proposed that the death of glutamatergic and cholinergic neurons is due to a combination of necrosis and apoptosis, caused by effects of too much or too little glutamatergic transmission.

Cholinergic and glutamatergic neurotransmission may have an interaction on behavior. For example, scopolamine (a cholinergic blocker) and MK-801 (a glutamate antagonist) at low doses impair delayed nonmatching-to-sample but do not affect this performance when given alone, suggesting that there is an interaction between the cholinegic and glutamatergic systems in visual recognition memory (Aigner 1995). Furthermore, physostigmine (a cholinesterase inhibitor) reverses the deficits induced by scopolamine but not by MK-801, indicating that the deficits observed following NMDA

blockade are independent of those produced by muscarinic receptor blockade. The basal forebrain and brainstem cholinegic nuclei can indirectly activate cortical areas via a projection to the thalamus that is thought to utilize glutamate (Aigner 1995, Levey et al. 1987). Acetylcholine has been shown to potentiate responses, induced by electrophysiological stimulus to NMDA receptors (Markham and Segal 1990). Antagonists of both muscarinic cholinergic and NMDA receptors are thought to block the induction of LTP (Bliss and Collingridge 1993).

The proposed connection of glutamatergic and cholinergic pathway (Francis 2003) is depicted in Figure 5).

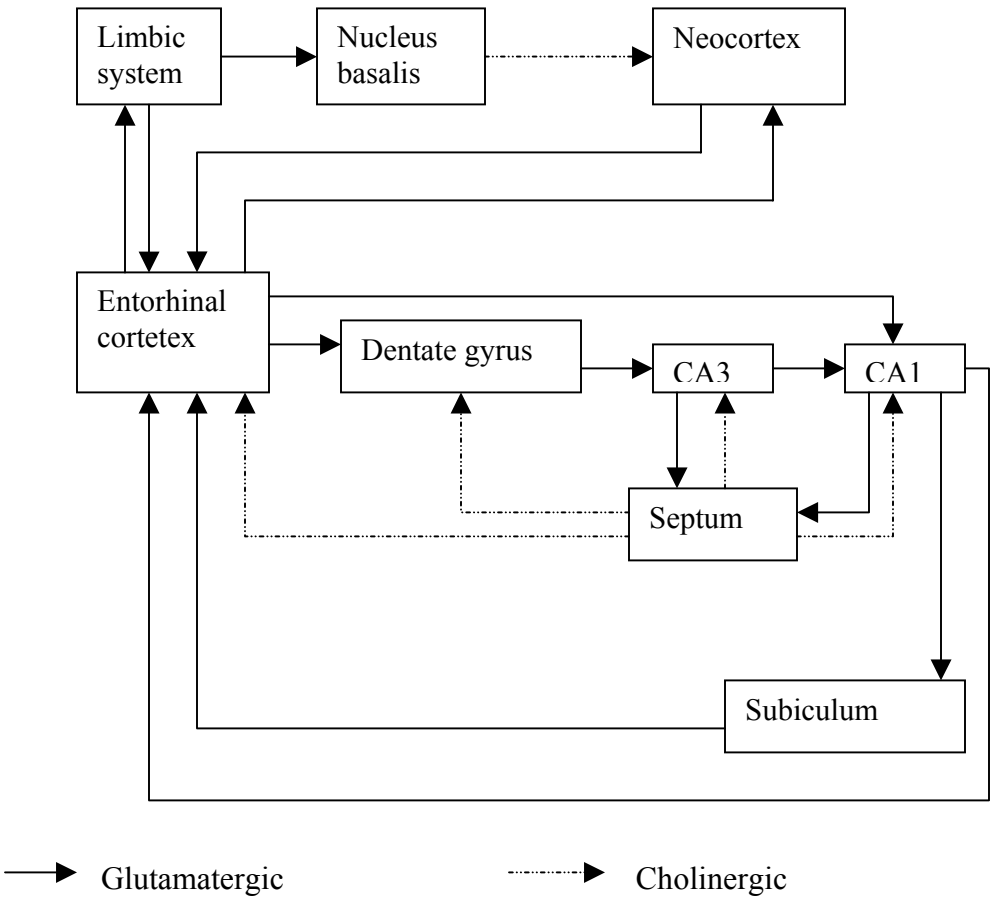


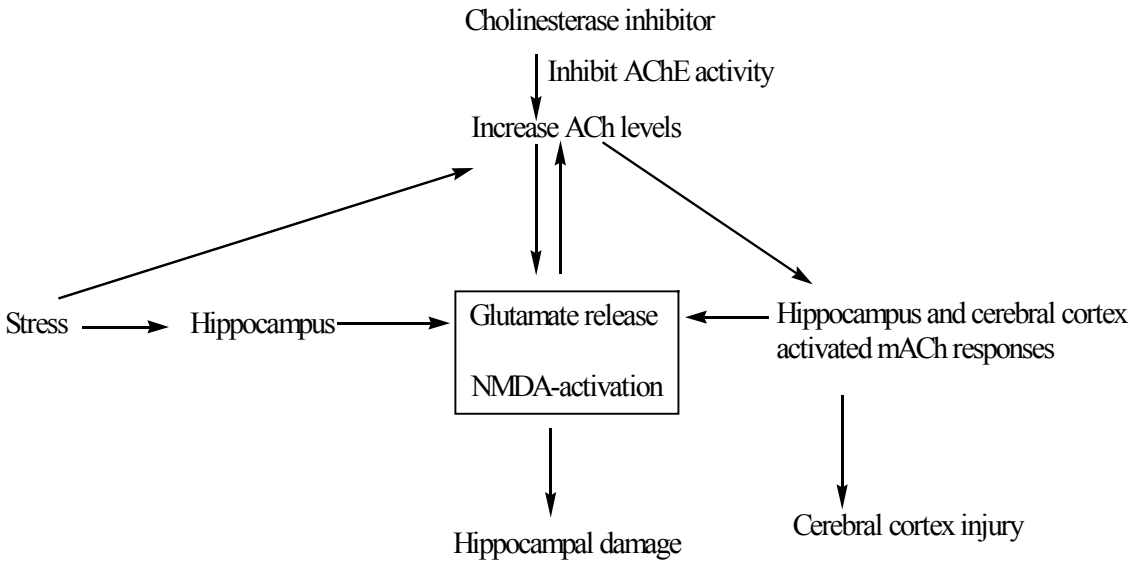
Figure 5. Proposed connection of glutamatergic and cholinergic pathways (Francis 2003)

Cholinergic pathways from the nucleus basalis of Meynert and medial septum innervate the neocortex and hippocampus, respectively. Both muscarinic and nicotinic receptors activate glutamatergic pyramidal neurons and increase glutamate release (Chessell and Humphrey 1995, Dijk et al. 1995, Francis 2003).

Glutamate facilitates the control of striatal cholinergic transmission via NMDA and non-NMDA receptor activation and agonists of both receptors have been reported to facilitate acetylcholine release from rat striatal slices (reviewed by Morari et al. 1998). NMDA receptors localize on the somatodendrite of the striatal cholinergic interneurons. Presynaptic glutamatergic modulation of ACh release was demonstrated in the cerebral cortex (reviewed by Morari et al. 1998). NMDA and non-NMDA ionotropic receptor activation increase ACh release from both rat striatal slices and synaptosomes. Furthermore, indirect evidence that an overactivation of NMDA receptors impaired striatal cholinergic transmission was demonstrated when ischemic conditions for 10 minutes caused a reversible, MK-801-sensitive, reduction of the striatal ACh release, induced by electrical stimulation (Badini et al. 1997).

In conclusion, stress and cholinesterase inhibition each affect glutamatergic and cholinergic systems in the hippocampus. It is likely that the glutamatergic system interacts with the cholinergic system in the hippocampus and cerebral cortex, as well. Proposed mechanisms examined in this dissertation are that stress affects the hypothalamic-pituitary-adrenal axis (HPA), causing release of adrenocorticotropin hormone and glucocorticoids. The excess of glucocorticoids causes negative feedback to the HPA and causes a release of glutamate and modulates NMDA responses (McEwen 1999). NMDA receptors are high densities in the hippocampus and cerebral cortex

(Cooper et al. 1996). Basal forebrain cholinergic pathways project to the entire telencephalon, including the cerebral cortex and hippocampus (Cooper et al. 1996) and muscarinic receptors (M_1 receptors) predominate in the hippocampus and cerebral cortex (Taylor and Brown 1999). Both glutamatergic and cholinergic systems are distributed in the hippocampus and cerebral cortex; therefore, an interaction of concurrent exposure to stress and cholinesterase inhibitors on the two systems is likely. Glutamatergic pathways not only innervate the hippocampal area, they also send fiber to neocortex (Francis 2003). Furthermore, both muscarinic and nicotinic receptors could activate glutamatergic pyramidal neurons and increase glutamate release (Francis 2003). The excess of glutamate can injure and/or cause cell death to neurons (McEwen et al. 1995); it could modulate the toxicity of cholinesterase inhibition. Therefore, the literatue suggests the proposed model for effects of concurrent exposure to chronic stress and cholinesterase inhibition is, as indicated previously in Figure 1 on page 6, reproduced below.



This proposed study used restraint stress as psychological stress (Magarinos et al. 1997), swim as acute stress (Kaufer et al. 1998) (physical and psychological stress), and restraint plus swim stress as unpredictable stress (Hancock et al 2003). Unpredictable stress will therefore, have both psychological and physical components. Chlorpyrifos was used as a cholinesterase inhibitor because it has only moderate toxicity and can penetrate into the brain. Concurrent exposure to stress and chlorpyrifos has been of concern in persons deployed in the Gulf War and there is a relatively high possibility of concurrent exposure in other situations.

The effects of repeated stress, CPF and interactions of repeated stress and CPF on the glutamatergic system by release of glutamate and aspartate, and modulation of NMDA receptors and also on the cholinergic system by inhibition of AChE, Cbxy and ChAT and modulation of total muscarinic receptors in the hippocampus and cerebral cortex were examined. The interactions between cholinergic enzymes, total muscarinic receptors, concentrations of excitatory amino acids and NMDA receptors in the same brain tissues within stress treatment and CPF were investigated, as well.

PART III: EXPERIMENTAL PROTOCOLS AND METHODS

CHAPTER 2

EXPERIMENTAL PROTOCOLS AND METHODS

EXPERIMENTAL PROTOCOLS AND METHODS

The studies were designed to test the hypothesis that concurrent exposure to repeated stress and a cholinesterase inhibitor would have an interaction on cholinergic and glutamatergic neurotransmitter systems. Repeated restraint, swim, and restraint with occasional swim stress were selected as stressors with handling of rats as a control. Repeated restraint stress could be suggested to represent psychological stress (Magarinos et al. 1997) that soldiers in the Gulf War experienced. Forced swimming causes acute stress (Kaufer et al. 1998) and was forced exercise, which makes it a phyical stress. Unpredictable stress (restraint with occasional swim) represents both physical and psychological stresses that could be suggested to relate to the stresses that troops experienced during the Gulf War. Chlorpyrifos was chosen as the cholinesterase inhibitor because it is widely used in agriculture and has moderate toxicity. Chlorpyrifos can cross the blood-brain barrier. Furthermore, chlorpyrifos was on the list of chemicals that were shipped to the Gulf War in 1991 (National Academy of Sciences, 2003).

Chapter 3

The study was to determine a dose of chlorpyrifos that would be useful for further studies. Four doses of chlorpyrifos (50, 100, 160, and 250 mg/kg) were subcutaneously injected in rats; whereas, control rats were given corn oil 1 ml/kg sc. Clinical signs and behaviors were observed after chlorpyrifos injection. Four days later, the rats were decapitated and hippocampus, cerebral cortex and hypothalamus were dissected. Activities of acetylcholinesterase, carboxylesterase, and choline acetyltransferase and

concentrations of glutamate were analyzed. The dose that decreased blood or brain acetylcholinesterase activity 50% or more with no clinical signs, a dose that altered concentrations of glutamate, or a dose of 60% the maximal tolerated dose were suggested as choices to examine the interaction between chronic stress and exposure to chlorpyrifos because this dose may not be too high to see effects during concurrent exposure with repeated stress. Further methodological details are included in Chapter 3.

Chapter 4

The study was to determine the effects of concurrent exposure to repeated stress and chlorpyrifos on activities of cholinergic enzymes and concentrations of glutamate and aspartate in the hippocampus and cerebral cortex. The effects of three different stressors were examined: repeated restraint stress, swim stress, and repeated unpredictable stress (restraint stress plus occasional swim). Handled rats were included as a control.

Repeated restraint stress was performed by having a rat stay in a Plexiglas tube for 1 hour per day, 5 days per week, for 28 days. Swim stress was performed by having rats swim in 22-23 °C water for 30 minutes for one day per week. Unpredictable stress was performed by four cycles of restraint stress of 1 hour per day for 4 days, swimming in 22-23 °C water for 30 minutes for one day and no stress for 2 days. Control rats, restraint rats, swim rats or restraint plus occasional swim rats were subcutaneously injected with corn oil or chlorpyrifos on day 24. Rats were continued on their stress model until day 28 when they were sacrificed by decapitation. Blood samples were collected for determination of whole blood acetylcholinesterase and plasma

corticosterone. Brains were dissected into hippocampus, and cerebral cortex for analysis of activities of acetylcholinesterase, carboxylesterase, choline acetyltransferase, and concentrations of glutamate and asparate. Further details on specific methods are found in Chapter 4.

Chapter 5

The study was designed to determine effects of concurrent exposure to repeated stress and chlorpyrifos on total muscarinic and NMDA receptors. The experimental design was similar to Chapter 4 except that hippocampus, cerebral cortex, and hypothalamus were assayed for maximal binding and equilibrium dissociation constants of NMDA and total muscarinic receptors. Further methodological details are found in Chapter 5.

Chapter 6

The study was designed to determine effects of concurrent exposure to repeated stress and chlorpyrifos on concentrations of monoamines and their metabolites. The experimental design was similar to Chapter 4 except that hippocampus, cerebral cortex and hypothalamus were analyzed for monoamines and their metabolites. HPLC was used for monamine determinations. Further methodological details are found in Chapter 6.

PART IV: RESULTS

CHAPTER 3

CHLORPYRIFOS DOSE FINDING EXPERIMENT

CHLORPYRIFOS DOSE FINDING EXPERIMENT

Abstract

Chlorpyrifos is a widely used organophosphate insecticide, which inhibits cholinesterase activity. This study determined a dose of chlorpyrifos for subsequent experiments that examined the effects of concurrent exposure of repeated stress and chlorpyrifos on the central nervous system. Rats were injected with a single dose of chlorpyrifos at 50, 100, 160, and 250 mg/kg sc (n=8). Control rats were injected with corn oil at 1 ml/kg (n=8). The lowest dose of chlorpyrifos (50 mg/kg) was high enough to maximally inhibit activities of both acetylcholinesterase and carboxylesterase. At the test doses, chlorpyrifos did not affect plasma corticosterone or concentrations of glutamate, glycine, aspartate, or norepinephrine in the hippocampus and cerebral cortex. There are no specific reports on doses of chlorpyrifos that affect glutamate concentrations in the brain. The reported maximal tolerated dose (MTD) of chlorpyrifos is 279 mg/kg sc. Therefore, a percentage of the maximal tolerated dose was chosen for subsequent experiments. The dose selected was 160 mg/kg sc (about 60% of MTD), which inhibited cortical acetylcholinesterase (80% of control) and carboxylesterase (50% of control).

Introduction

Chlorpyrifos (CPF) is an organophosphate insecticide commonly used in the United States and other countries to control agricultural and urban pests (Pope 1998,

Pope 1999). It causes an acute toxicosis through the inhibition of acetylcholinesterase (EC 3.1.1.7), the enzyme that degrades the neurotransmitter, acetylcholine. Inhibition of acetylcholinesterase causes an accumulation of acetylcholine cholinergic synapses. Overstimulation by acetylcholine of postsynaptic cholinergic receptors causes signs such as salivation, lacrimation, urination and diarrhea (Ecobichon 2001).

Pope et al. (1991) reported the subcutaneous maximal tolerated dose of CPF for adult rats is 279 mg/kg and for neonates is 45 mg/kg. At that dose, brain cholinesterase was inhibited 82-89% in adult rats. Recovery was very slow in the adult rats, with inhibition still noted 7 days after dosing, which acetylcholinesterase activities in brain, plasma or red blood cells were 10% or less of control. In contrast, adult rats given parathion had recovered brain and plasma acetylcholinesterase to about 20-30% of control at that time.

Organophosphates also bind to other esterase enzymes such as carboxylesterases (EC 3.1.1.1) without causing any obvious effects (Chanda et al. 1997). Carboxylesterases are able to alter the toxicity of several nerve gases (Maxwell 1992, Fonnum et al. 1985) and some organophosphate pesticides (Gupta and Kadel 1989). Chanda et al. (1997) demonstrated that there were tissue (brain, liver and plasma) and gender related differences for basal acetylcholinesterase and carboxylesterase activities. For example, rats given CPF at 80 mg/kg po had brain and plasma acetylcholinesterase activities that were more inhibited than carboxylesterase. *In vitro* incubation of brain tissue with chlorpyrifos-oxon indicated that acetylcholinesterase was more sensitive than carboxylesterase to inhibition. However, *in vitro* plasma acetylcholinesterase was less sensitive to chlorpyrifos-oxon than plasma carboxylesterase, but *in vivo* plasma

acetylcholinesterase was more inhibited than carboxylesterase. They proposed that the carboxylesterase activity would modify the toxicity of CPF (Chanda et al. 1997). This assumption is supported by Karanth and Pope (2000), as the MTD of CPF is highly correlated with age-related differences in both carboxylesterase and A-esterase activities in liver, plasma and lung.

CPF, parathion, and methyl parathion inhibit acetylcholinesterase in the cerebral cortex and medulla oblongata and carboxylesterase in the liver and plasma (Chambers and Carr 1993). CPF at a dose of 50 mg/kg po in rats inhibits acetylcholinesterase 43-57% when measured at time points from 2 hours to 14 days. There are no significant differences in the inhibition between days after treatment; however, the peak of inhibition occurs on day 2 and 4. CPF at a higher dose of 70 mg/kg results in more inhibition of acetylcholinesterase activity with peaks of 86% and 81% inhibition on days 1 and 2 (Chambers and Carr 1993).

Some organophosphate insecticides inhibit acetylcholinesterase activity and also release glutamate in the central nervous system (Lallement et al 1992). An accumulation of acetylcholine in synapses is thought to have a major role in convulsions induced by the nerve gas soman, a potent acetylcholinesterase inhibitor. Lallement et al. (1992) reported that subcutaneous injection of soman inhibited acetylcholinesterase activity in the hippocampus leading to increase the levels of acetylcholine in the CA1, CA3 and dentate gyrus of the hippocampus, and in the amygdala as well. Glutamate is thought to be involved in soman-induced seizures (Lallement et al. 1991). Subcutaneous injection of soman at a dose of 90% of LD_{50} transiently increases the extracellular glutamate levels in the CA3 of the hippocampus within 30 minutes of seizures. The authors reported that the

concentrations of extracellular glutamate in soman-treated animals both without seizures and during the preseizure period were not different from baseline. Furthermore, uptake of glutamate was also related to the levels of extracellular glutamate. The early excess of acetylcholine and, subsequently, the high levels of glutamate suggest that acetylcholine elicits seizure activity and glutamate may maintain the seizure. Glutamate has been implicated in induced-seizures by cholinergic agonists (McDonough and Shih 1997), but the association with the long-term acetylcholinesterase inhibition induced by chlorpyrifos has not been examined.

The sc single doses of CPF in this study were 50, 100, 160, and 250 mg/kg because the MTD is 279 mg/kg (Pope et al. 1991) and the dose of 50 mg/kg of CPF can inhibit acetylcholinesterase. Four days after CPF injection, we assessed acetylcholinesterase activity in the whole blood and activities of cholinergic enzymes in the cerebral cortex, and concentrations of amino acids and monoamines in the cerebral cortex and hippocampus previously shown to be associated with inhibition of cholinergic enzymes (Pope et al. 1992, Lewis 2003). We were converging on a dose that was not too high to cause severe toxicosis when it was combined with repeated stress.

A purpose of this study was to find a dose of CPF that would be suitable to use in subsequent studies to examine the interaction between the stress and exposure to anticholinesterase agents. An optimal dose would be one that inhibits blood acetylcholinesterase activity 50% or more with no noticeable signs of poisoning, but also one that alters activity of acetylcholinesterase and concentrations of glutamate in the hippocampus or cerebral cortex. An alternative would be a dose of 60% of the MTD

because this intermediate dose may show effects on the cholinergic and glutamatergic systems with concurrent exposures to repeated stress.

Materials and methods

A) Animals

Adult male Long-Evans rats aged 90-120 days were obtained from Harlan Sprague Dawley (Indianapolis, IN) and shipped to Laboratory Animal Resources at Virginia Tech. Rats were allowed to acclimatize to their surroundings for 7 days. They were housed individually with food and water provided *ad libitum*. Room temperature was kept at 21-23 °C with a light cycle of 7 pm to 7 am. All procedures involving animals were in accordance with the Virginia Polytechnic Institute and State University guidelines and approved by the Virginia Tech Animal Care Committee.

Experimental design was completely randomized design with 4 doses of chlorpyrifos (CPF) sc injection and one corn oil group as a control. Rats were randomly divided into 5 groups with 8 rats per group. CPF was suspended in corn oil to give a concentration of 200 mg/ml. Treatment rats were subcutaneously injected with CPF on the back behind the neck with single doses of 50, 100, 160, and 250 mg/kg body weight. Control rats were subcutaneously injected with corn oil at 1 ml/kg. After dosing, rats were casually observed for clinical signs such as tremor, salivation, lacrimation, urination, defecation, weakness and need for treatment with atropine, 0.1-0.2 mg/kg, if signs became severe. Rats were decapitated on day 4 and blood samples were collected. The brains were dissected for removal of the hippocampus and cerebral cortex and the

tissues were kept at -70°C until assayed. Whole blood samples were assayed for acetylcholinesterase acitivity. Blood samples were spun on a Beckman Microfuge Lite® centrifuge at 3,500 x g for 3 minutes. Plasma samples were collected to determine concentration of corticosterone and kept in the freezer at -70 °C until assayed.

B) Chemicals

Chlorpyrifos (*O,O´*-diethyl-3,5,6-trichloro-2-pyridinyl-phosphorothioate, 99.5% pure) was obtained from Chemical Services (West Chester, PA). Corticosterone radioimmunoassay kits were purchased from ICN Biomedicals, Inc., Costa Mesa, CA. Catecholamine and amino acids reagents were purchased from Sigma Aldrich (St. Louis, MO). Other chemicals were HPLC or analytical grade. They were purchased from Sigma Aldrich (St. Louis, MO), VWR (Suwanee, GA) and Fisher (Suwanee, GA).

C) Acetylcholinesterase assay

Acetylcholinesterase activities in the whole blood and cerebral cortex were measured by spectrophotometry at 412 nm using acetylthiocholine iodide as a substrate (Ellman et al.1961, Correll and Ehrich 1991). The whole blood was diluted to 1:1000 with phosphate buffer pH 8.0. For true acetylcholinesterase activity, iso-OMPA (N,N´,N´´,N´´´-tetraisopropylpyrophosphamide) was added at a final concentration of 10^{-8} M to inhibit psuedocholinesterase activity. The enzyme activity was determined as a difference of absorbance between incubation time at 30 minutes and time zero. Cerebral cortex was homogenized in 50 mM Tris-HCl-10 mM EDTA pH 7.5 using a 16.7 dilution factor. The homogenized samples were diluted 1:9 by 50 mM Tris buffer-0.2 mM EDTA,

pH 8.0. This stock homogenate was further diluted 1:6.9 with 0.1M phosphate buffer pH 8.0. Fifty µl of working dilution was added to microplate wells along with 150 µl of 0.1 M phosphate buffer pH 8.0, 50 µl of 6 mM of 5,5′-dithio-bis-2-nitrobenzoic acid, and 50 µl of 4.5 mM of acetylthiocholine iodide. Samples were assayed in triplicate.

D) Brain carboxylesterase

Carboxylesterase activity in the cerebral cortex was determined by the methods of Sprague et al. (1981) and Correll and Ehrich (1991). The spectrophotometric microassay was performed in a microplate with 96 wells. Cerebral cortex was homogenized in 50 mM Tris-HCl-10 mM EDTA pH 7.5 in a dilution factor of 16.7 and diluted further 1:9 by 50 mM Tris buffer-0.2 mM EDTA pH 8.0. These diluted samples of 25 µl were added to a microplate along with 125 µl of 50 mM Tris buffer-0.2mM EDTA in triplicate and then incubated at 37 °C for 20 minutes. Then, 50 µl of phenyl valerate (0.0011 g/ml in 0.03% Triton X-100) was added to all wells and incubated at 37 °C for 15 minutes. Fifty microliters of 5% sodium lauryl sulfate (including 0.02% of 4-aminoantipyrine and 0.05 M Tris-base) was added to stop the reaction and 50 µl of 0.4% potassium ferricyanide was added to initiate the redox reaction. The reaction was run for 15 minutes before reading the absorbance at 510 nm. Carboxylesterase activity was calculated on the standard curve of hydrolyzed phenol because carboxylesterase hydrolyzes phenyl valerate to give phenol, which would react with potassium ferricyanide to produce orange color. Protein was determined by using the BioRad assay kit (Bio-Rad Laboratories, Richmond, CA).

E) Plasma corticosterone

Concentration of corticosterone was measured using a radioimmunoassay and auto-gamma detection (Sousa et al. 1998, ICN Biomedicals, Costa Mesa, CA). Plasma was diluted with a steroid diluent to 1:200. The diluted plasma (100 μl) was mixed with 200 μl of antiserum to corticosterone and incubated at room temperature for 2 hours. Then, 500 μl of a precipitating solution was added and vigorously mixed to stop the reaction. The precipitate was centrifuged at 1,000 x g for 15 minutes and supernatant was decanted. A CROBA II auto-gamma counter (Packard Bioscience Company, Meriden, CT) counted the precipitate.

F) Concentrations of glutamate, aspartate and glycine

Concentrations of glutamate, aspartate, and glycine in the hippocampus and cerebral cortex were analyzed using high performance liquid chromatography (HPLC) and fluorescence detection (Piepponen and Skujins 2001, Phillips and Cox 1997). Brain tissues were homogenized in ice-cold Ringer's solution (147 mM NaCl, 1.2 mM $CaCl_2$, 2.7 mM KCl, 1.0 mM $MgCl_2$, 0.04 mM ascorbic acid) along with an internal standard (homoserine with a final concentration of 2 μM) using a Cell Disruptor Sonicator (Heat System-Ultrasonic, Farmingdale, NY) for 10 seconds before centrifugation at 13,000 x g, 4 °C for 5 minutes. Supernatants were filtered through Acrodisc LC13 mm syringe filters and diluted 20,000 fold with Ringer's solution prior to amino acid analysis. The working derivatization reagent was freshly prepared as 5 mg of *O*-pthaladehyde in 5 ml of 0.10 M sodium tetraborate and 15 μl of 14.3 mol/l of β-mercaptoethanol. The filtrate was manually mixed with the derivatization reagent (1:2 by volume) 2 minutes prior to its

injection for each sample on the analytical column. The external standard series of glutamate, aspartate, glycine and homoserine were run for quantitative analysis. A Hewlett Packard Series 1100 Quaternary Pump with a degasser and autosampler HPLC (Agilent Technologies, Wilmington, DA) was used. The separation was performed on reversed phase analytical column (Luna C18 (2) 5 μ, 150x4.6 mm, Phenomenex, Torrance, CA). Fluorescence detection was used with the excitation wavelength of 330 nm and emission wavelength of 425 nm. The mobile phase consisted of 88% of 0.05 M disodium phosphate pH 6.1 (pH adjusted with phosphoric acid) and 12% of acetonitrile. Flow rate of mobile phase was 1.2 ml/min until glycine (the last analyte) came out at 28 minutes. Then, the column was washed with deionized water for 12 minutes, followed by 30% acetonitrile in deionized water for 5 minutes and 100% acetonitrile for 8 minutes to wash out other amino acids. Then, the column was equilibrated with mobile phase for 14 minutes for the next assay.

G) Brain norepinephrine

Concentrations of norepinephrine and epinephrine in the hippocampus and cerebral cortex were simultaneously determined by high performance liquid chromatography (HPLC) and electrochemical detection (Jussofie et al. 1993). Brain samples (0.02-0.05 g) were homogenized in 200 μl of mobile phase pH 4.7 and an internal standard (isoproterenol at a final concentration of 1 μM) was added. Brain tissues were homogenized by using a Cell Disruptor Sonicator with speed at 6 cycles for 30 seconds. Homogenates were centrifuged by a Beckman Microfuge Lite® at 10,100 x g for 5 minutes and supernatants were kept in vials at –20 °C until assayed. Supernatants

were thawed on ice on the assay days and then filtered through an Acrodisc LC 13 mm syringe filter with 0.2 μm PVDF membrane into HPLC vials. The chromatographic instrument was a Hewlett Packard Series 1100 Quaternary Pump with a degasser and autosampler. The separation was performed on a reverse-phased C18 analytical column (Nucleosil 100 3μ, 250x4 mm), preceded by a guard column (Nucleosil 100 C18 3μ 8x4 mm). Electrochemical detection was used in the oxidation mode with +0.35 volt. Isocratic elution was performed at flow rate of 0.6 ml/min. The mobile phase was citric buffer pH 4.7 composed of 0.1 M sodium acetate, 25 mM citric acid, 134 μM ethylenediaminetetraacetic acid (EDTA), 230 μM octanesulfonic acid, and 6% methanol. The external standard solutions of norepinephrine bitartrate, epinephrine hydrochloride, dopamine hydrochloride, 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptamine oxalate (serotonin), 5-hydroxyindole-3-acetic acid (5-HIAA), and isoproterenol hydrochloride were purchased from Sigma used for quantitative analysis.

H) Statistical analysis

Differences of mean were analyzed by one-way analysis of variance (ANOVA) using SAS program (version 8.2, SAS Institute Inc., Cary, NC). Blood acetylcholinesterase and plasma corticosterone were subsampling variables whereas brain acetylcholinesterase, carboxylesterase, glutamate, and norepinephrine were single sampling. Post hoc comparisons were tests of Tukey's HSD.

Results

Chlorpyrifos-treated rats did not show any outward signs of cholinergic stimulation (salivation, lacrimation, urination, diarrhea, or tremor) after the injection during 4 days after dosing. No atropine treatment was required.

Chlorpyrifos (CPF) sc single doses of 50, 100, 160, and 250 mg/kg inhibited blood acetylcholinesterase (AChE) in the range of 75 to 81% of control (Table 1). CPF at 50 mg/kg was able to decrease blood AChE activity by 75%. Chlorpyrifos also inhibited cerebral cortex AChE and carboxylesterase activities at doses of 50 mg/kg to 250 mg/kg. Esterase inhibitions did not necessarily correlate with doses. It appeared that the dose of 50 mg/kg sc CPF was high enough to maximally inhibit cerebral cortex AChE and carboxylesterase activities (Figure 1 and 2).

To investigate whether test doses of CPF caused stress to the rats 4 days after injection, plasma corticosterone was assayed. However, plasma corticosterone concentrations were not affected by any dose of CPF (Figure 3).

There were no significant differences in effects of CPF on concentrations of glutamate, aspartate, and glycine in the hippocampus and cerebral cortex when samples from treated rats were compared to controls (Figure 4 and 5).

CPF did not alter concentrations of norepinephrine in the hippocampus and cerebral cortex (Figure 6). Epinephrine results were not reported because their peaks were not consistent and near the detection limit.

Discussion

Chlorpyrifos (CPF) at doses of 50, 100, 160 and 250 mg/kg sc inhibited blood AChE, and AChE and carboxylesterase activities in the cerebral cortex. The inhibition was maximal at the lowest dose and did not follow a dose response. Therefore, results suggested that chlorpyrifos doses of more than 50 mg/kg sc had sufficient effects on cholinergic enzyme activities that they could be used in further experiments.

CPF at the test doses did not alter plasma corticosterone concentrations 4 days after treatment. Therefore, stress as indicated by plasma corticosterone concentrations could not be determined after dosing with CPF.

Since CPF did not affect concentrations of glutamate, aspartate, glycine, or norepinephrine in the hippocampus and cerebral cortex, the results did not support the choice of a dose to be used for the next experiments. However, CPF-treated rats, even the highest dose, did not show any cholinergic signs or convulsions. The doses may not have been high enough or the brain samples may have been collected too late to demonstrate alterations in these amino acid neurotransmitters.

Although there is no specific report that CPF affects glutamate concentrations in the brain, concentrations of glutamate were expected to be higher in the CPF groups, especially at 250 mg/kg, as some cholinesterase inhibitors induce the release of glutamate in the hippocampus (Lallement et al. 1991). Organophosphate compounds have the potential to induce seizures at high dosages (Lallement et al. 1991, El-Etri et al. 1992), although none were seen in the CPF-dosed rats in this study. Chronic stress has been shown to increase concentrations of glutamate in the hippocampus (McEwen 2000, Sunanda et al. 2000).

The maximal tolerated dose of CPF in adult rats is 279 mg/kg sc with the time of maximal brain cholinesterase inhibition of CPF in adult rats being 4 days after treatment (Pope and Chakraborti 1992, Pope et al. 1991, Mary 2003). At the maximal tolerated dose, CPF reduces total muscarinic receptor binding in the cortex and striatum (Chaudhuri et al. 1993). Therefore, a percentage of the maximal tolerated dose (MTD) was chosen for further experiments on the interaction of stress and organophosphate toxicity. Sixty percent of the MTD of CPF (160 mg/kg) was selected because this dose inhibited AChE (85%) and Cbxy (50 %). When CPF is concurrently administrated to rats undergoing repeated stress, this intermediate dose may be optimal for exhibiting interactive effects on the glutamatergic and cholinergic systems.

Acknowledgment

The author would like to thank the staff of the toxicology, neurotoxicology, and biochemistry laboratories of Virginia Tech for technical support. Statistical analysis was assisted by Dan Ward. This work was granted by DAMD 17-99-1-9489 from the U.S. Army Medical Research and Materiel Command and by Virginia-Maryland Regional College of Veterinary Medicine. This work does not necessarily reflect the position or policy of the U.S. Government.

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Table 1. Effect of chlorpyrifos on acetylcholinesterase activity of whole blood

Dose (mg/kg)	Blood AChE (μmole/ml/min)	% control of blood AChE	% inhibition
0	3.226 ± 0.112	100 ± 0	0
50	0.806 ± 0.064*	24.6 ± 1.9*	75.4
100	0.614 ± 0.050*	18.8 ± 1.5*	81.2
160	0.755 ± 0.053*	23.1 ± 1.6*	76.9
250	0.655 ± 0.036*	20.0 ± 1.1*	80.0

Rats were sc injected with chlorpyrifos at ssingle doses of 0, 50, 100, 160 and 250 mg/kg. They were sacrificed 4 days after dosing. Data are presented as mean ± SEM as μmole/ml/min and percent of control. (* indicates $p < 0.001$).

Figure legend

Figure 1. Effect of chlorpyrifos on acetylcholinesterase activity of cerebral cortex. Rats were injected with chlorpyrifos at single doses of 0, 50, 100, 160 and 250 mg/kg sc. They were sacrificed 4 days after dosing. Data are presented as mean ± SEM (nmole of acetylthiocholine hydrolyzed/min/mg protein). * Significant difference from control ($p < 0.001$).

Figure 2. Effects of chlorpyrifos on carboxylesterase activity of the cerebral cortex. Rats were injected with chlorpyrifos at single doses of 0, 50, 100, 160 and 250 mg/kg sc. They were sacrificed 4 days after dosing. Data are represented as mean ± SEM in nmole phenyl valerate hydrolyzed/min/mg protein. * Significant difference from control ($p < 0.001$).

Figure 3. Effects of chlorpyrifos on plasma concentrations of corticosterone. Rats were injected with chlorpyrifos at single doses of 0, 50, 100, 160 and 250 mg/kg sc. They were sacrificed 4 days after dosing. Data are represented as mean ± SEM in ng/ml.

Figure 4. Effect of chlorpyrifos on concentrations of glutamate, aspartate and glycine in the hippocampus. Rats were injected with chlorpyrifos at single doses of 0, 50, 100, 160 and 250 mg/kg sc. They were sacrificed 4 days after dosing. Data are presented as mean ± SEM in μmole/g tissue.

Figure 5. Effect of chlorpyrifos on concentrations of glutamate, aspartate and glycine in the cerebral cortex. Rats were injected with chlorpyrifos at single doses of 0, 50, 100, 160 and 250 mg/kg sc. They were sacrificed 4 days after dosing. Data are presented as mean ± SEM in μmole/g tissue.

Figure 6. Effects of chlorpyrifos on concentrations of norepinephrine in the hippocampus and cerebral cortex. Rats were injected with chlorpyrifos at single doses of 0, 50, 100,

160 and 250 mg/kg sc. They were sacrificed 4 days after dosing. Data are presented as mean ± SEM in nmole/g tissue.

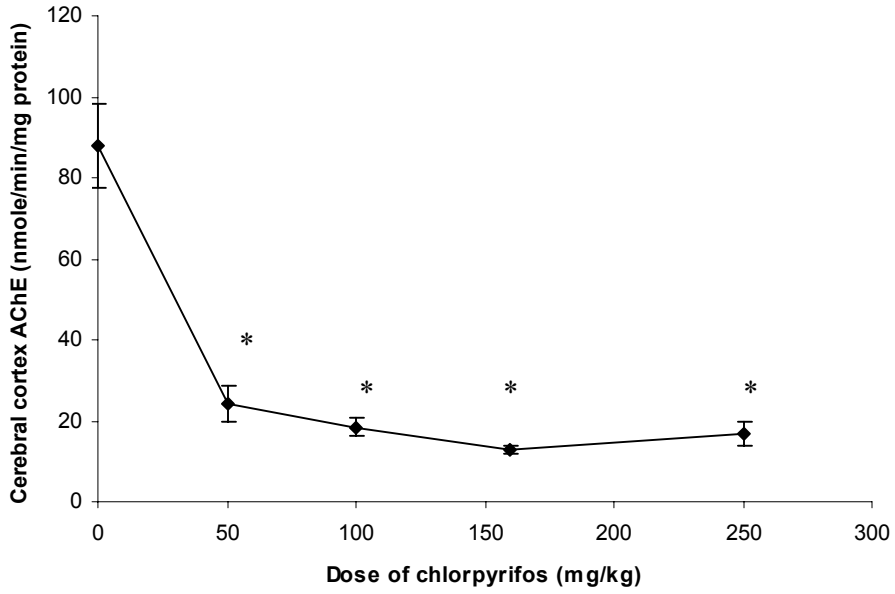

Figure 1.

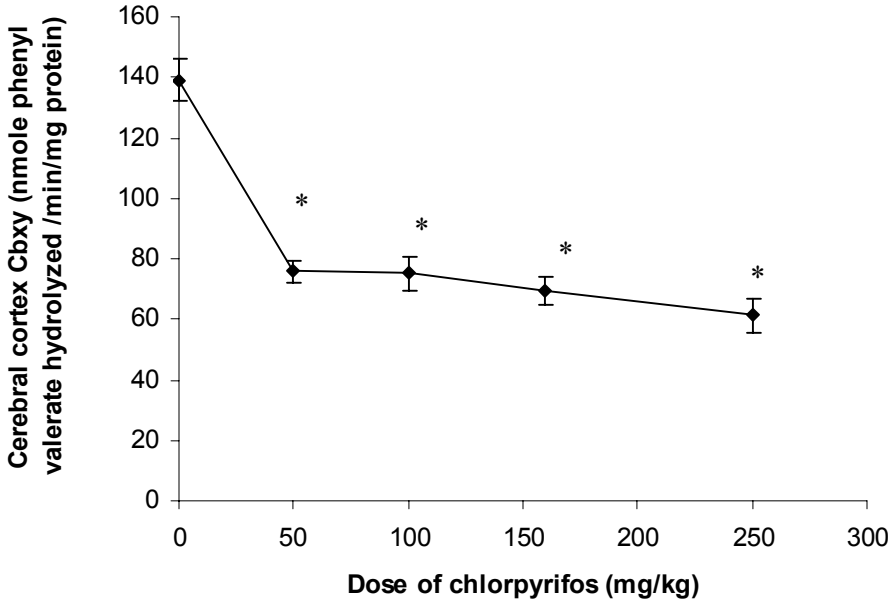

Figure 2.

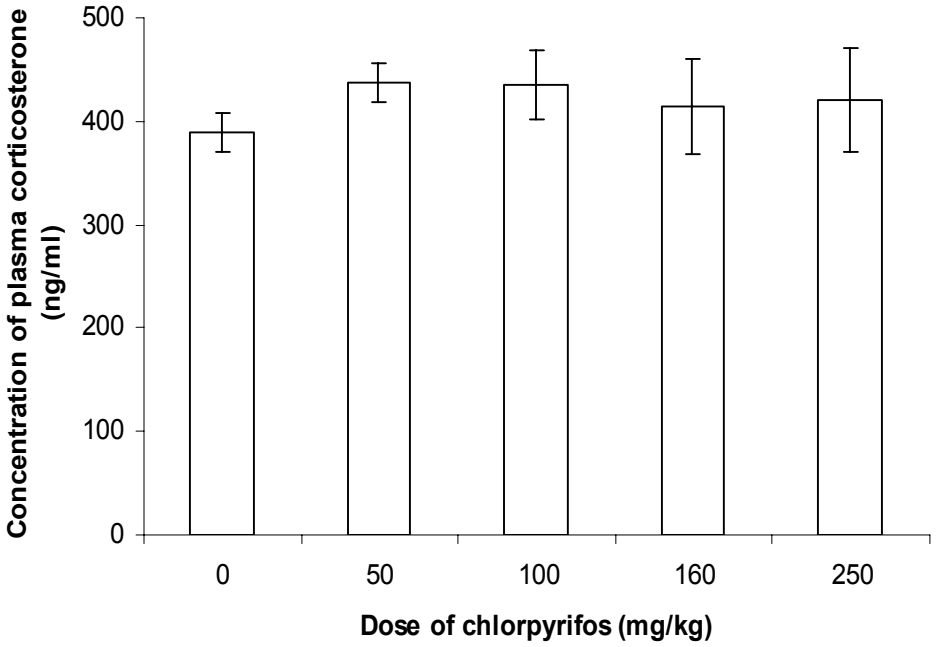
Concentration of plasma corticosterone (ng/ml)
500
400
300
200
100
0
0
50
100
160
250
Dose of chlorpyrifos (mg/kg)

Figure 3.

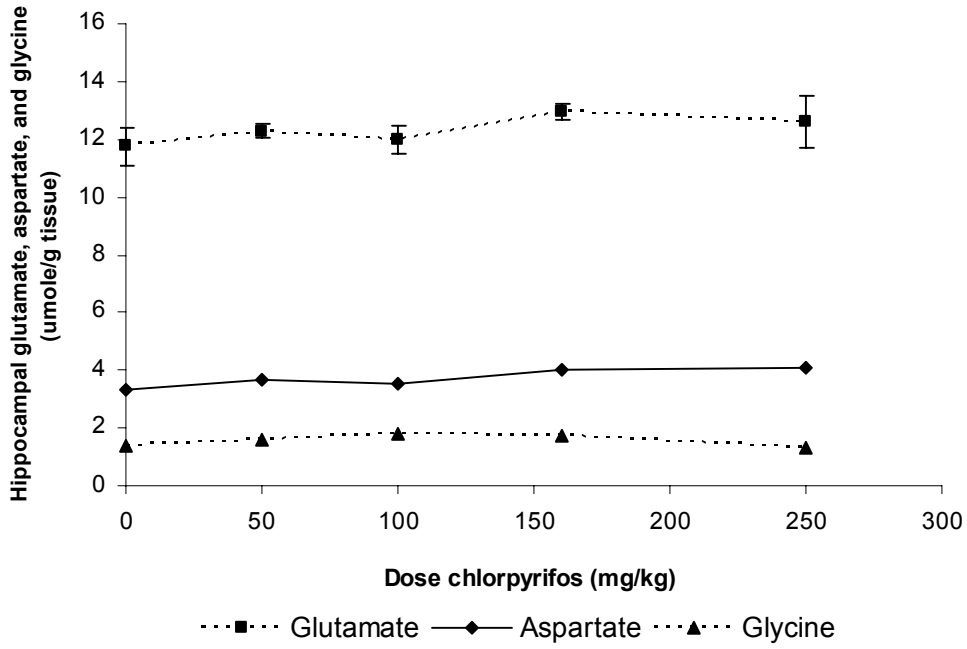

Figure 4.

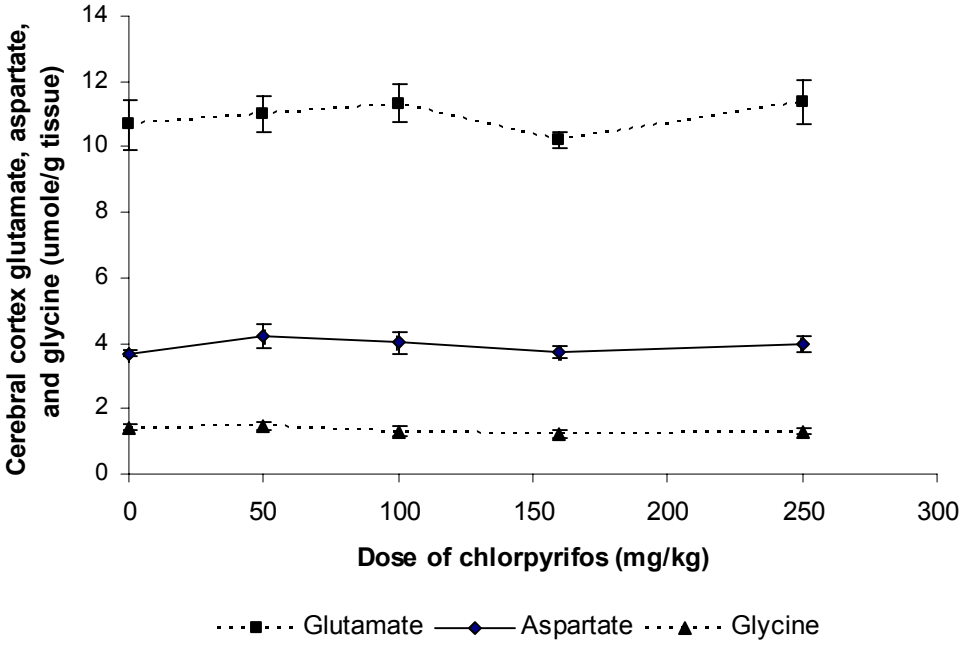

Figure 5.

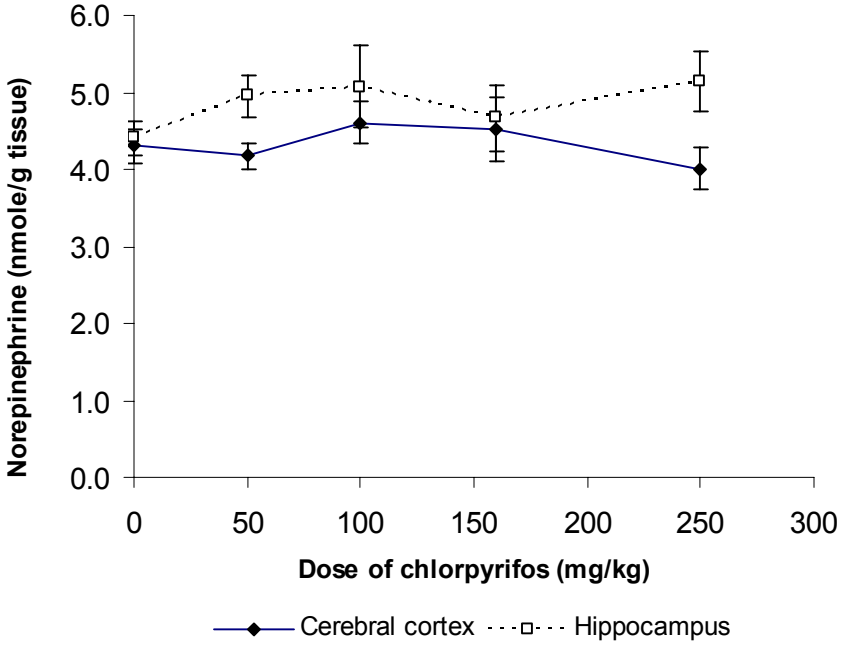
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Norepinephrine (nmole/g tissue)
0
50
100
150
200
250
300
Dose of chlorpyrifos (mg/kg)
Cerebral cortex
Hippocampus

Figure 6.

CHAPTER 4

EFFECTS OF REPEATED STRESS AND CHLORPYRIFOS ON CHOLINERGIC ENZYMES AND GLUTAMATE CONCENTRATIONS

EXAMINATION OF CONCURRENT EXPOSURE TO REPEATED STRESS AND CHLORPYRIFOS ON CHOLINERGIC ENZYMES AND GLUTAMATE IN THE HIPPOCAMPUS AND CEREBRAL CORTEX

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Abstract

Repeated stress has been reported to cause reversible impairment of the hippocampus. Cholinergic and glutamatergic systems were proposed to change following stress and exposure to cholinesterase inhibitors. Effects of concurrent exposure to repeated stress and chlorpyrifos (CPF) on swimming behavior, enzymes of acetylcholine synthesis and metabolism and concentrations of glutamate and aspartate in brain were studied in Long Evans rats. Groups of rats (n=7) were handled 5 days/week; restrained 1 hour/day for 5 days/week; swum 30 minutes for 1 day/week; or restrained 4 days/week and swum for 1 day/week, for 28 days. On day 24, each group was injected either with corn oil or CPF 160 mg/kg sc 4 hours after restraint. On day 28, blood samples were collected for acteylcholinesterase (AChE) activity. Brains were dissected into hippocampus (HP) and cerebral cortex (CC) for determination of AChE, carboxylesterase (Cbxy), and choline acetyltransferase (ChAT) activities, and glutamate and aspartate concentrations. On day 28, CPF-treated rats that were swum and CPF-treated rats that were restrained and swum had average swim times of 27.1 minutes. Rats that were swum and that were restrained and swum but not given CPF had average swim times of 29.4 and 30 minutes, respectively. CPF inhibited AChE activity in blood, CC and HP, but stress did not affect blood AChE activity. CPF inhibited Cbxy activity in CC and HP. Restraint with swim stress also inhibited cortical Cbxy (40.05 ± 2.21 compared to ranges of 53.49-56.54 nmole phenyl valerate hydrolyzed /min/mg protein in other groups). Repeated stress and CPF individually inhibited Cbxy. Neither repeated stress nor CPF affected ChAT activity in CC and HP. Restraint with swim had a statistical trend toward increased concentrations of glutamate in hippocampus more than swim alone ($p = 0.064$).

CPF did not affect concentrations of glutamate. However, CPF decreased the elevated concentrations of aspartate in the hippocampus of rats that were restrained and swum. In summary, restraint with swim inhibited of Cbxy and increased glutamate in the HP. The interaction of restraint with swim and CPF was demonstrated on aspartate concentrations of the HP. Table 4 summarizes the overall results of this study.

Keywords: Stress, chlorpyrifos, cholinergic enzymes and glutamate.

Introduction

Exposure to sustained stress has numerous pathological effects, especially in the nervous system. Long-term exposure to stress can cause physical and mental illnesses such as hypertension, headache, gastric ulcer, inflammation, and neuronal atrophy (McEwen 2001). Chronic restraint stress leads to atrophy of apical dendrites in the pyramidal CA3 of the hippocampus of rats and tree shrews (Magarinos and McEwen 1995, Magarinos et al. 1996, Magarinos et al. 1997). Concurrent exposure to repeated stress and cholinesterase inhibitors is of concern because Gulf War veterans were exposed to a combination of these agents, including nerve gases and pesticides (Sapolsky et al. 1998, National Academy of Sciences, 2003). Other individuals under stress may be exposed to pesticides as well. Mechanisms for atrophy of hippocampal neurons have not been well defined. We hypothesized that concurrent exposure to repeated stress and cholinesterase inhibitors may lead to additive, synergistic, or antagonistic effects on the glutamatergic and cholinergic systems and that such changes could be responsible for hippocampal atrophy. This was suggested from other studies. For example, stress elevates

glucocorticoid hormones (Sapolsky et al. 1986, Herman and Cullinan 1997, Mizuguchi et al. 2001). Glucocorticoid hormones mediate a variety of effects on neuronal excitability, neurochemistry, and structural plasticity (DeKloet et al. 1998), including effects associated with the glutamatergic system of excitatory amino acids. This can occur because glucocorticoid hormones modulate excitatory amino acids, and their *N*-methyl-D-aspartate (NMDA) receptors (McEwen 1999, McEwen 2000). Excitatory amino acids (including glutamate and aspartate) and NMDA receptors are involved in neuronal damage and death, especially in pyramidal neurons in the hippocampus and cerebral cortex after seizures. A similar mechanism may contribute to the impairment of the hippocampus following severe and chronic psychosocial stress (McEwen 2000).

Restraint, swim, and restraint with occasional swim were selected stress models. Restraint stress relates to psychological stress (Magarinos et al. 1997) and leads to release of glutamate and activates NMDA receptors in the hippocampus (McEwen 2000, Sunanda et al. 2000). Swim causes both physical and psychological stress and modified gene expression of acetylcholine enzymes (Kaufer et al. 1999). Restraint with occasional swim is a model of unpredictable physical and psychological stress.

The toxic agent we investigated was a cholinesterase inhibitor. Cholinesterase inhibitors are used as medications, pesticides, or warfare agents (Ecobichon 2001). In addition to inhibiting cholinesterase enzymes (Ecobichon 2001), cholinesterase inhibitors can also modulate the glutamatergic system. For example, some cholinesterase inhibitors increase extracellular glutamate concentrations (Lallement et al. 1991) and pyridostigmine enhances glutamate transmission in the hippocampus (Pavlovsky et al. 2003). Chlorpyrifos, the organophosphate used in our study, inhibits cholinesterase

activity, including acetylcholinesterase, in both peripheral and central nervous systems (Pope and Chakraborti 1992, Ecobichon 2001). Carboxylesterases detoxify chlorpyrifos by stoichiometrically binding to chlorpyrifos-oxon, resulting in a decrease of free chlorpyrifos-oxon available for inhibiting AChE (Fonnum et al. 1985, Chambers and Carr 1993). A-esterase (chlorpyrifos-oxonase) catalytically inactivates chlorpyrifos-oxon to protect from cholinergic toxicity (Li et al. 1995). Our study examined activities of acetylcholinesterase and carboxylesterase in the hippocampus and cerebral cortex. We did not examine chlorpyrifos-oxonase because this enzyme is mainly present in liver and plasma, but less in the brain. Excess acetylcholine mediates glutamate concentrations (Savolianen 2001). Caramiphen, an antagonist at muscarinic M1 receptors prevents soman-induced seizures and attenuates alterations of the equilibrium dissociation rate constant (Kd) and maximum number of receptors (Bmax) for NMDA in the brain of soman-treated rats (Raveh et al. 1999).

Restraint and swim stress modulate the cholinergic system (Friedman et al. 1996, Kaufer et al. 1998, Kaufer et al. 1999, Sunanda et al. 2000). Swim stress modulates expression of genes that regulate acetylcholinesterase and choline acetyltransferase (Kaufer et al. 1998). Furthermore, glutamatergic receptors, especially NMDA receptors, have high density in the hippocampus and cholinergic pathways that innervate the hippocampus and cerebral cortex (Cooper et al. 1996). The hippocampus and cerebral cortex function in learning and memory (Cooper et al. 1996) and are affected by stress (Magarinos et al. 1997).

As mentioned above, chronic stress and CPF individually affect glutamatergic and cholinergic systems. We hypothesized that interactions of our stress models (restraint,

swim, and restraint with swim) and CPF would modify the responses of the cholinergic and glutamatergic systems in the hippocampus and cerebral cortex. The first major objective of this study was to assess the effect of repeated stress models and chlorpyrifos on plasma corticosterone and swimming time. The second major objective was to examine the effects of repeated stress and chlorpyrifos and their interactions on enzyme activities of the cholinergic system, including acetylcholinesterase, carboxylesterase, and choline acetyltransferase in the hippocampus and cerebral cortex. The third major objective was to determine the effects of repeated stress, chlorpyrifos, and the interaction on concentrations of glutamate and asparate in the hippocampus and cerebral cortex.

Materials and methods

A) Animals

Fifty-six adult male Long-Evans rats aged 100-120 days, weighing 230-265 g, were obtained from Harlan Sprague Dawley (Indianapolis, IN) and shipped to Laboratory Animal Resources at Virginia Tech. Rats acclimated to their surroundings for 7 days. They were housed individually with food and water provided *ad libitum*. Room temperature was kept at 21-23 °C with a light cycle of 7 pm to 7 am. All procedures involving animals were in accordance with the Virginia Polytechnic Institute and State University guidelines and approved by the Virginia Tech Animal Care Committee.

B) Experimental design

The experimental design was generalized randomized complete block design. Treatment structure was 4x2, which were 4 types of repeated stress (handling, restraint, swim, and restraint with occasional swim) and subcutaneous injections of either corn oil or chlorpyrifos (CPF). The handling group was used as the control group because rats in other groups were also handled during the experiment. Treatment and sacrifice were performed in two experimental blocks separated by 1 day. Seven rats were randomly grouped into a 4x2 treatment design. Samplings of body weight and plasma corticosterone were repeated measurements on various days. On day 24, rats were injected with either corn oil or CPF 160 mg/kg sc. On day 28, swimming time, blood acetylcholinesterase, brain activities of acetylcholinesterase, carboxylesterase, choline acetyltransferase, and concentrations of brain glutamate and asparate were single measurements.

Rats were handled 5 days/week, restrained 1 hour/day for 5 days/week, swum 30 minutes for 1 day/week, or restrained 4 days and randomly swum 1 day/week, for 28 days. Body weights were measured on days –4, 0, 7, 14, 21, 24, and 28 when day 0 was the start of treatment. Blood samples were collected from the orbital sinus, using isofluorane (Abbott Animal Health, North Chicago, IL) anesthesia, on day –3 to determine basal concentrations of plasma corticosterone, and on days 8 and 17, ten minutes after the stress treatment. On day 24, rats were stressed at 8:15-10:00 am and then rats in each group were injected either with corn oil or CPF 160 mg/kg sc 4 hours after restraint (1:10-2:45 pm). CPF was suspended in corn oil to give a concentration of 200 mg/ml and the volumes of corn oil or CPF given to each rat were 0.25-0.35 ml. After dosing, rats were casually observed for clinical signs such as tremor, salivation,

lacrimation, urination, defecation, weakness and need for treatment with atropine, 0.1-0.2 mg/kg, if signs became severe. Blood samples were collected three hours after CPF dosing (4:15-5:30 pm) to determine plasma concentrations of corticosterone and activity of blood acetylcholinesterase (AChE). On day 28, rats were stressed from 8 am to 12 pm. Immediately (10-30 minutes) after stress, rats were sacrificed by decapitatation and blood samples were collected in heparinized tubes to determine blood AChE activity. Brains were dissected into hippocampus and cerebral cortex for activities of AChE, carboxylesterase (Cbxy), choline acetyltransferase (ChAT) and concentrations of glutamate and aspartate. Brain tissues were kept at -70°C until assayed.

C) Stress equipment and chemicals

Restraint stress was performed using a plexiglass cylinder with air holes, 6.5 cm in diameter and 22 cm in length. Rats were held in these cylinders for 60 minutes in the morning between 9 am and 1 pm. Restraint stress was conducted 5 days per week. Swim stress was done by forcing rats to swim for 30 minutes between 9 am to 12 pm in 21-23 °C water in a 30-gallon fish tank that was divided equally into 4 sections.

Chlorpyrifos (*O,O'*-diethyl-3,5,6-trichloro-2-pyridinyl-phosphorothioate, 99.5% pure) was obtained from Chemical Services (West Chester, PA). Corticosterone radioimmunoassay kits were purchased from ICN Biomedicals, Inc., Costa Mesa, CA. [Acetyl-1-^{14}C]-Coenzyme A was used as a radioactive substrate for ChAT activity. It had specific activity of 50 mCi/mmol and concentration of 100 μCi/ml (ICN Biomedicals, Inc.). Other chemicals were high performance liquid chromatography (HPLC) or

analytical grade. They were purchased from Sigma Aldrich (St. Louis, MO), VWR (Suwanee, GA) and Fisher (Suwanee, GA).

D) Dissection of rat brain

Hippocampus and cerebral cortex were selected. Brain dissection was performed following the location of these regions (Palkovits and Brownstein 1988). To remove the brain, the calvarium was removed and the foramen magnum was exposed. The medulla was cut *in situ* and the dura matter was removed. The brain was removed in a rostro-caudal direction, transecting any cranial nerves attaching the brain to the skull. The brain was placed in a brain mold. The brain was dissected by making two transverse section cuts. One cut was just caudal to the optic chiasm; the other cut was at caudal aspect of mammillary body. The hippocampus and cerebral cortex were removed by blunt dissection from the above section and the rostal and caudal segments of the brain.

E) Blood acetylcholinesterase (AChE)

Acetylcholinesterase activity in blood was analyzed using acetylthiocholine iodide as a substrate. The enzyme hydrolyzes acetylthiocholine to thiocholine and acetate. Thiocholine reacted with dithiobisnitrobenzoate to give a yellow colored solution that was measured at absorbance of 412 nm (Ellman et al. 1961). The whole blood was diluted to 1:1000 with phosphate buffer pH 8.00. Iso-OMPA (*N,N′,N″,N‴*-tretaisopropylpyrophosphamide) was added to the diluted blood at a concentration of 10^{-8} M to inhibit pseudocholinesterase activity. The enzyme reaction was performed in a microplate and equilibrated at room temperature for 5 minutes before the absorbance was

read at 412 nm for time zero. The reaction was incubated at room temperature for 30 minutes and then the absorbance was read again.

F) Brain acetylcholinesterase activity

Acetylcholinesterase activity in the brain regions (hippocampus and cerebral cortex) was analyzed by spectrophotometry at 412 nm using acetylthiocholine iodide as a substrate (Ellman et al. 1961, Correll and Ehrich 1991). Brain tissues (~ 0.05-0.5 gm) were homogenized in 50 mM Tris-HCl-10 mM EDTA, pH 7.5 using a 16.7 dilution factor. The homogenized samples were diluted 1:9 by 50 mM Tris buffer-0.2 mM EDTA, pH 8.0. This stock homogenate was further diluted 1:6.9 with 0.1M phosphate buffer, pH 8.0. The working dilution of 50 µl was added to microplate wells along with 150 µl of 0.1 M phosphate buffer at pH 8.0, 50 µl of 6 mM of 5,5′-dithio-bis-2-nitrobenzoic acid, and 50 µl of 4.5 mM of acetylthiocholine in triplicate. A microplate reader (SoftMax, Molecular Devices, Sunnyvale, CA) measured the absorbance at 412 nm and the absorbance was read at time zero after 5 minutes lag time. Then, the enzyme reaction was incubated at room temperature for 30 minutes. Detection limit of acetylcholinesterase activity is in the range of nmole/min/mg protein. Protein was determined by using the BioRad assay kit (Bio-Rad Laboratories, Richmond, CA).

G) Brain choline acetyltransferase

Choline acetyltransferase synthesizes acetylcholine from choline and acetyl CoenzymeA (CoA). Choline acetyltransferase activity was determined using a radiochemical microassay (Fonnum 1975, Chambers and Chambers 1989). This assay

was modified in our toxicology laboratory (Flory and Correll 2002, unpublished). Brain samples were homogenized with 50 mM Tris-HCl-5 mM EDTA buffer pH 7.5 at a ratio of 1:16.7. EDTA 10 mM was added to each sample at a ratio of 1:1 to dilute the homogenate and 70 μl of this suspension was kept for protein assay. Then, the suspension was further diluted with 2.5% Triton X-100 in 10 mM EDTA at a ratio of 4:1. The incubation mixture in a total volume of 7 μl consisted of the following reagents in millimolar concentrations: sodium phosphate buffer, pH 7.4, 50; sodium chloride, 300; choline bromide, 8; disodium EDTA, 20; eserine sulfate, 0.1; [^{14}C] acetyl-CoA, 0.2 and homogenate containing 8-14 μg of protein. The reaction was incubated at 37 °C for 15 minutes and stopped by adding 0.4 ml of 70 mM cold phosphate buffer pH 7.4. Each sample was then washed with 1.6 ml of phosphate buffer. The washing solution was transferred to a scintillation vial that contained 2 ml of 0.5% sodium tetraphenylborate in butyronitrile and 10 ml of Scintilene (scintilation cocktail). Gentle shaking extracted the [^{14}C] ACh product into the cocktail, leaving the [^{14}C] acetyl-CoA in the aqueous phase. Each vial had beta radiation counted by a scintillation counter (Beckman LS6500, Beckman Coulter, Inc. Fullerton, CA). Detection limit of ChAT activity was in the range of pmole/min/mg protein.

H) Brain carboxylesterase activity

Carboxylesterase (Cbxy) activity was determined using spectrophotometry at 510 nm (Sprague et al. 1981, Correll and Ehrich 1991). Phenyl valerate (an ester substrate) was hydrolyzed by Cbxy to phenol and valeric acid. A redox reaction of phenol and potassium ferricyanide was measured using a microplate reader (Correll and Ehrich

1991). Brain tissues were homogenized in 50 mM Tris-HCl-10 mM ethylenediamine-tetraacetate disodium dihydrate (EDTA) pH 7.5 in a dilution of 16.7 and prepared with the same dilution as done for the AChE assay. The homogenized samples were diluted by taking 110 µl of homogenate and adding 880 µl of 50 mM Tris buffer-0.2 mM EDTA pH 8.0. Tris buffer 125 µl of 50 mM was added to microplate wells along with 25 µl of working diluted sample and then incubated at 37 °C for 20 minutes in triplicate for each sample. Then, 50 µl of phenyl valerate (1.1 mg/ml in 0.03% Triton X-100 was added to all wells. The microplate was further incubated at 37 °C for 15 minutes. Sodium lauryl sulfate 5% was added to stop the reaction and 0.4% potassium ferricyanide was added to initiate the redox reaction. The reaction was run for 15 minutes before reading the absorbance at 510 nm. Cbxy activity was calculated using the standard curve created by reaction of phenol and potassium ferricyanide. Detection limit of Cbxy activity was in the range of nmole phenyl valerate hydrolyzed/ min/mg protein.

I) Concentration of plasma corticosterone

Corticosterone concentrations were assayed using a radioimmunoassay method and autogamma detection (Sousa et al 1998, ICN Biomedicals, Inc., Costa Mesa, CA). Serum or plasma samples were diluted 1 to 200 with steroid diluent. The diluted plasma samples (100 µl) were added into test tubes followed by 200 µl of ^{125} I corticosterone, and 200 µl of antiserum to corticosterone. The reaction was mixed and incubated at room temperature for 2 hours. After incubation, 500 µl of a precipitating solution was added, mixed and centrifuged at 1000 x g for 15 minutes. The supernatants were decanted. A

CROBA II auto-gamma counter (Packard Bioscience Company, Meriden, CT) counted gamma activity of the precipitates. Detection limit was in the range of ng/ml of plasma.

J) Brain concentrations of glutamate and aspartate

Glutamate concentrations in brain tissues were determined using high performance liquid chromatography (HPLC) and fluorescence detection (Phillips and Cox 1997, Piepponen and Skujins 2001). The method was modified from Piepponen and Skujins (2001). Brain tissues were homogenized in ice-cold Ringer's solution (147 mM NaCl, 1.2 mM $CaCl_2$, 2.7 mM KCl, 1.0 mM $MgCl_2$, 0.04 mM ascorbic acid) along with an internal standard (homoserine at a final concentration of 2 μM) using a Cell Disruptor Sonicator (Heat System-Ultrasonic, Farmingdale, NY) before centrifugation at 13000 x g, 4 °C for 5 minutes. Supernatants were filtered through Acrodisc LC13 mm syringe filters and diluted 20,000-24,000 fold with Ringer's solution prior to amino acid analysis. The working derivatization reagent was freshly prepared using 5 mg of *O*-pthaladehyde in 5 ml of 0.10 M sodium tetraborate and 15 μl of 14.3 mol/l of *β*-mercaptoethanol. The filtrate was mixed manually with the derivatization reagent (1:2 by volume) 2-4 minutes prior to its injection for each sample on the analytical column. The external standard series of glutamate, aspartate, glycine and homoserine were prepared in 1:1 water-methanol and run for quantitative analysis. A Hewlett Packard Series 1100 Quaternary Pump with a degasser and autosampler HPLC (Agilent Technologies, Wilmington, DA) was used. The separation was performed on a C18 reversed phase analytical column (Luna C18 (2) 5 μ, 150x4.6 mm, Phenomenex, Torrance, CA). Flow rate of mobile phase was 1.2 ml/min until glycine (the last analyte) came out at 28 minutes. Then, the column

was washed with deionized water for 12 minutes, followed by 30% acetonitrile in deionized water for 5 minutes and 100% acetonitrile for 8 minutes to wash out other amino acids. Then, the column was equilibrated with mobile phase for 14 minutes for the next assay. Fluorescence detection was used with the excitation wavelength of 330 nm and emission wavelength of 425 nm. The mobile phase consisted of 88% of 0.05 M disodium phosphate pH 6.1 (pH adjusted with phosphoric acid) and 12% of acetonitrile. The mobile phase was filtered through 0.2 μm membrane filter and degassed before use. Detection limit was in the range of 0.05 μM per 20 μl of injection and tissue sample needed was 0.02-0.05 g.

K) Statistical analysis

Data were analyzed by two-way ANOVA using the SAS program (version 8.2, SAS Institute Inc., Cary, NC). To test the effects of repeated stress on body weight and concentrations of plasma corticosterone before injection of CPF from day 0 to day 24, the change of body weight and concentrations of plasma corticosterone were analyzed by mixed model ANOVA and post hoc comparisons were tests of simple main effect of repeated stress on separate days. To hold the experimentwise error rate to 0.05, the comparisonwise alpha level was 0.00833 (Bonferroni-corrected). The main effects of repeated stress and CPF on body weight of day 28 and on plasma corticosterone on day 24 were analyzed by two-way ANOVA. Post hoc comparisons of stress effect were tested by Tukey's HSD and the interaction of repeated stress and CPF was tested by simple main effect within stress. The main effects of repeated stress, CPF, and the interaction of repeated stress and CPF on day 28 on blood acetylcholinesterase, activities of brain

acetylcholinesterase, carboxylesterase, choline acetyltransferase, and concentrations of brain glutamate and aspartate were analyzed by two-way ANOVA. Post hoc comparison was by Tukey's HSD for stress effects and by simple main effect for the interaction of stress and chlorpyrifos within stress treatment. Significant difference was considered when $p < 0.05$ and a statistical trend to significant different was arbitrarily considered when $p \leq 0.08$.

Results

Prior to CPF injection from day 1 to day 24, there were interactions of repeated stress and day on body weight (df= 1, 349, $p = 0.0296$). However, from post hoc comparison, there were no differences of body weight among handling, restraint, swim and restraint with swim within day (Figure 1).

Blood samples taken 10-40 minutes after swim stress or restraint with swim stress on days 8 and 17 demonstrated increased concentrations of plasma corticosterone more than samples from control rats or rats exposed to restraint alone (df = 1, 96, $p < 0.0001$, for both days and all comparisons of swim, control; swim, restraint; restraint with swim, control; restraint with swim, restraint, Figure 2). Concentrations of plasma corticosterone in restrained rats were not different from those in control rats on days 8 and 17 (Figure 2).

On day 24, there were no differences of repeated stress, CPF, or the interaction of repeated stress and CPF on concentrations of plasma corticosterone (df = 3, 99, $p = 0.0954$, df = 1, 99, $p = 0.2317$; df = 3, 99, $p = 0.2029$, respectively) when the blood samples were taken 7 hours after restraint stress and 3 hours after CPF injection. Since

there was no interaction of repeated stress and CPF, concentrations of plasma corticosterone of day 24 were included in Figure 2. CPF in this study did not affect concentrations of plasma corticosterone 3 hours after dosing. On day 28, plasma corticosterone concentrations were not determined. However, we examined concentrations of plasma corticosterone on day 28 in the following experiment (Chapter 5), which monitored effects of repeated stress and CPF on NMDA and total muscarinic receptors. In summary, swim and restraint with swim were effective stressors as demonstrated by increase of plasma corticosterone. Restraint stress for 1 hour/day for 28 days (psychological stress) may cause less stress based on plasma corticosterone concentrations.

On day 28, 4 days after CPF injection, neither repeated stress, CPF, nor the interaction of repeated stress and CPF affected body weight. Since there was no interaction of stress and CPF, the body weights of day 28 were included in Figure 1.

CPF adminstration resulted in a statistical trend toward a decrease in swimming time of the swimming rats on day 28 (df = 1, 23, $p = 0.0846$, Table 1). CPF-treated rats that were swum or restrained with swim were less able to tolerate being in the water than rats that were given corn oil because some CPF-treated rats had to be rescued before the end of the 30 minute swimming period. The average swimming time of both stress groups was 27.1 minutes after CPF treatment. Rats given corn oil swam an average of 29.4 and 30 minutes for those who swam and those who were restrained with swim, respectively (Table 1).

Repeated stress did not affect activity of blood acetylcholinesterase (AChE), or activities of brain AChE and carboxylesterase (Cbxy) in the hippocampus (Table 2),

whereas CPF inhibited activities of these enzymes in the hippocampus (df = 1, 45, $p <$ 0.0001 for all three comparisons, Table 3). However, there were no interactions of repeated stress and CPF on activities of these esterases in the hippocampus. Repeated stress did not affect activities of AChE in the cerebral cortex (Table 2). In contrast, restraint with swim stress decreased activities of Cbxy in the cerebral cortex when compared to control, restraint, and swim (df = 1, 45, $p < 0.0001$; df = 1, 45, $p < 0.0001$; df = 1, 45, $p = 0.0004$, respectively) as shown in Table 2 and Figure 3. CPF inhibited activities of AChE and Cbxy in the cerebral cortex (df = 1, 45, $p < 0.0001$; df = 1, 45, $p <$ 0.0001, Table 3). Even though restraint with swim stress and CPF inhibited Cbxy activity in the cerebral cortex, there was no interaction of restraint with swim stress and CPF on activities of this enzyme (df = 1, 45, $p = 0.233$). Repeated stress, CPF, and the interaction of repeated stress and CPF did not affect the activity of ChAT in both brain tissues (Table 2 and 3).

Restraint with swim stress resulted in a statistical trend toward an increase in concentrations of glutamate in the hippocampus (11.139 ± 0.315 μmole/g tissue) more than swim stress (10.018 ± 0.304 μmole/g tissue, df = 1, 45, $p = 0.064$) but did not differ from control as shown in Figure 4. CPF did not affect concentrations of hippocampal glutamate (Figure 5). There was no interaction of restraint with swim stress and CPF on the concentrations of hippocampal glutamate (Figure 5). There were no effects of repeated stress, CPF, nor was there an interaction of stress and CPF on concentrations of glutamate in the cerebral cortex.

CPF reduced the increase of hippocampal aspartate of rats that were restrained and swum (df = 1, 45, $p = 0.0063$, Figure 6) but not swum rats (df = 1, 45, $p = 0.1863$).

Swim and restraint with swim increased hippcampal aspartate when compared to handling (df = 1, 45, p = 0.0035; df = 1, 45, p = 0.0003). Repeated stress, CPF, or exposure to both repeated stress and CPF did not affect concentrations of aspartate in the cerebral cortex.

Discussion

Repeated stress and CPF each have been reported to affect glutamatergic and cholinergic systems. Chronic restraint stress releases glutamate in the hippocampus (Sunanda et al. 2000) and causes reversible dendritic atrophy of CA3 of the hippocampus (Magarinos et al. 1997, McEwen 2000). There have been debates of effects of stress on the cholinergic system (Friedman et al. 1996, Kaufer et al. 1998, Song et al. 2002, Tian et al. 2002). CPF inhibits activities of AChE and Cbxy (Pope et al. 1991, Chanda et al. 1997). Some cholinesterase inhibitors modulate glutamate concentrations in the hippocampus (Lallement et al. 1991). We hypothesized that repeated stress (restraint, swim or restraint with swim), CPF or interaction of repeated stress and CPF modulate activities of cholinergic enzymes and/or concentration of glutamate in the hippocampus and cerebral cortex. Our results demonstrated that restraint with swim inhibited the activity of carboxylesterase in the cerebral cortex compared to other stressors; however, there was no interaction of stress and CPF on this enzyme activity. Restraint with swim resulted in a statistical trend toward an increase in concentrations of glutamate in the hippocampus when compared with swim alone, but restraint with swim did not differ

from control. Furthermore, CPF attenuated the elevated concentrations of aspartate in the hippocampus of restrained with swim rats.

Effects of repeated stress and chlorpyrifos on body weight and swimming behavior

Effects of repeated stress and CPF on body weight are important because loss of body weight is a common response of the whole physiological system to many stressors. In this study, repeated stress did not alter body weight within day. Neither CPF nor an interaction of repeated stress and CPF affected body weight. Hancock et al. (2003) also reported that rats that underwent restraint and restraint with swim stress did not have body weights different from rats that were only handled. However, the swum rats in Hancock's study had lower body weight than other groups of rats. The discrepancy could be due to the amount of swimming. Rats in this study were swum one day per week, whereas in Hancock's experiment the rats were swum 5 days per week. Restraint of rats for 30 minutes daily for up to 26 days was previously reported to decrease body weight and diminish weight gain, when compared to handling (Konarska et al. 1989, Konarska et al. 1990). Another study (Magarinos and McEwen 1995) showed that restraint for 6 hours/day over 21 days reduced body weight. In their study, concentrations of plasma corticosterone were very high on days 1, 7, and 14, but were decreased on day 21. This suggests rats could acclimate to restraint. However, restraint with water immersion in rats for 2 hour/day for 4 weeks reduces body weight (Mizoguchi et al. 2001). In summary, our stress models were not sufficient to cause body weight change.

Four days after injection of the CPF dose of 160 mg/kg, body weight was slightly but nonsignificantly decreased and rats did not show any clinical signs. Karanth and Pope

(2003) demonstrated that CPF at the maximal tolerated dose (MTD) (279 mg/kg) decreased body weight of neonatal, juvenile and adult rats, while causing few overt signs of cholinergic toxicosis.

From our observation of swimming time, CPF had a statistical trend toward enhancing exhaustion of the swimming rats. On day 28, CPF-treated rats that were swum or restrained and swum were less able to tolerate being in the water and they had to be rescued before the 30 minute time period for swimming was over. Since CPF tended to affect swimming time, our results suggested that forced swimming in CPF-treated rats caused physical stress but the combined effects of swimming and CPF were insufficient to lower body weight.

Effects of repeated stress and chlorpyrifos on plasma corticosterone

Swim stress and restraint with swim stress increased concentrations of plasma corticosterone more than control and restraint alone on days 8 and 17 when blood samples were drawn 10-40 minutes after stress. Hancock et al. (2003) also reported that swim and restraint with swim stress elevated concentrations of plasma corticosterone above those seen in rats that underwent handling and restraint only. However, in our study, when blood samples on day 24 were taken seven hours after restraint stress, or 3 hours after CPF, plasma corticosterone concentrations were unaffected. Our results were similar to those of Magarinos and McEwen (1995), Konstandi et al (2000), and Mizoguchi et al (2001), who also demonstrated increases in plasma corticosterone after restraint and/or swim stress. The study of Mizoguchi et al (2001) suggested that concentrations of plasma corticosterone remained high for 5 hours. The high

concentrations of plasma corticosterone could induce negative feedback of the hypothalamic-pituitary adrenal axis (Sapolsky et al. 1986). Therefore, swim and restraint with swim in our study were effective stressors.

Effects of repeated stress and chlorpyrifos on cholinergic enzymes

Restraint or swim stress have been reported to modulate the cholinergic system (Friedman et al. 1996, Kaufer et al. 1998, Sunanda et al. 2000). This, however, is a controversial issue. Our results demonstrated that repeated restraint, swim, or restraint with occasional swim did not affect activities of blood acetylcholinesterase (AChE), or activities of AChE, carboxylesterase (Cbxy) and choline acetyltransferase (ChAT) in the hippocampus.

CPF 160 mg/kg sc in this study inhibited activity of AChE in blood, and activities of AChE and Cbxy in the hippocampus and cerebral cortex 4 days after injection. This corresponds to previous studies (Pope et al 1992, Chanda et al. 1997). Our results most clearly correspond to those of Karanth and Pope (2003), who also showed that CPF at the maximal tolerated dose of 279 mg/kg inhibited cholinesterase activity by 85-95%. The maximal cholinesterase inhibition in their adult rats was observed 96 hours after dosing. In our studies, CPF did not affect the activity of ChAT. Therefore, CPF at 160 mg/kg sc decreased activities of degradatory enzymes of acetylcholine but did not affect the synthetic enzyme. Karanth and Pope (2003) also reported that CPF had little effect on acetylcholine synthesis.

Restraint with swim stress decreased activity of Cbxy in the cerebral cortex more than other stressors. Cbxy was not determined during the stress treatments done in other

laboratories. Cbxy detoxifies foreign compounds and protects AChE in organophosphate poisoning (Chanda et al. 1997). The reduction of Cbxy in rats undergoing restraint with swim could enhance the toxicity of CPF because CPF would be less likely to be hydrolyzed and would be free to distribute to cholinergic synapses. However, analysis indicated there was no interaction of restraint with swim and CPF on the activity of Cbxy in the cerebral cortex. The concurrent exposure of repeated stress and CPF did not alter the cholinergic enzyme activities. In summary, repeated stress had only a few effects on the enzymes of the cholinergic system.

The effects of stress on the cholinergic system are variable. Pyridostigmine causes more inhibition of AChE in mice swum for 4 minutes than in unstressed mice (Friedman et al. 1996). The acute 4-minute swim was also reported to increase brain *c-fos* mRNA level for AChE in brain tissue and induce a disruption of the blood brain barrier. In a later study, Kaufer et al. (1998) reported that two 4 minutes swim sessions of mice cause bi-directional modulation of genes that regulate acetylcholine availability. These results encouraged several researchers to investigate the effects of stress along with pyridostigmine on the central nervous system.

The enhanced ability of pyridostigmine to cause inhibition of AChE when animals are stressed has not been demonstrated in all cases. For example, Song et al. (2002) reported that repeated restraint stress of rats for 1 hour daily over 14 days did not enhance cholinesterase inhibition after po administration of pyridostigmine at 10 mg/kg/day immediately before rats were placed in restraint tubes. The doses of pyridostigmine were sufficient to inhibit cholinesterase activity in the blood and diaphragm, but they had little effect on brain cholinesterase activity. In another study,

intermittent holding for 1 hour and tailshock to rats before treatment with pyridostigmine ip lowered AChE activity in the basal forebrain/striatum (Beck et al. 2003). Furthermore, neither forced swimming for 15 minutes nor forced running for 90 minutes affected acute toxicity of pyridostigmine or brain regional cholinesterase inhibition of rats (Tian et al. 2002). Swim for two 4-minute periods prior to pyridostigmine or physostigmine im did not affect the inhibition of brain cholinesterase caused by these carbamates (Grauer et al. 2000).

In summary, our studies demonstrated results more similar to Song et al. (2002), Tian et al (2002), Grauer et al (2000), and Hancock et al. (2003) than Friedman et al. (1996), Beck et al. (2003) and Sunanda et al. (2001). Restraint, swim, and restraint with swim stress were not sufficient to inhibit AChE in blood or AChE, Cbxy, and ChAT in the hippocampus, nor did they further enhance the AChE inhibition caused by CPF. However, restraint with swim and CPF individually inhibited Cbxy activity in the cerebral cortex.

Effects of repeated stress and chlorpyrifos on concentrations of glutamate and aspartate

Restraint with swim stress had a statistical trend to increase concentrations of hippocampal glutamate more than swim stress. Swim and restraint with swim stress increased concentrations of aspartate in the hippocampus when compared to handling. Other studies have also noted increases in glutamate or aspartate with stress. For example, restraint for 6 hours daily for 21 days increases concentrations of glutamate in the hippocampus of rats (Sunanda et al. 2001). In another study, a 20-minute restraint increased extracellular glutamate and aspartate concentrations in the prefrontal cortex,

hippocampus, striatum, and nucleus accumbens of rats (Moghaddam 1993, Moghaddam et al. 1994). Swim stress also elevated extracellular glutamate and aspartate concentrations in the prefrontal cortex and striatum (Moghaddam 1993). In contrast, our results demonstrated that repeated restraint stress did not alter concentrations of glutamate in the hippocampus, but our experiments differed from those of Moghaddam (1993) and Moghaddam et al. (1994). In their studies, rats were restrained for 20 minutes and the concentrations of extracellular glutamate were measured immediately, but rats in our study were restrained for 1 hour/day over 28 days and glutamate concentrations were determined from post-mortem brain tissues. Moreover, it appeared that rats restrained for 28 days became acclimated to the restraint tubes.

CPF did not affect hippocampal glutamate concentrations. These results contrast with studies conducted with another organophosphate compound, soman. An injection of soman at a dose of 0.9 LD_{50} sc transiently elevated the extracellular glutamate concentrations in the hippocampus within 30 minutes; but this dose also caused seizures (Lallement et al. 1991). Our dose of CPF was 60% of the maximum tolerated dose sc and it did not cause seizures. Lack of convulsions may explain the lack of release of glutamate in our study. There was no interaction of repeated stress and CPF on concentrations of glutamate in the hippocampus. Swim and restraint with swim increased hippocampal asparate when compared to handling (control), and CPF reduced concentrations of aspartate in the hippocampus of restraint with swim rats but did not affect this excitatory amino acid of swum rats. These results suggested that there was an antagonism between restraint with swim and CPF on the hippocampal aspartate. Since

aspartate can modulate NMDA receptors (Cooper et al. 1996), the alteration of asparate may modulate NMDA receptor responses.

Our stress models did not affect AChE activity nor alter the inhibition of this enzyme in the response to CPF. Both restraint with swim stress and CPF decreased activities of Cbxy in the cerebral cortex even though there was no interaction of them on Cbxy, suggesting that restraint with swim may indirectly increase the availability of acetylcholine. The two major cholinergic regions consist of the cholinergic nuclei in the forebrain and brainstem (Aigner 1995). The basal forebrain nuclei (median septum) innervate the cortex and hippocampus and nucleus basilis innervates the entire cerebral cortex, whereas the brainstem (dorsal pons) innervates the hippocampus. Individual cholinergic neurons in the basal forebrain and brainstem are uniquely positioned to directly and indirectly affect the level of cortical and limbic activity. Inhibition of Cbxy in the cerebral cortex and cholinergic pathways suggested the cholinergic system may be vulnerable to stress effects.

Concentrations of hippocampal glutamate exhibited a statistical trend toward an increase in the restrained with swim rats compared to swum rats. CPF decreased concentrations of aspartate in the hippocampus in the restrained with swim rats. These findings indicated repeated stress increases the release of excitatory amino acids in the hippocampus, suggesting that this stressor may affect glutamatergic response. Glutamate facilitates the control of striatal cholinergic transmission via NMDA and non-NMDA receptor activation and agonists of both receptors facilitate acetylcholine release from rat striatal slices (review in Morari et al. 1998). Presynaptic glutamatergic modulation of ACh release was demonstrated in the cerebral cortex (review Morari et al. 1998).

The finding that CPF decreased concentrations of elevated aspartate in the hippocampus of the restrained-with swim rats was the only interaction of stress and CPF found. Cholinomimetics increase extracellular glutamate; for example, physostigmine (an acetylcholinesterase inhibitor) at dose of 0.3 mg/kg im increases concentrations of extracellular glutamate but not aspartate in the striatum of anaesthetized rats (Dijk et al. 1995). Antagonists of both muscarinic cholinergic and NMDA receptors are thought to block the induction of long-term potentiation (Bliss and Collingridge 1993). NMDA receptors are involved in memory amd learning (Cooper et al. 1996).

In conclusion, our study demonstrated that swum and restrained with swim rats were stressed as they had elevated plasma corticosterone and they were exhausted when swum after CPF injection. Repeated stress did not affect acetylcholinesterase activity. However, restraint with swim and CPF individually inhibited Cbxy in the cerebral cortex. The inhibition of Cbxy in the restrained with swim rats could enhance the toxicity of CPF bcause CPF would be less hydrolyzed. Therefore, it would be free to distribute to cholinergic synapses to inhibit AChE, suggesting that restraint with swim may indirectly increase acetylcholine in the cholinergic synapses. This may result in modification of cholinergic receptors and vulnerability of cholinergic response after exposure to cholinesterase inhibitors. However, there was no interaction of stress and CPF on the enzyme activity. The effects of concurrent exposure to stress and CPF on cholinergic muscarinic receptors remain to be studied.

Concentrations of glutamate in the hippocampus demonstrated a statistical trend to increase in the restrained with swim rats more than in the swum rats. Glutamate distributed in the hippocampus is thought to be involved in long-term potentiation and

memory; therefore, elevated glutamate may modify long-term potentiation and memory. Restraint with swim may lead the hippocampus to be vulnerable to toxic agents because excess glutamate may cause excitotoxicity. Antagonism of the effects of restraint with swim by CPF on hippocampal asparate was found. CPF reduced concentrations of aspartate in the hippocampus, suggesting there may be a decrease of excitability of neurons in the hippocampus after CPF administration. Asparate usually coexists with glutamate and it can modulate NMDA receptors. The antagonism of restraint stress and CPF on aspartate concentrations may modify the responses of glutamatergic and or cholinergic receptors that are located in this region.

Acknowledgments

The authors would like to acknowledge the assistance of Kristin Knight, Geraldine Magnin, Kristel Fuhrman, Jonathan Hinckley, David Moore, Sandy Hancock, Linda Correll, Delbert Jones, Barbara Wise, Ellen Uri and Daniel Ward. Appreciations are to the Faculty of Liberal Arts and Science, Kasetsart University, and the Thai government for T. Pung's scholarship. This work was supported by DAMD 17-99-1-9489 from the U.S. Army Medical Research and Materiel Command and by Virginia-Maryland Regional College of Veterinary Medicine.

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Table 1. Effects of repeated stress and chlorpyrifos on swimming time on day 28. Rats were injected sc on day 24 with either corn oil or chlorpyrifos (CPF) at 160 mg/kg.

Type of treatment	Swimming Time (min)	
	Corn oil	Chlorpyrifos
Swim	29.4 ± 0.6	27.1 ± 1.9
Restraint with swim	30.0 ± 0	27.1 ± 1.9

Data represent mean ± SEM (n=14). The main effect of CPF had a statistical trend toward a decrease in swimming time ($p = 0.08$).

Table 2. Effects of repeated stress on activities of acetylcholinesterase (AChE), carboxylesterase (Cbxy), and choline acetyltransferase (ChAT) on day 28

Type of stress	Blood AChE	Hippocampus			Cerebral cortex		
		AChE	Cbxy	ChAT	AChE	Cbxy	ChAT
Handling (control)	1.80±0.15	32.45±2.81	47.87±1.72	2.11±0.08	20.48±2.33	56.54±2.13	1.42±0.08
Restraint	1.99±0.16	36.60±2.92	49.77±1.79	2.08±0.09	21.92±2.42	55.22±2.21	1.54±0.09
Swim	1.81±0.15	37.45±2.81	47.37±1.72	2.12±0.08	19.89±2.33	53.49±2.13	1.57±0.08
Restraint with swim	1.81±0.16	33.17±2.92	44.84±1.79	2.08±0.09	21.75±2.42	40.05±2.21**	1.51±0.09

Blood AChE is expressed as nmol/ml. Hippocampal and cerebral cortex AChE is expressed as nmol/min/mg protein. Cbxy is expressed as nmole of phenyl valerate hydrolyzed/min/mg protein. ChAT is expressed as pmol/min/mg protein. All values were not significantly different from control ($p > 0.05$), except that restraint with swim stress decreased levels of Cbxy in the cerebral cortex (** $p < 0.001$). Data are presented as mean ± SEM (n=7).

Table 3. Effects of chlorpyrifos on activities of acetylcholinesterase (AChE), carboxylesterase (Cbxy), and choline acetyltransferase (ChAT) on day 28

Type of injection	Blood AChE	Hippocampus			Cerebral cortex		
		AChE	Cbxy	ChAT	AChE	Cbxy	ChAT
Corn oil	2.87±0.11	58.42±2.03	61.46±1.24	2.08±0.06	36.48±1.68	67.52±1.54	1.58±0.06
Chlorpyrifos	0.84±0.11***	11.42±2.03***	33.47±1.24***	2.12±0.06	5.54±1.68***	35.13±1.54***	1.45±0.06

Blood AChE is expressed as nmol/ml. Hippocampal and cerebral cortex AChE is expressed as nmol /min/mg protein. Cbxy is expressed as nmole of phenyl valerate hydrolyzed/min/mg protein. ChAT is expressed as pmol/min/mg protein. *** indicates significantly different from control ($p < 0.0001$). Chlorpyrifos inhibited AChE in blood, hippocampus and cerebral cortex. Chlorpyrifos also inhibited Cbxy in the hippocampus and cerebral cortex, but did not affect ChAT in the hippocampus and cerebral cortex. Data are presented as mean ± SEM (n=7).

Table 4. Effects of repeated stress, CPF, and interaction of stress and CPF on concentrations of excitatory amino acids and cholinergic enzymes

Treatment	Glutamatergic system		Cholinergic system		
	Glutamate	Aspartate	AChE	Cbxy	ChAT
Stress					
Handling	No effect	No effect	No effect	No effect	No effect
Restraint	No effect	No effect	No effect	No effect	No effect
Swim	No effect		No effect	No effect	No effect
Restraint with swim	Increased glutamate in the HP more than swim		No effect	Inhibited Cbxy in the CC more than handling and restraint	No effect
CPF	No effect		Inhibited AChE in the HP and CC	Inhibited Cbxy in the HP and CC	No effect
Stress *CPF					
Handling	No effect	No effect	No effect	No effect	No effect
Restraint*CPF	No effect	No effect	No effect	No effect	No effect
Swim*CPF	No effect	No effect	No effect	No effect	No effect
Restraint*CPF	No effect	CPF attenuated the elevated aspartate in the HP of restraint with swim rats	No effect	No effect	No effect

HP= hippocampus, CC= cerebral cortex, HT= hypothalamus

Figure legends

Figure 1. Change of body weight after the rats were either handled, restrained, swam, or restrained with swimming during 28-day treatment. They were injected with either corn oil or chlorpyrifos at a dose of 160 mg/kg sc on day 24. There were no significant interactions of chronic stress and chlorpyrifos on body weight on day 28 ($p > 0.05$). The data points represent mean of body weight change from day –4.

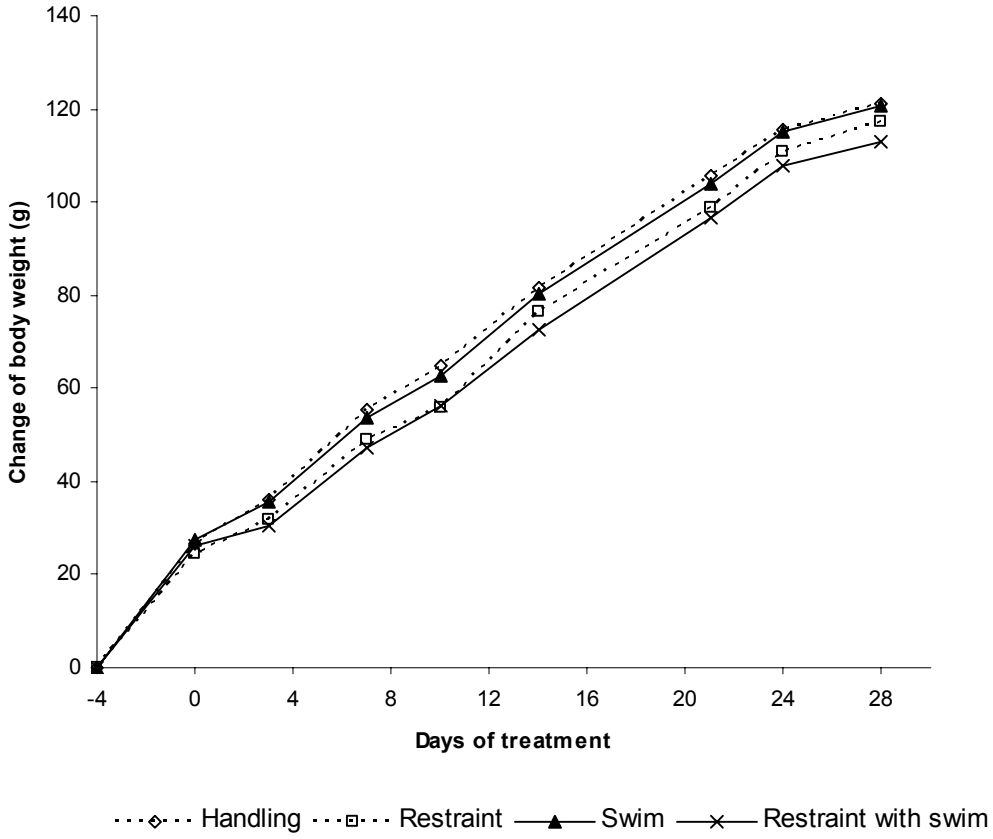
Figure 2. Plasma corticosterone concentrations were measured during stress treatment in combination with either corn oil or chlorpyrifos 160 mg/kg sc. Data are expressed as mean ± SEM (n=14). Baseline of plasma corticosterone was determined on day –4. On days 8 and 17, plasma samples were taken 10-40 minutes after the stress treatment. On day 24, chlorpyrifos was injected 4 hours after stress treatment and plasma samples were taken 7 hours after stress treatment. ^{a,b} indicate significantly different mean values with no letters in common were significantly different at $\alpha = 0.05$ according to Bonferroni's corrected multiple comparison.

Figure 3. Effects of repeated stress and chlorpyrifos (CPF) on carboxylesterase activity in the cerebral cortex. Data are presented as mean ± SEM (n=7). *** indicates significant difference in cerebral cortex of restraint with swim from handling ($p < 0.0001$). ### indicates significant difference of chlorpyrifos injection from corn oil ($p < 0.0001$). The interaction of restraint with swim and chlorpyrifos on Cbxy in the cerebral cortex was not significant ($p = 0.233$).

Figure 4. Effects of repeated stress on concentrations of hippocampal glutamate. Data are presented as mean ± SEM (n=14). * indicates a statistical trend toward an increase in glutamate of restraint with swim more than swim stress ($p = 0.064$).

Figure 5. Effects of repeated stress and chlorpyrifos (CPF) on concentrations of glutamate in the hippocampus. Data are presented as mean ± SEM (n=7). Neither chlorpyrifos nor the interaction of chronic stress and chlorpyrifos affect concentrations of glutamate in the hippocampus.

Figure 6. Effects of repeated stress and chlorpyrifos (CPF) on concentrations of aspartate in the hippocampus. Data are presented as mean ± SEM (n=7). * indicates significantly different effects of CPF from corn oil within the restraint with swim rats. Chlorpyrifos attenuated the response of aspartate of the restrained with swim rats ($p = 0.0063$). Stress alone increased aspartate concentrations in rats that were swum or restrained and swum ($p < 0.05$).


140
120
100
80
60
40
20
0
Change of body weight (g)
-4
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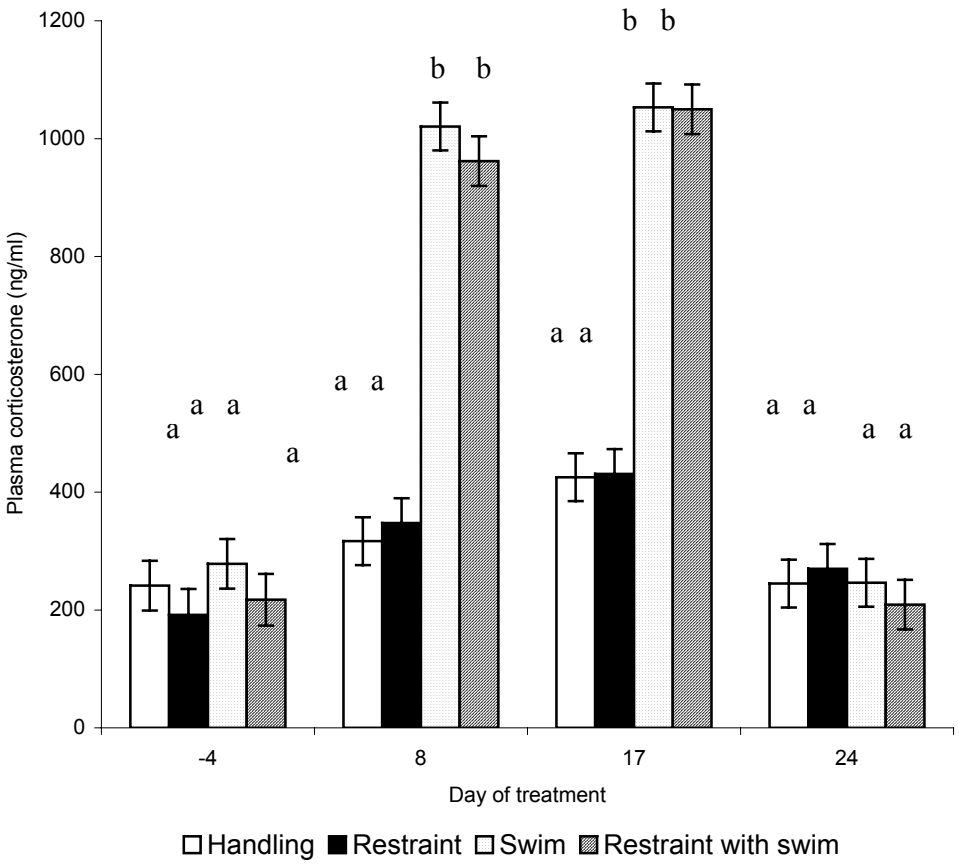
4
8
12
16
20
24
28
Days of treatment
Handling
Restraint
Swim
Restraint with swim

Figure 1.


1200
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800
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400
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0
Plasma corticosterone (ng/ml)
a a a a
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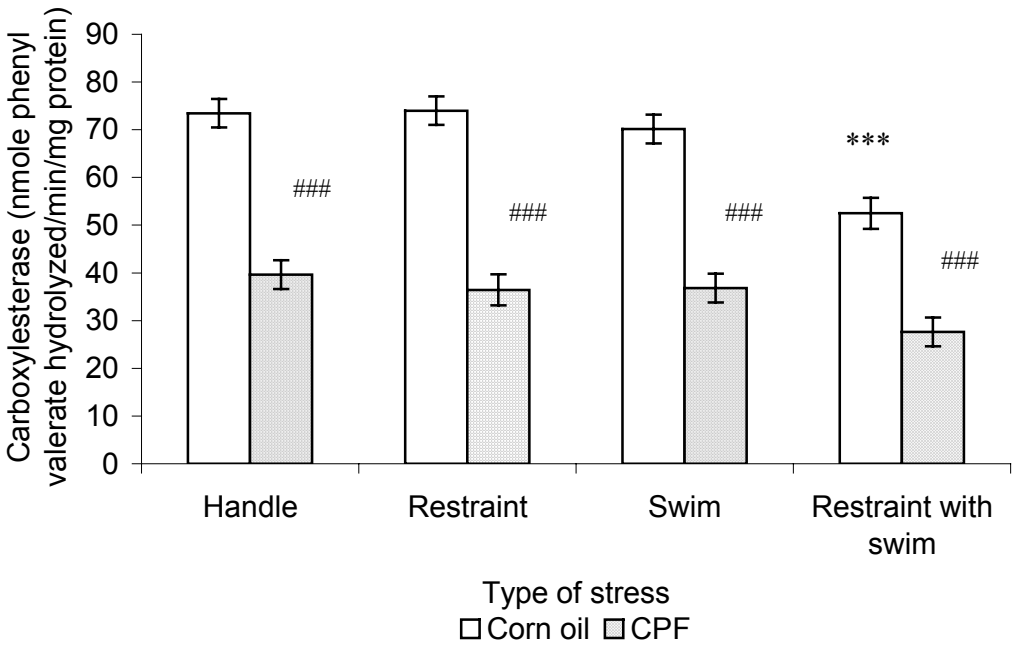
-4
8
17
24
Day of treatment
Handling
Restraint
Swim
Restraint with swim

Figure 2.


Carboxylesterase (nmole phenyl valerate hydrolyzed/min/mg protein)
90
80
70
60
50
40
30
20
10
0
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###
###
***
###
Handle
Restraint
Swim
Restraint with swim
Type of stress
Corn oil
CPF

Figure 3.


*
12
10
8
6
4
2
0
Hippocampal glutamate (umole/g tissue)
Handle
Restraint
Swim
Restraint with swim
Type of stress

Figure 4.

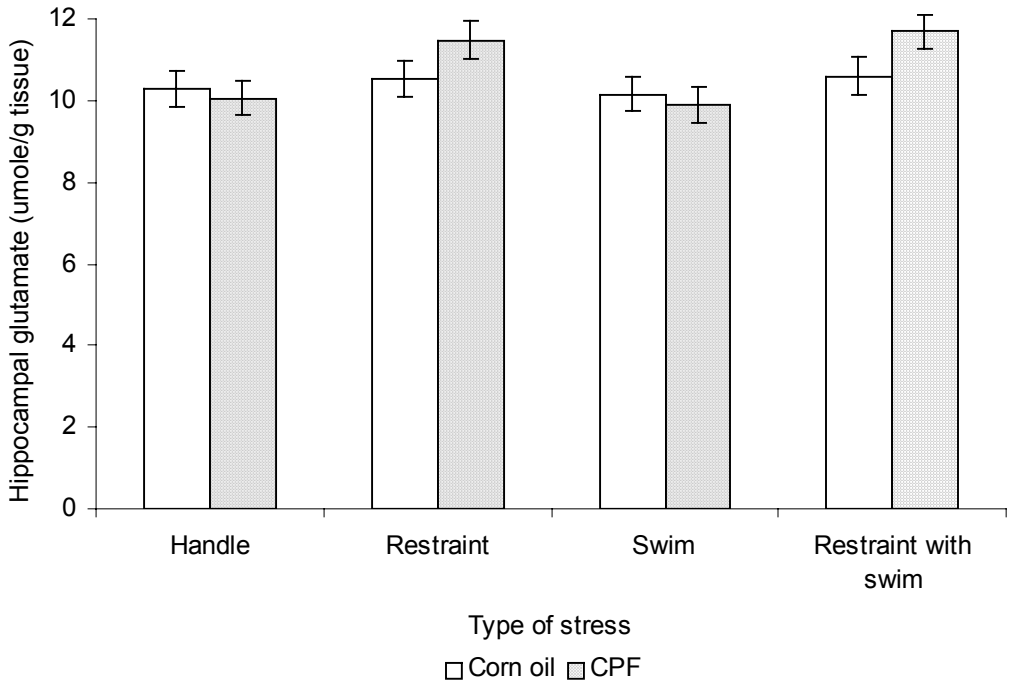
Hippocampal glutamate (umole/g tissue)
12
10
8
6
4
2
0
Handle
Restraint
Swim
Restraint with swim
Type of stress
Corn oil
CPF

Figure 5.

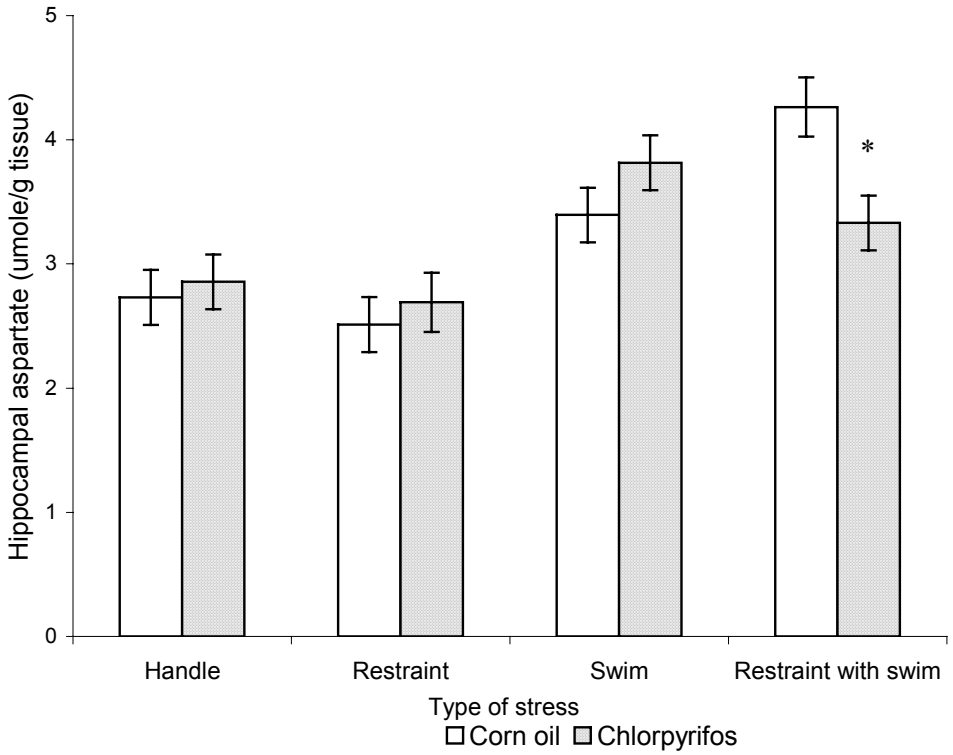
Hippocampal aspartate (umole/g tissue)
5
4
3
2
1
0
*
Handle
Restraint
Swim
Restraint with swim
Type of stress
Corn oil
Chlorpyrifos

Figure 6.

CHAPTER 5

EFFECTS OF REPEATED STRESS AND CPF ON NMDA AND TOTAL MUSCARINIC RECEPTORS

EXAMINATION OF CONCURRENT EXPOSURE TO REPEATED STRESS AND CHLORPYRIFOS ON NMDA AND TOTAL MUSCARINIC RECEPTORS IN HIPPOCAMPUS, CEREBRAL CORTEX AND HYPOTHALAMUS.

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Abstract

Sustained stress causes reversible impairment to the hippocampus, possibly related to glutamatergic effects. Effects of concurrent repeated stress and chlorpyrifos (CPF) on NMDA and total muscarinic receptors in the brain were studied in Long-Evans rats. Groups of rats (n=8) were handled 5 days/week (control); restrained 1 hour/day for 5 days/week; swum 30 minutes for 1 day/week; or restrained 4 days/week and swum for 1 day/week, for 28 days. On day 24, each group was injected either with corn oil or CPF 160 mg/kg sc 4 hours after restraint. On day 28, blood samples were collected for plasma corticosterone, norepinephrine, and epinephrine. Brains were dissected into hippocampus, cerebral cortex, and hypothalamus to determine maximum binding density (Bmax) and equilibrium dissociation rate constant (Kd) of NMDA and total muscarinic receptors. Swim and restraint with swim elevated plasma corticosterone more than handling and restraint alone. Swim and restraint with swim decreased plasma norepinephrine and epinephrine more than handling. Restrained rats (1.839 ± 0.140 nM) had higher Kd of NMDA receptors in the hippocampus than control (1.386 ± 0.150 nM) and restrained with swim rats (1.333 ± 0.140 nM); however, Bmax was similar. CPF decreased Bmax and Kd of total muscarinic receptors in the cerebral cortex of swum rats (237.64 ± 17.36 fmol/mg protein, 0.216 ± 0.023 nM) and CPF also decreased Bmax of total muscarinic receptors in the cerebral cortex of restrained rats (229.08 ± 17.36 fmol/mg protein). There were no effects of stress, CPF, and interactions of stress and CPF on NMDA receptors in the cerebral cortex and on total muscarinic receptors in the hippocampus although CPF down-regulated total muscarinic receptors of swum and restrained rats. Therefore,

restraint stress increased binding affinity of NMDA receptors in the hippocampus and decreased Bmax of total muscarinic receptors in the cerebral cortex. Restraint with swim decreased both Bmax and Kd of total muscarinic receptors in the cerebral cortex. Table 6 summarized overall results of this study.

Keywords: repeated stress, chlorpyrifos, NMDA receptors, total muscarinic receptors

Introduction

The major targets of stress are the hypothalamic-pituitary adrenal axis and the sympathetic adrenal medulla system, both of which are important to maintain physiological homeostasis. However, repeated stress causes reversible impairment to other brain regions such as the hippocampus (McEwen 1999). Furthermore, glucocorticoids, excitatory amino acids, along with *N*-methyl-D-aspartate (NMDA, ionotropic glutamatergic receptors) receptors are involved in the stress response (Magarinos and McEwen 1995). NMDA receptors are located with high density in the cerebral cortex, hippocampus, striatum, septum, and amygdala and these areas are thought to be involved in cognition and emotion (Petrie et al. 2000). Excessive stimulation of NMDA receptors can cause neuronal cell death. This may contribute to several neuropathological disorders including Alzheimer's disease, brain ischemia, and epilepsy (Petrie et al. 2000). Overstimulation of NMDA receptors may cause neurons to be more vulnerable to toxic agents.

The cholinergic system has also been reported to be involved in the stress response. For example, repeated restraint stress reduces acetylcholinesterase activity (Sunanda et al. 2000). Cholinesterase inhibitors not only inhibit cholinesterase activity, but they also down-regulate muscarinic and nicotinic receptors (Ecobichon 2001). Some acetylcholinesterase-inhibiting organophosphates, such as soman, increase extracellular glutamate concentrations in the hippocampus during seizures (Lallement et al 1991, Lallement et al 1992).). NMDA and /or muscarinic receptors may have been modified in the hippocampus and cerebral cortex of Gulf War veterans who were exposed to chronic stress and toxic agents. Modulation of NMDA and muscarinic receptors may lead the hippocampus and cerebral cortex to be vulnerable to organophospohate insecticides. Our study was designed to assess the effects of repeated stress and CPF (a cholinesterase inhibitor) on NMDA and total muscarinic receptors.

Effects of repeated stress on the hippocampus appear to be reversible and are mediated by glucocorticoid hormones working together with excitatory amino acids (EAA) (such as glutamate) and NMDA receptors, along with transmitters such as serotonin and GABA. Glucocorticoids, EAA, and NMDA receptors are involved in neuronal damage and death in the pyramidal neurons of the hippocampus associated with seizures, ischemia, and severe and prolonged psychosocial stress (McEwen 2000). For example, psychosocial stress and NMDA activation regulate adult neurogenesis in the tree shrew (Gould 1997). Glucocorticoids and NMDA activation regulate neurogenesis in the dentate gyrus of developing and adult rats (Cameron et at 1998). The NMDA receptor antagonist (MK801) prevents the corticosterone-induced decrease of the proliferation of granule neurons in the dentate gyrus of adult rats (Cameron et al 1998). CGP43487, a

competitive antagonist of NMDA receptors, similarly blocks restraint-induced dendritic atrophy (Magarinos and McEwen 1995). This suggests the atrophy of CA3 neurons in restrained rats is reversible and NMDA receptors are major factors in the enhancement of atrophy. Our study examined effects of stress and CPF on NMDA receptors.

Cholinesterase inhibitors increase concentrations of acetylcholine in synapses of both peripheral and central nervous systems via inhibition of acetylcholinesterase enzyme activities (Ecobichon 2001). Cholinergic pathways in the basal forebrain project to the entire telencephalon, including the cerebral cortex and hippocampus (Cooper et al. 1996) and muscarinic receptors (M_1 receptors) predominate in the hippocampus and cerebral cortex (Taylor and Brown 1999). There are a few studies demonstrating that stress disrupts the cholinergic system (Sunanda et al. 2000, Kaufer et al. 1998). Both glutamatergic and cholinergic systems are distributed in the hippocampus and cerebral cortex. Furthermore, excess glutamate, an excitatory neurotransmitter, can endanger and/or cause cell death of neurons (McEwen et al. 1995); it may modulate the toxicity of cholinesterase inhibition. Previous studies suggested the possibility of stress and CPF effects on muscarinic as well as glutamatergic receptors.

As noted, repeated stress increases glutamate and modulates NMDA receptors. CPF inhibits acetylcholinesterase activity and down-regulates muscarinic receptors. Furthermore, there are interconnections of glutamatergic and cholinergic systems in the hippocampus and cerebral cortex. Therefore, it is likely that NMDA and muscarinic receptors are affected by stress and CPF. The major objective of our study was to examine the effects of our stress models and CPF on the NMDA and total muscarinic receptors in the hippocampus, cerebral cortex and hypothalamus. We had investigated the

effects of our stress models (handling as a control, restraint, swim, restraint with swim) and CPF 160 mg/kg sc on the activity of cholinergic enzymes and concentrations of glutamate (Chapter 4). Results demonstrated that restraint with swim stress reduced carboxylesterase activity in the cerebral cortex, but there was no interaction of stress and CPF. Restraint with swim also increased concentrations of glutamate more than swim stress; however, this stress effect was independent from CPF effect. This study assessed the same stressors and the same dose of CPF on NMDA and total muscarinic receptors. On day 28, plasma concentrations of corticosterone, norepinephrine, and epinephrine were also examined because they were indicators of stress situations.

Materials and methods

A) Animals

Sixty-four adult male Long-Evans rats aged 54-82 days, weighing 246-422 g, were obtained from Harlan Sprague Dawley (Indianapolis, IN) and shipped to Laboratory Animal Resources at Virginia Tech. Rats were allowed to acclimate to their surroundings for 7 days. They were housed individually with food and water provided *ad libitum*. Room temperature was kept at 21-23 °C with a light cycle of 7 pm to 7 am. All procedures involving animals were in accordance with the Virginia Polytechnic Institute and State University guidelines and approved by the Virginia Tech Animal Care Committee.

B) Experimental design

The experimental design was a generalized, randomized, complete block design. Treatment structure was 4x2, which consisted of 4 types of chronic stress (handling as a control, restraint, swim, and restraint with occasional swim) and subcutanous injection either with corn oil or chlorpyrifos. Eight rats were randomly allocated into the 4x2 treatment design. Treatment and sacrifice were performed in two experimental blocks separated by 8 days. Body weights were analyzed as repeated measurement on various days. Concentrations of plasma corticosterone, norepinephrine and epinephrine, and NMDA and total muscarinic receptors in the hippocampus and cerebral cortex, and NMDA in the hypothalamus were assessed with a single measurement at sacrifice.

Rats were handled 5 days/week, restrained 1 hour/day for 5 days/week, swum 30 minutes for 1 day/week, or restrained for 4 days and swum for 1 day/week, for 28 days. Body weights were measured on days 0, 3, 7, 14, 21, 24, 26, and 28. On day 24, rats were stressed at 8:15-10:00 am and then rats in each group were injected either with corn oil or CPF 160 mg/kg sc 4 hours after restraint. CPF was suspended in corn oil to give a concentration of 200 mg/ml and the volumes of corn oil or CPF given to each rat were 0.27-0.41 ml. After dosing, rats were casually observed for clinical signs such as tremor, salivation, lacrimation, urination, defecation, weakness and need for treatment with atropine, 0.1-0.2 mg/kg, if signs became severe. On day 28, rats were stressed from 8 am to 12 pm. Immediately (10-30 minutes) after stress, rats were sacrificed by decapitatation and blood samples were collected in heparinized tubes to determine blood acetylcholinesterase (AChE) activity and plasma concentrations of corticosterone, norepinephrine, and epinephrine. Brains were dissected into hippocampus, cerebral cortex, and hypothalamus to examine the maximum binding density (Bmax) and

dissociation equilibrium rate constant (Kd) of NMDA and total muscarinic receptors. Brain tissues were kept at -70°C until assayed.

C) Stress equipment and chemicals

Restraint stress was performed using a plexiglass cylinder, 6.5 cm in diameter and 22 cm in length. Rats were held in these cylinders for 60 minutes in the morning between 9 am and 1 pm. Restraint stress was performed 5 days per week. Swim stress was performed by forcing rats to swim for 30 minutes in 21-23 °C water in a 30-gallon fish tank that was divided equally into 4 sections.

Chlorpyrifos (*O,O'*-diethyl-3,5,6-trichloro-2-pyridinyl-phosphorothioate, 99.5% pure) was obtained from Chemical Services (West Chester, PA). Corticosterone radioimmunoassay kits were purchased from ICN Biomedicals, Inc., Costa Mesa, CA. Norepinephrine bitartrate, epinephrine hydrochloride, dopamine hydrochloride, 3,4-dihydroxyphenylacetic acid (DOPAC), and isoproterenol hydrochloride (an internal standard of monoamines in the high performance liquid chromatography assay) were purchased from Sigma Aldrich (St. Louis, MO). [^{3}H] MK-801 (5H-dibenzo [a,d] cyclohepten-5,10-imine,10,11-dihydro-5-methyl-, (5S)-, (Z)-2-butenedioate(1:1), dizocilpinemaleate) is the *N*-methyl-D-aspartate antagonist. It had specific activity of 25 Ci/mmol and concentration of 1 mCi/ml (American Radiolabeled Chemicals Inc. St. Louis MO). l-Quinuclidinyl[phenyl-4-^{3}H]benzylate ([^{3}H] QNB) is a nonselective muscarinic receptor antagonist. It had specific activity of 42 Ci/mmol and concentration of 1.0 mCi/ml (Amersham Biosciences, Piscataway, NJ). Radioactive material experiments were performed in accordance with Environmental Health and Safety

Services, Virginia Tech guidelines. Other chemicals were high performance liquid chromatography (HPLC) or analytical grade. They were purchased from Sigma Aldrich (St. Louis, MO), VWR (Suwanee, GA) and Fisher (Suwanee, GA).

D) Dissection of rat brain

Hippocampus, cerebral cortex and hypothalamus were selected. Brain dissection was performed following the location of these regions (Palkovits and Brownstein 1988). To remove the brain, the calvarium was removed and the foramen magnum was exposed. The medulla was cut *in situ* and the dura matter was removed. The brain was removed in a rostro-caudal direction, transecting any cranial nerves attaching the brain to the skull. The brain was placed in a brain mold. The brain was dissected by making two transverse section cuts. One cut was just caudal to the optic chiasm; the other part was at the caudal aspect of the mammillary body. The hippocampus was removed by blunt dissection from the above section and the caudal segment of the brain.

E) Determination of plasma corticosterone levels

Corticosterone concentrations were determined using blood samples collected from the orbital sinus in heparinized microcentrifuge tubes and spun on a Beckman microfuge Lite® centrifuge at 3500 x g for 3 minutes. Plasma samples were kept at -70 °C until assayed. Plasma corticosterone concentrations were measured using a ^{125}I radioimmunoassay kit for rats and mice (ICN Biomedicals, Inc., Costa Mesa, CA). In brief, plasma was diluted with a steroid diluent to 1:200. The diluted plasma (100 μl) was mixed with 200 μl of antiserum to corticosterone and incubated at room temperature for 2

hours. Then, 500 μl of a precipitating solution was added to stop the reaction, and the suspension was vigorously mixed. The precipitate was centrifuged at 1000 x g for 15 minutes. A COBRA II Auto-gamma was used for gamma counting of the precipitate. Detection limit was in the range of ng/ml of plasma.

F) Determination of plasma norepinephrine and epinephrine

Plasma norepinephrine and epinephrine were analyzed by reversed-phase HPLC coupled with electrochemical detection (Wang et al 1999). The method provides sufficient sensitivity in a range of ng/ml plasma. Norepinephrine and epinephrine were extracted from plasma by acid-washed alumina in Tris buffer pH 8.6. Isoproterenol, an internal standard (final concentration of 0.5 μM), was added to the plasma before the extraction. Acid-washed alumina was washed twice with distilled water and desorbed with a mixture of 0.04 M phosphoric acid-0.2 M acetic acid (20:80, v/v, pH 1.5-2.0). Then, the supernatant was filtered through an Acrodisc LC 13 mm syringe filter with 0.2 μm PVDF membrane into HPLC vials. HPLC conditions were modified from Wang et al (1999). The HPLC system consisted of a Hewlett Packard Series 1100 Quaternary pump, degasser, autosampler, and electrochemical detector. Separation was performed on a reversed phase C18 analytical column (Nucleosil 100 3μ, 250x4 mm) preceded by a guard column (Nucleosil 100 C18 3μ, 8x4 mm, Macherey-Nagel Gmbh & Co., Düren, Germany). Electrochemical detection was used in the oxidation mode with +0.35 volt. Isocratic elution was performed at a flow rate of 0.6 ml/min. The mobile phase consisted of 0.1 M sodium acetate, 25 mM citric acid, 134 μM ethylenediaminetetraacetic acid

disodium dihydrate salt (EDTA), 230 μM octanesulfonic acid, and 6 % methanol at pH 4.7.

G) Determination of the maximal number of receptors and the equilibrium dissociation constant of neurotransmitter receptors

The maximal number of receptors (Bmax) and equilibrium dissociation constant (Kd) of neurotransmitter receptors were determined using a radioligand-binding method (Motulsky and Neubig 1997, Betancourt and Carr 2004, Bellinger et al. 2002). The methods were performed as saturation binding experiments of antagonists to the receptors for total muscarinic receptors (Betancourt and Carr 2004) and for NMDA receptors (Bellinger et al. 2002). Saturation binding experiments determine receptor number and affinity by measuring specific binding at various concentrations of the radioligand (Motulsky and Neubig 1997). The assumption of this assay was that the incubation had reached equilibrium. The radioligand at various concentrations was incubated with membrane suspensions to determine total binding. Nonspecific binding was determined from incubation of the radioligand with membrane suspensions and an unlabeled compound that binds to essentially all the receptors. Specific binding was determined as total binding minus nonspecific binding at each concentration (Motulsky and Neubig 1997).

Brain tissues were thawed in about 10 volumes of 0.32 M sucrose for assay of muscarinic and NMDA receptors. The tissue was homogenized using a Polytron Brinkmann (Brinkmann Instrument Westbury, NY) at speed of 5-6 cycles for 30 seconds. This was followed by centrifugation using a refrigerated centrifuge (International

Equipment Company, Needham Hts, MA) at 1000 x g, 4°C, for 10 minutes. The homogenized suspension was divided into two parts for the muscarinic and NMDA receptor binding assays. For the muscarinic binding assay, the suspension was centrifuged at 48,000 x g, 4°C for 10 min by an ultracentrifuge Beckman L8-80M (Palo Alto, CA). The pellet was washed twice with cold 50 mM Tris-HCl buffer (containing 120 mM NaCl; 5 mM KCl; 2 mM $CaCl_2$; 1 mM $MgCl_2$) by resuspending the pellet in fresh cold buffer and centrifuging. The final pellet was resuspended in cold buffer to have 0.1-0.4 mg of membrane protein. The mAChR binding was determined with 6 concentrations of [^{3}H] QNB (0.1-10 nM). The assay was performed in duplicate in a 96-well microplate. Atropine sulfate 1 μM (25 μl) was used as an unlabeled antagonist. Membrane suspension (100 μl) was incubated without atropine (total binding) or with atropine (nonspecific binding) at 37 °C for 60 minutes. [^{3}H] QNB 25 μl was added to give final concentrations of 0.1-10 nM. The incubation was terminated by rapid filtration over FilterMat (filter paper for receptor binding, 1μm, Molecular Devices, Sunnyvale, CA) using a Cell Harvesters Instrument (Molecular Devices, Sunnyvale, CA). The filters were dried in an oven at 50 °C for 1 hour before they were punched into scintillation vials. Liquid scintillation cocktail (Ultima Gold F, PerkinElmer Life and Analytical Sciences, Boston, MA) was added at a volume of 3 ml. Radioactivity was measured using a liquid scintillation counter, Beckman LS6500 (Beckman Coulter. Fullerton, CA.)

For the NMDA receptor assay, brain suspensions were centrifuged at 40,000 x g, 4 °C, for 10 minutes. The pellet was washed with 5 mM Tris-HCl pH 7.4 by resuspending and centrifuging at 40,000 x g, 4 °C, for 30 minutes. The pellet was washed with deionized water and this pellet was resuspened in a small amount of 5 mM Tris-HCl

and kept at -70°C until time for the assay. On the assay day, the pellet was thawed and washed twice with 5 mM Tris-HCl pH 7.4 by resuspending and centrifuging. Then, it was resuspended in 5mM Tris-HCl to have 0.1-0.4 mg of membrane protein. Membrane suspension of 25 μl was added to microplate wells containing 5 mM Tris-HCl pH 7.4 (125 μl). Glutamate, glycine and spermidine (all at final concentration of 10 μM) were present in the buffer for maximum activation of NMDA receptors. [^{3}H] MK-801 in a volume of 25 μl at 6 final concentrations between 0.1-15 nM were added to the microplate. Membrane suspensions were incubated with 50 μM of MK-801 unlabeled (nonspecific binding) or without MK-801 unlabeled (total binding) at 25 °C for 120 minutes. The incubation was terminated by rapid filtration over FilterMat (filter paper for receptor binding, 1μm) by Cell Harvesters Instrument. The filters were dried and punched into scintillation vials. The scintillation cocktail (Ultima Gold F) was added and radioactivity was measured using a liquid scintillation counter Beckman LS6500. Count per minute of each sample was calculated to fmol/mg protein. Bmax and Kd were calculated by GraphPad Prism Software package version 4 (Motulsky 2003).

H) Statistical analysis

Data were analyzed by two-way ANOVA using the SAS program (version 8.2, SAS Institute Inc., Cary, NC). To test the effects of repeated stress on body weight before injection of CPF from day 0 to day 24, the change of body weight was analyzed by mixed model ANOVA and post hoc comparisons were tests of simple main effect of repeated stress on separate days. To hold the experimentwise error rate to 0.05, the comparisonwise alpha level was 0.00833 (Bonferroni-corrected). On days 26 and 28, the

main effects of chronic stress and CPF on body weight were analyzed by two-way ANOVA and post hoc comparisons of stress effect and the interaction of repeated stress with CPF were tested by Tukey's HSD because the data were analyzed on each day, and two treatments (stress and CPF) contributed. The main effects of repeated stress, CPF, and the interaction of repeated stress and CPF on day 28 on plasma concentrations of corticosterone, norepinephrine, and epinephrine, Bmax and Kd of NMDA and total muscarinic receptors in the hippocampus, cerebral cortex, and hypothalamus were analyzed by two-way ANOVA. Post hoc comparisons were analyzed by Tukey's HSD for stress effects and the interaction of stress and CPF within stress treatment. Significant difference was considered when $p < 0.05$.

Results

Prior to CPF injection from day 3 to day 24, repeated stress did not affect body weight (Figure 1). On day 26 and 28, repeated stress and the interaction of chronic stress and CPF did not affect body weight. Therefore, no main effects of repeated stress on body weight were detected. CPF significantly decreased change of body weight on day 26 ($df = 1, 55$, $p = 0.0178$); however, it did not significantly decrease change of body weight on day 28 ($df = 1, 55$, $p = 0.0990$, Table 1).

Plasma corticosterone is an indicator of stress. Swim and restraint with swim increased plasma corticosterone more than control or restraint stress ($df = 1, 53$, $p < 0.0001$ for all comparisons of swim, control; swim, restraint; restraint with swim, control; restraint with swim, restraint, Table 2). Swim and restraint with swim decreased plasma

norepinephrine and epinephrine more than control (norepinephrine, swim, control, df = 1, 47, p = 0.0275; norepinephrine, restraint with swim, control, df = 1, 47, p = 0.0033; epinephrine, swim, control, df = 1, 47, p = 0.0132, epinephrine, restraint with swim, control, df = 1, 47, p = 0.0019, Table 2). CPF did not alter plasma corticosterone, norepinephrine or epinephrine. There were no interactions of repeated stress and CPF on plasma corticosterone, norepinephrine, or epinephrine.

NMDA receptors play an important role in impairment of the hippocampus. Repeated stress did not alter Bmax of NMDA receptors in the hippocampus; however, Kd of samples from restrained rats was higher than samples from control (df = 1, 52, p = 0.0318) and samples from restrained with swim rats (df = 1, 52, p = 0.0137, Table 3). CPF or interactions of CPF and repeated stress did not alter Bmax and Kd of NMDA receptors in the hippocampus. Furthermore, repeated stress, CPF, or interactions of repeated stress and CPF did not affect Bmax or Kd of total muscarinic receptors in the hippocampus (Table 3) or Bmax or Kd of NMDA receptors in the hypothalamus (Table 4) and cerebral cortex (Table 5).

Interestingly, ANOVA analysis showed that there were interactions of repeated stress and CPF on Bmax and Kd of total muscarinic receptors in the cerebral cortex (df = 3, 54, p = 0.0226; df = 3, 55, p = 0.0119, Table 4). Post hoc analysis showed that CPF decreased Bmax of total muscarinic receptors in the cerebral cortex in the restrained rats (df = 1, 54, p = 0.0094) and the swum rats (df = 1, 54, p = 0.0013) when compared to CPF given to control rats (df = 1, 54, p = 0.0740). CPF also decreased Kd of total muscarinic receptors in the cerebral cortex in the swum rats (df = 1, 55, p = 0.0069) when compared to control rats also given CPF (df = 1, 55, p = 0.4186).

Discussion

NMDA receptors in the hippocampus are involved in stress responses (McEwen 1999). Cholinesterase inhibitors not only inhibit acetylcholineseterase activity but also down-regulate muscarinic receptors (Ecobochon 2001). Furthermore, glutamatergic and cholinergic systems interact (Giovannini et al. 1997, Castellano et al. 1996). Our results demonstrated that restraint for 1 hour, 5 days/week for 28 days increased Kd of NMDA receptors in the hippocampus, but there was no interaction of stress and CPF on NMDA receptors. The interaction of stress and CPF was demonstrated because CPF reduced Bmax and Kd of the total muscarinic receptors in the cerebral cortex of the swum rats and Kd of the restrained rats.

Swim and restraint with swim increased plasma corticosterone more than control and restraint stress, suggesting that swim and restraint with swim were sufficient stressors. The elevation of plasma corticosterone indicated that the hypothalamic-pituitary adrenal axis was stimulated. Swim and restraint with swim decreased plasma norepinephrine and epinephrine more than control. The plasma concentrations of norepinephrine and epinephrine were anticipated to be elevated because the sympathetic adrenal medulla was supposed to be activated (Axelrod and Reisine 1984, Toates 1995). This reduction may be because repeated swim and restraint with swim caused exhaustion in rats and modified the sympathetic adrenal medulla system. The hypothalamic-pituitary-adrenal axis and the sympathetic adrenal medulla interact at the hypothalamus, pituitary, and adrenal gland (Toates 1995). Glucocorticoids influence synthesis of norepinephrine and epinephrine in the adrenal medulla (Toates 1995). The high levels of

corticosterone may modulate the synthesis of norepinephrine and epinephrine in the adrenal medulla. Therefore, the high concentrations of plasma corticosterone of the swum and restrained with swim rats may modify the release of norepinephrine and epinephrine from adrenal medulla to blood circulation.

Repeated stress did not alter Bmax of NMDA receptors in the hippocampus and cerebral cortex, suggesting our stress treatments were insufficient to affect maximum number of NMDA receptors. Our data demonstrated that the Kd of NMDA receptors in the hippocampus of restrained rats was higher than that observed in control and restrained with swim rats. This suggests that restraint stress was sufficient to elevate binding affinity of NMDA receptors of the hippocampal neurons. NMDA receptors in the hippocampus are involved in memory and learning functions. The elevated binding affinity of these receptors may contribute to modulation of memory or learning in these rats. While behavioral study of this effect needs to be investigated, we noticed that restraint rats were familiar with plexiglass tubes over time. NMDA receptors have been reported to be involved in stress response (Magarinos and McEwen 1995). McEwen (1999) proposed that stress affects glucocorticoids and NMDA receptors in the hippocampus. NMDA together with glucocorticoids and serotonin are participants in neuronal impairment and death in the pyramidal neurons following ischemia, seizures, and in severe and long-term psychosocial stress (McEwen 2000). Therefore, the increase of Kd of NMDA receptors to noncompetitive antagonist (MK-801) induced by repeated restraint stress may participate in neuronal impairment. However, the histopathology of the hippocampus remains to be examined in this model.

CPF did not alter Bmax and Kd of NMDA receptors in the hippocampus, cerebral cortex and hypothalamus. Some cholinesterase inhibitors such as soman are associated with other adverse effects on the central nervous system modulated by NMDA receptors, such as seizures (Raveh et al.1999). For example, caramiphen (a muscarinic M1 antagonist) protects rats from soman-induced seizures by decreasing Bmax of [^{3}H] MK-801 in rat brain membranes 24 hours following soman intoxication (Raveh et al. 1999). CPF at the dose used in our experiments did not cause any signs of seizures and did not affect NMDA responses, suggesting the dose of CPF was insufficient to modulate NMDA receptors. There were no interactions of CPF and repeated stress on NMDA receptors in either the hippocampus or cerebral cortex.

Repeated stress, CPF or the interaction of repeated stress and CPF did not affect total muscarinic receptors in the hippocampus, indicating that our stressors and CPF were not sufficient to modulate the muscarinic receptors in the hippocampus. This suggests that unaffected muscarinic receptors could not injure the hippocampus structure.

Down-regulation of total muscarinic receptors by CPF depended on type of stressor and brain region. Statistical analysis indicated that CPF decreased the elevated Bmax and Kd of total muscarinic receptors in the cerebral cortex of the swum rats. CPF also decreased Bmax of total muscarinic receptors in the cerebral cortex in the restrained rats. When compared to control rats, CPF did not significantly decrease the Bmax or Kd of total muscarinic receptors, suggesting that there were additive effects of CPF and swim and restraint stressors. The results agree with a previous study using autoradiographic technic in which repeated immobilization of rats for 2 hours/day for 21 days increased the

Bmax of muscarinic receptors in several brain regions such as cerebral cortex, hippocampus, and caudate-putamen but did not affect Kd (Gonzaiez and Pazos 1992).

Glutamate facilitates the control of striatal cholinergic transmission via NMDA and non-NMDA receptor activation and agonists of both receptors have been reported to facilitate acetylcholine release from rat striatal slices (reviewed by Morari et al. 1998). NMDA and muscarinic receptors are mainly located in the hippocampus and cerebral cortex; therefore, activation of one type of these receptors may modulate other receptors. Presynaptic glutamatergic modulation of ACh release was demonstrated in the cerebral cortex (reviewed by Morari et al. 1998). However, our results showed no effects of stress or CPF on NMDA receptors.

The cholinergic system involved in learning and memory performance appears to be modulated by other neuronal systems including the glutamatergic system (reviewed by Li et al. 1996). Our results demonstrated that concurrent exposure to repeated swim and restraint or CPF reduced the response to stress of the total muscarinic receptors suggesting that learning and memory may be modified. Gulf War veterans have self-reported alterations of learning and memory that might be related to modulation of muscarinic and NMDA receptors.

In summary, CPF and the interaction of CPF and repeated stress did not affect NMDA receptors in both the hippocampus and cerebral cortex. This study used restraint for 1 hour, 5 days/week for 28 days. With this regimen, Kd of NMDA receptors was increased in the hippocampus, but there was no interaction of stress and CPF on NMDA receptors. The elevated Kd of NMDA receptors may modulate memory functions of the hippocampus; otherwise, it may impair hippocampal structure. The additive effects of

repeated stress and CPF were demonstrated as CPF reduced Bmax and Kd of the total muscarinic receptors in the cerebral cortex of the swum rats and Kd of the restrained rats, whereas CPF did not decrease Bmax or Kd of control rats.

Acknowledgments

The authors would like to acknowledge the assistance of Kristel Fuhrman, Geraldine Magnin, Kristin Knight, Jonathan Hinckley, Daniel Ward, Sandy Hancock, Delbert Jones, Barbara Wise, Ellen Uri and Nikola Agatic. Appreciations are to the Faculty of Liberal Arts and Science, Kasetsart University, and the Thai government for T. Pung's scholarship. This work was supported by DAMD 17-99-1-9489 from the U.S. Army Medical Research and Materiel Command and Virginia-Maryland Regional College of Veterinary Medicine.

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Table 1. Effects of chlorpyrifos 160 mg/kg sc given on day 24 on body weight gains measured on days 26 and 28

Type of injection	Change of body weight from day 0 (g)	
	Day 26	Day 28
Corn oil	74.63 ± 3.65	77.93 ± 3.87
Chlorpyrifos	62.03 ± 3.65*	68.75 ± 3.87

Data are presented as mean ± SEM (n=32). * indicates significant difference from corn oil injection ($p = 0.0178$).

Table 2. Effects of repeated stress on concentrations of plasma corticosterone, norepinephrine, and epinephrine

Type of stress	Corticosterone	Norepinephrine	Epinephrine
Handling (control)	463.06 ± 59.07	58.35 ± 8.49	92.98 ±12.08
Restraint	464.99 ± 59.07	38.23 ± 8.42	63.16 ± 11.97
Swim	1195.97 ± 59.07***	32.09 ± 7.90*	50.71 ± 11.24**
Restraint with swim	1136.13 ± 63.30***	23.55 ± 7.37*	40.40 ± 10.49**

Data are presented as mean ± SEM (n=16). Plasma corticosterone is expressed as ng/ml. Plasma norepinephrine and epinepnrine are expressed as nmole/ml. * indicates significant difference of swim (p = 0.0275) and restraint with swim (p = 0.0033) from control. ** indicates significant difference of swim (p = 0.0132) and restraint with swim (p = 0.0019) from control. *** indicates significant difference of swim and restraint with swim from control and restraint ($p < 0.0001$)

Table 3. Effects of repeated stress on NMDA and total muscarinic receptors in the hippocampus

Type of stress	NMDA receptors		Total muscarinic receptors	
	Bmax	Kd	Bmax	Kd
Handling	177.22 ± 10.74	1.386 ± 0.150	288.68 ± 32.35	0.282 ± 0.041
Restraint	182.71 ± 10.37	1.839 ± 0.140*	305.23 ± 33.50	0.354 ± 0.042
Swim	186.20 ± 10.74	1.643 ± 0.145	327.85 ± 32.35	0.321 ± 0.042
Restraint with swim	179.49 ± 10.37	1.333 ± 0.140	310.32 ± 32.35	0.353 ± 0.041

Data are presented as mean ± SEM (n=16). * indicates significantly different from control ($p = 0.0318$) and restraint with swim stress ($p = 0.0137$). Bmax is expressed as fmol/mg protein. Kd is expressed as nM.

Table 4. Effects of repeated stress on NMDA receptors in the hypothalamus

Type of stress	NMDA receptors	
	Bmax	Kd
Handling	40.96 ± 9.69	2.813 ± 1.036
Restraint	57.90 ± 10.17	4.113 ± 1.088
Swim	38.72 ± 8.34	3.486 ± 0.892
Restraint with swim	39.41 ± 8.64	2.794 ± 0.924

Data are presented as mean ± SEM (n=16). No significant differences were noted. Bmax is expressed as fmol/mg protein and Kd is expressed as nM.

Table 5. Effects of repeated stress and chlorpyrifos (CPF) on NMDA and total muscarinic receptors in the cerebral cortex.

Type of stress	Type of injection	NMDA receptors		Total muscarinic receptors	
		Bmax	Kd	Bmax	Kd
Handling	Corn oil	157.06 ± 14.57	1.862 ± 0.348	272.00 ± 17.36	0.250 ± 0.023
	CPF	128.66 ± 14.57	2.582 ± 0.348	227.27 ± 17.36	0.223 ± 0.023
Restraint	Corn oil	125.19 ± 14.57	2.186 ± 0.348	295.19 ± 17.36	0.263 ± 0.023
	CPF	134.93 ± 15.59	2.391 ± 0.348	229.08 ± 17.36*	0.201 ± 0.023
Swim	Corn oil	143.04 ± 14.57	2.362 ± 0.348	323.93 ± 18.58	0.308 ± 0.023
	CPF	152.47 ± 14.57	1.831 ± 0.348	237.64 ± 17.36*	0.216 ± 0.023*
Restraint with swim	Corn oil	141.59 ± 14.57	1.515 ± 0.348	273.62 ± 17.36	0.252 ± 0.023
	CPF	161.30 ± 15.59	2.250 ± 0.373	293.59 ± 17.36	0.312 ± 0.023

Data are presented as mean ± SEM (n=8). Bmax is expressed as fmol/mg protein. Kd is expressed as nM. * indicates significant difference from corn oil within stress treatment ($p < 0.05$, according to simple main effect).

Table 6. Summary of effects of repeated stress, CPF, and interaction of stress and CPF on NMDA and mAChR receptors

Treatment	NMDA	mAChR
Stress		
Handling	No effect	
Restraint	Increased Kd of NMDA in the HP.	
Swim	No effect	
Restraint with swim	No effect	
CPF	No effect	
Stress *CPF		
Handling	No effect	No effect
Restraint*CPF	No effect	CPF decreased the elevated Kd of mAChR in the CC of restrained rats.
Swim*CPF	No effect	CPF decreased the elevated Bmax and Kd in the CC of mAChR of swum rats.
Restraint*CPF	No effect	No effect

HP= hippocampus, CC= cerebral cortex

Figure legends

Figure 1. Change of body weight after rats were either handled, restrained, swam, or restrained with swimming during 24-day treatment. Rats were injected with either corn oil or chlorpyrifos 160 mg/kg sc on day 24. The data are presented as mean of body weight change from day 0. No significant effects were noted ($p > 0.05$).

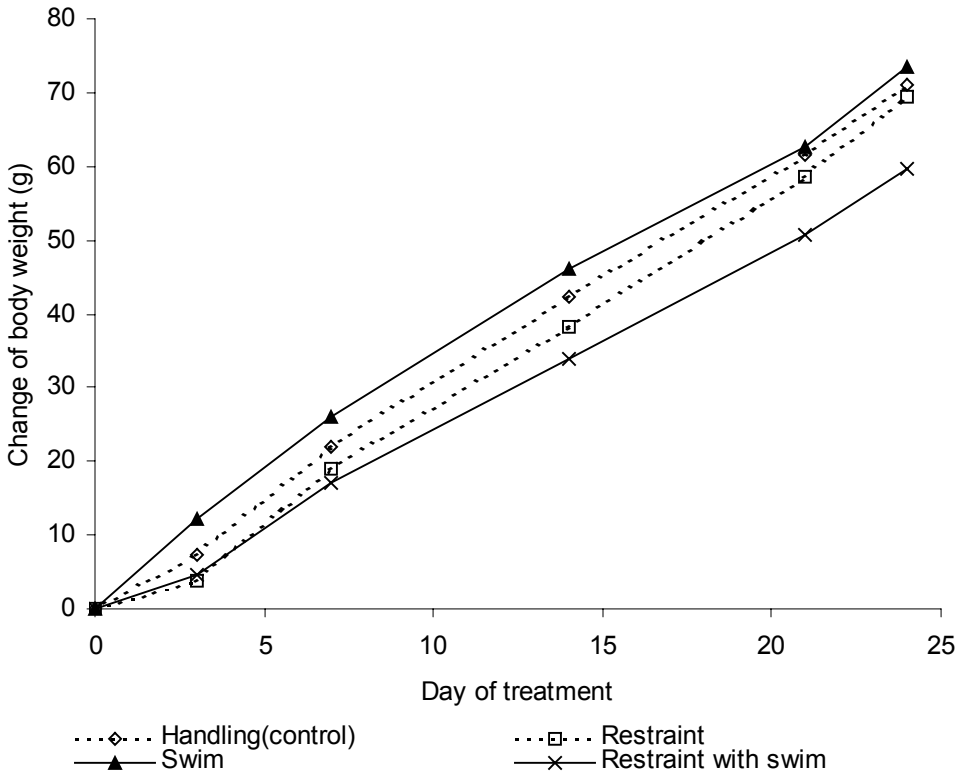
80
70
60
50
40
30
20
10
0
Change of body weight (g)
0
5
10
15
20
25
Day of treatment
Handling(control)
Restraint
Swim
Restraint with swim

Figure 1.

CHAPTER 6

EFFECTS OF REPEATED STRESS AND CHLORPYRIFOS ON CONCENTRATIONS OF MONOAMINES

EXAMINATION OF CONCURRRENT CHRONIC STRESS AND CHLORPYRIFOS ON MONOAMINES AND THEIR METABOLITES IN THE BRAIN

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Abstract

Repeated stress has been reported to alter concentrations of monoamines and their metabolites in parts of the brain. Such alteration could potentially modulate the toxicity of some toxic agents, e.g., cholinesterase inhibitors. This study investigated effects of repeated stress, chlorpyrifos, and interaction of repeated stress and chlorpyrifos on concentrations of monoamines and their metabolites in the hippocampus, cerebral cortex, and hypothalamus. Adult male Long-Evans rats were randomly divided into 4x2 treatments. The four types of chronic stress were: 1) handling as a control; 2) restraint 1 hour/day for 5 days/week; 3) swim 30 minutes/day for 1 day/week; and 4) restraint 1 hour/day for 4 days/week and random swim 1 day/week for 28 days. On day 24, rats in each group were injected either with corn oil or chlorpyrifos 160 mg/kg sc. Swim stress decreased concentrations of norepinephrine in the hippocampus more than control ($p < 0.05$) but not in the cerebral cortex. Chlorpyrifos decreased concentrations of norepinephrine in the hippocampus; however, there was no interaction of stress and chlorpyrifos on norepinephrine concentrations. Swim and restraint with swim decreased concentrations of norepinephrine but increased concentrations of dihydroxyphenyl acetic acid (DOPAC) in the hypothalamus more than control or restraint alone. Swim stress increased concentrations of dopamine in the hypothalamus more than control or restraint. Chlorpyrifos did not alter concentrations of norepinephrine, dopamine, or DOPAC in the hypothalamus. There were statistical trends of interactions of repeated stress and chlorpyrifos on serotonin in the cerebral cortex ($p = 0.06$) and hypothalamus ($p = 0.08$). Chlorpyrifos increased serotonin concentrations in the hypotahalmus of rats that were

handled and restrained but not swum. Chlorpyrifos reduced the elevated concentrations of serotonin in rats that were subjected to restraint and restraint with swim stress ($p < 0.05$). In summary, swim and restraint with swim were potential stress models that altered noradrenergic and dopaminergic responses in the hypothalamus. Table 3 summarizes overall results of this study.

Keywords: chronic stress, chlorpyrifos, monoamines

Introduction

Living organisms survive by maintaining homeostasis. When higher organisms face physical or emotional stressors, their physiological responses change to reduce or remove the threats. Two pivotal responses to stress are mediated through the hypothalamic-pituitary-adrenal axis (HPA) and sympathetic-adrenal medulla (DeKloet 1998). Stress systems interact with each other and also interact with other neurotransmitters including monoamines of the central nervous system. This influences the integration of information, initiation of specific action, and control of emotion (Konstandi et al. 2000). Even though the stress response tends to protect the organism from threats, long-term exposure to stressors can decrease defense mechanisms including detoxifying enzymes (Konstandi et al. 2000). Organophosphate insecticides not only inhibit cholinesterase activity but some of them alter concentrations of monoamines (Dam et al. 1999, Bloomquist et al. 2002, Lewis 2003). Veterans from the 1991 Gulf War and others who have experienced repeated stress might also be exposed to

organophosphate insecticides (National Academy of Sciences, 2003). The concurrent exposure to stress and insecticides may alter the monoaminergic response to stress. Furthermore, cholinergic and monoaminergic pathways innervate the hippocampus, cerebral cortex, and hypothalamus (Cooper et al. 1996). Our study assessed effects of repeated stress and chlopyrifos on concentrations of monoamines in the hippocampus, cerebral cortex, and hypothalamus.

Although interactions with an organophosphate have not been examined, chronic stress has been reported to alter concentrations of monoamines, norepinephrine, dopamine, serotonin, and their metabolites in the brain. For example, repeated restraint stress in rats decreases norepinephrine, dopamine, and serotonin in the hippocampus and hypothalamus (Konstandi et al. 2000, Sunanda et al. 2000). Repeated restraint stress of rats for 14 days after the rats had been injected into the frontal cortex with 6-hydroxydopamine delayed regeneration of serotonergic axons in the cerebral cortex (Liu et al. 2003). Swim stress in rats lowers concentrations of norepinephrine in the hypothalamus, pons, and midbrain; however, it does not alter concentrations of dopamine in striatum or midbrain (Sudo 1983). Therefore, we hypothesized that repeated stress-modified concentrations of monoamines would alter the response to CPF.

Repeated stress in our study was expected to modify concentrations of monoamines in the central nervous system. Functions and location of monoamines (norepinephrine, dopamine, and serotonin) also suggest that the stress may have effects on neurotransmitters. For example, brain catecholamines participate in the regulation of sympathetic neuronal outflow and regulation of adrenal medullary secretion (Goldstein and Pacak 2001). How catecholamines in the central nervous system control sympathetic

function in response to a stressful situation is incompletely understood (Goldstein and Pacak 2001). Furthermore, serotonin, an indole amine, plays a critical role in neurogenesis of the nervous system and in the regulation of appetite, sleep, emotion, and cognition (Frazer and Hensler 1999). Chaolouff (2000) reviewed effects of different stressors on serotonin release and reuptake, and serotonergic receptors. In brief, both acute and chronic stressors affect the serotonergic system in regions that control fear, anxiety and memory: dorsal and median raphe nuclei, frontal cortex, amygdala and hippocampus. The HPA axis also modulates serotonin transmission during exposure to stress.

Monoaminergic systems are interrelated because their innervations terminate in the same regions. Effects of stress on one monoamine may modulate the other. For example, there are two major cholinergic pathways: the basal forebrain complexes project nerve tracts to the entire nonstriatal cortex; pontomesencephalotegmental complexes send nerve tracts to the thalamus, diencephalon (including hypothalamus), and pons (Cooper et al. 1996). Noradrenergic pathways originate from clusters of neurons in the locus coeruleus and send major nornoradrenergic tracts to all cortices, thalamus, hypothalamus, olfactory bulb, cerebral cortex, brain stem and spinal cord (Cooper et al. 1996). Dopaminergic pathways are found in three systems (Cooper et al. 1996). The interplexiform amacrine-like neurons innervate the retina and olfactory bulb. The dopaminergic neurons in the tuberohypophysial, hypothalamus, and medullary periventricular that project nerve tracts to intermediate lobe of the pituitary and the median eminence of the hypothalamus and vagus nerve. The ventral tegmental and substantia nigra connects nerve transmission to neostriatum and limbic structures

including the hippocampus (Cooper et al. 1996). Serotonergic neurons originate in the dorsal and median raphe nucleus and terminate in the dorsal and ventral parts of the hippocampus and cerebral cortex (Frazer and Hensler 1999, Cooper et al.1996). Since monoaminergic systems locate and terminate their nerve tracts in the same regions as the HPA axis and cholinergic system, activation and/or inhibition of HPA axis and cholinergic system would be expected to modulate monoaminergic responses.

Cholinesterase inhibitors mainly inhibit activities of acetylcholinesterase, and other B-esterases (Ecobichon 2001). Chlorpyrifos, a cholinesterase inhibitor used in our study, affects not only the cholinergic system but also the noradrenergic, dopaminergic and serotonergic systems in the central nervous system. Dam et al. (1999) reported that *in vitro* concentrations of 50 μg/ml chlorpyrifos release about 20% of labeled norepinephrine from synaptosomes of whole rat brain. Chlorpyrifos 100 mg/kg sc to mice decreases dopamine uptake in *ex vivo* striatal synaptosomes by 90% maximum rate (Vmax) of control (Karen et al. 2001, Bloomquist et al. 2002). Even though chlorpyrifos does not cause cytotoxicity in the striatum, dopamine turnover increases (Karen et al. 2001). The mechanism of dopamine release by chlorpyrifos is not clearly related to the inhibition of acetylcholinesterase.

Hippocampus, cerebral cortex and hypothalamus were selected brain regions for the present study because hippocampal neurons receive considerable input mediated by acetylcholine and the biogenic amines, such as norepinephrine and serotonin (Cooper et al. 1996, Webster 2001, Stanford 2001a, Stanford 2001b). Serotonin evokes many different effects in CA1 hippocampal neurons (De Kloet 1998). The cerebral cortex is innervated by cholinergic, noradrenergic, dopaminergic, and serotonergic systems

(Cooper et al. 1996, Webster 2001b). Some stressors affect dopaminergic and serotonergic systems in the cerebral cortex (Mizoguchi et al. 2000, Mangiavacchi et al. 2001). The hypothalamus is a crucial region in HPA axis and its functions are controlled by many neurotransmitters including monoamines (Lowry et al. 2003, Akil et al. 1999).

As mentioned above, monoamines are associated with regulation of the stress response. Chlorpyrifos inhibits acetylcholinesterase, resulting in activation of the cholinergic system. The cholinergic system is associated and connected with monoaminergic systems, which would be expected to alter concentrations of monoamines and their metabolites. The major objective of this study was to assess effects of our repeated stress models and effects of chlorpyrifos and concurrent exposure to stress and chlorpyrifos on concentrations of monoamines. We previously investigated the effects of repeated stress models (handling as a control, restraint, swim, and restraint with occasional swim) and chlorpyrifos 160 mg/kg sc on the cholinergic and glutamatergic systems (Chapter 4 and Chapter 5). Our stress models indicated that swim and restraint with swim were effective stressors because they elevated plasma corticosterone. The present study examined effects of chronic stress and chlorpyrifos on concentrations of monoamines.

Materials and methods

A) Animals

Fifty-six adult male Long-Evans rats aged 100-120 days, 230-265 g, were obtained from Harlan Sprague Dawley (Indianapolis, IN) and shipped to Laboratory of

Animal Resources at Virginia Tech. Rats were acclimated to their surroundings for 7 days. They were housed individually with food and water provided *ad libitum*. Room temperature was kept at 21-23 °C with a light cycle of 7 pm to 7 am. All procedures involving animals were in accordance with Virginia Polytechnic Institute and State University guidelines and approved by the Virginia Tech Animal Care Committee.

B) Experimental design

The experimental design was a generalized, randomized, complete block design. Treatment structure was 4x2, which consisted of 4 types of repeated stress (handling as a control, restraint, swim, and restraint with occasional swim) and subcutanous injections of either corn oil or chlorpyrifos (CPF). Seven rats were randomly grouped into a 4x2 treatment design. Treatment and sacrifice were performed in two experimental blocks separated by 1 day. Samplings of monoamines and their metabolites were single measurements.

Rats were handled 5 days/week, restrained 1 hour/day for 5 days/week, swum 30 minutes for 1 day/week, or restrained 4 days with swimming 1 day/week, from 9:00 am to 12:00 pm for 28 days. On day 24, rats were stressed at 8:15-10:00 am and then rats in each group were injected either with corn oil or CPF 160 mg/kg sc 4 hours after restraint (1:10-2:45 pm). CPF was suspended in corn oil to give a concentration of 200 mg/ml and volumes of corn oil or CPF given to each rat were 0.25-0.35 ml. After dosing, rats were casually observed for clinical signs such as tremor, salivation, lacrimation, urination, defecation, weakness and need for treatment with atropine, 0.1-0.2 mg/kg, if signs became severe. On day 28, rats were stressed from 8 am to 12 pm. Immediately after

stress (10-30 minutes), rats were sacrificed by decapitation and brains were dissected into hippocampus, cerebral cortex and hypothalamus to determine concentrations of monoamines and their metabolites. The brain tissues were kept at -70°C until assayed.

C) Stress equipment and chemicals

Restraint stress was performed using a plexiglass cylinder, 6.5 cm in diameter and 22 cm in length. Rats were held in these cylinders for 60 minutes from 9 am to 1 pm. Restraint stress was performed 5 days per week. Swim stress was performed by forcing rats to swim for 30 minutes in 21-23 °C water in a 30-gallon fish tank that was divided equally into 4 sections.

Chlorpyrifos (*O,O'*-diethyl-3,5,6-trichloro-2-pyridinyl-phosphorothioate, 99.5% pure) was obtained from Chemical Services (West Chester, PA). Monoamine reagents norepinephrine bitartrate, epinephrine hydrochloride, dopamine hydrochloride, 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptamine oxalate (serotonin), 5-hydroxyindole-3-acetic acid (5-HIAA), and isoproterenol hydrochloride were purchased from Sigma (St. Louis, MO). Other chemicals were high performance liquid chromatography (HPLC) or analytical grade. They were purchased from Sigma Aldrich (St. Louis, MO), VWR (Suwanee, GA) or Fisher (Suwanee, GA).

D) Dissection of rat brain

Hippocampus, cerebral cortex and hypothalamus were selected. Brain dissection was performed following the location of these regions (Palkovits and Brownstein 1988). To remove the brain, the calvarium was removed and the foramen magnum was exposed.

The medulla was cut *in situ* and the dura matter was removed. The brain was removed in a rostro-caudal direction, transecting any cranial nerves attaching the brain to the skull. The brain was placed in a brain mold. The brain was dissected by making two transverse section cuts. These cuts were just caudal to the optic chiasm and at the caudal aspect of mammillary body. The hypothalamus was removed bilaterally from this section. The cerebral cortex was blunt dissected from the above section and the rostral and caudal segments of this section. The hippocampus was removed by blunt dissection from the above section and the caudal segment of the brain.

E) Concentrations of brain monoamines and their metabolites

Concentrations of monoamines and their metabolites in the hippocampus, cerebral cortex, and hypothalamus were determined by using high performance liquid chromatography combined with electrochemical detection (HPLC-ECD) (Jussofie et al. 1993). Brain samples (0.02-0.05 g) were homogenized in 250 μl of mobile phase pH 4.7 and an internal standard (isoproterenol hydrochloride, Sigma, St. Louis, MO, with a final concentration of 1 μM) was added. Brain tissues were homogenized by using a Cell Disruptor Sonicator (Heat System-Ultrasonic, Farmingdale, New York) with speed set at 6 cycles for 30 seconds. Homogenates were centrifuged by a Beckman Microfuge Lite® at 10,100 x g for 5 minutes and supernatants were transferred and kept in HPLC-light protective vials at –20 °C until assayed. Supernatants were thawed on ice on the assay days and then filtered through an Acrodisc LC 13 mm syringe filter with 0.2 μm PVDF membrane into HPLC vials. The chromatographic instrument was a Hewlett Packard Series 1100 Quaternary Pump with a degasser and autosampler (Agilent Technologies,

Wilmington, DE). Separation was performed on a reverse-phased C18 analytical column (Nucleosil 100 3μ, 250x4 mm, Macherey-Nagel, Easton, PA), preceded by a guard column (Nucleosil 100 C18 3μ 8x4 mm, Macherey-Nagel, Easton, PA). Electrochemical detection (Hewlett Packard, model 1049A) was used in the oxidation mode with +0.35 volt. Isocratic elution was performed at flow rate of 0.6 ml/min. The mobile phase was citric buffer pH 4.7 composed of 0.1 M sodium acetate, 25 mM citric acid, 134 μM ethylenediaminetetraacetic acid (EDTA), 230 μM octanesulfonic acid, and 6% of methanol (HPLC grade). External standard solutions of norepinephrine bitartrate, epinephrine hydrochloride, dopamine hydrochloride, 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptamine oxalate (serotonin), 5-hydroxyindole-3-acetic acid (5-HIAA), and isoproterenol hydrochloride were used for quantitative analysis. Range of concentrations used was $5x10^{-8}$ M to $1x10^{-5}$ M.

F) Statistical analysis

Data were analyzed by two-way ANOVA using the SAS program (version 8.2, SAS Institute Inc., Cary, NC). The main effects of repeated stress, CPF, and interactions of repeated stress and CPF on concentrations of monoamines and their metabolites in the hippocampus, cerebral cortex and hypothalamus were analyzed by two-way ANOVA. Comparisons of means of repeated stress were performed by Tukey's HSD test. Post hoc comparisons of the interaction of chronic stress and CPF were analyzed by simple main effect within stress treatment. Significant difference was considered when $p < 0.05$ or a statistical trend of difference was considerd when $p \leq 0.08$.

Results

Effects of repeated stress on monoamines and their metabolites

Repeated stress in our study did not alter concentrations of norepinephrine, dopamine, serotonin, DOPAC, or 5-HIAA in the hippocampus and cerebral cortex, except swim stress decreased concentrations of norepinephrine in the hippocampus more than handling (df = 1, 45, $p = 0.003$, Table 1).

Repeated stress affected concentrations of norepinephrine, dopamine, DOPAC and serotonin ($p < 0.01$), but not the concentration of 5-HIAA in the hypothalamus. Swim and restraint with swim stress decreased concentrations of norepinephrine in the hypothalamus more than control (df = 1, 44, $p < 0.0001$) or restraint (df = 1, 44, $p \leq 0.002$, Table 1). Swim stress increased concentrations of dopamine in the hypothalamus more than control (df = 1, 44, $p = 0.054$) and restraint (df = 1, 44, $p = 0.010$). Swim and restraint with swim increased concentrations of DOPAC in the hypothalamus more than control (df = 1, 44, $p \leq 0.001$) and restraint (df = 1, 44, $p \leq 0.002$). Restraint stress decreased concentrations of serotonin in the hypothalamus more than swim (df = 1, 44, $p = 0.0186$) and restraint with swim (df = 1, 44, $p = 0.0142$), but did not differ from control (df = 1, 44, $p = 0.0991$, Table 1).

Effects of chlorpyrifos on monoamines and their metabolites

Chlorpyrifos decreased concentrations of norepinephrine in the hippocampus when compared to corn oil (df = 1, 45, $p = 0.005$; df = 1, 45, $p = 0.035$, respectively) as shown in Table 2. However, chlorpyrifos did not affect concentrations of dopamine,

DOPAC, or 5-HIAA in the hippocampus. Chlorpyrifos did not affect concentrations of norepinephrine, dopamine, DOPAC, and 5-HIAA in the cerebral cortex and hypothalamus (Table 2). Chlorpyrifos decreased concentrations of serotonin in the hippocampus and hypothalamus (df = 1, 45, p = 0.0357; df = 1, 44, p = 0.0175) but increased concentrations of serotonin in the cerebral cortex (df = 1, 45, p = 0.0227).

Effects of repeated stress and chlorpyrifos on monoamines and their metabolites

The interaction test using ANOVA indicated there were two statistical trends of interactions of concurrent exposure to repeated stress and chlorpyrifos. These effects were on concentrations of serotonin in the cerebral cortex (df = 3, 45, p = 0.06) and hypothalamus (df = 3, 44, p = 0.08). Chlorpyrifos affected concentrations of serotonin in these tissues depending on types of stress. Chlorpyrifos increased concentrations of serotonin in the cerebral cortex in the control rats (df = 1, 44, p = 0.0124) and restrained-rats (df = 1, 44, p = 0.0155), but not in the swum and restrained with swim rats (Figure 1). Chlorpyrifos decreased concentrations of serotonin in the hypothalamus in the restrained rats (df = 1, 44, p = 0.0271) and restrained with swim rats (df = 1, 44, p = 0.0159), but not in the control or swum rats (Figure 2).

Discussion

Our previous study indicated that swim and restraint with swim increased the concentrations of plasma corticosterone on days 8 and 17 (Chapter 4). Chlorpyrifos did not affect concentrations of plasma corticosterone, but CPF did inhibit activity of blood

acetylcholinesterase, and activities of acetylcholinesterase and carboxylesterase in the hippocampus and cerebral cortex. The amount of hypothalamic tissue was insufficient to assay activities of cholinergic enzymes (Chapter 4).

Brain monoamines in the central nervous system in response to stress have been investigated (Konstandi et al. 2000, Goldstein and Pacak 2001, Koob 1999, Chaouloff 2000). Norepinephrine, dopamine, serotonin and their metabolites play an important role in coping with stressful situations. Our results demonstrated that swim stress decreased concentration of norepinephrine in the hippocampus. Swim and restraint with swim decreased norepinephrine but increased DOPAC in the hypothalamus. Furthermore, CPF reduced the elevated serotonin in the hypothalamus of restrained and restrained with swim rats.

Our results indicated that restraint stress for 1 hour/day, 5 days/week for 28 days did not alter concentrations of norepinephrine in the hippocampus, cerebral cortex or hypothalamus when compared to handling. These results were in agreement with Hellriegel and D'Mello (1997) and Campmany et al. (1996) but differed from Sunanda et al. (2000) and Konstanndi et al. (2000). Chronic immobilization for 1 hour/day for 14 days and chronic intermittent immobilization for 14 days over a period of 60 days do not alter norepinephrine concentrations in the hypothalamus, hippocampus, cerebral cortex, and locus ceruleus (Hellriegel and D'Mello 1997). Restraint 1 hour daily for 27 days increases concentrations of norepinephrine in the hypothalamus but does not alter concentrations of norepinephrine in the hippocampus or frontal cortex (Campmany et al. 1996). In contrast, restraint stress in rats for 6 hours/day for 21 days decreases concentrations of norepinephrine in the hippocampus (Sunanda et al. 2000). Restraint

stress for 2 hours/day for 4 days in rats decreases concentrations of norepinephrine in the hypothalamus (Konstandi et al. 2000). The discrepancy may be the period of restraint. The findings demonstrated that our restraint stress was insufficient to influence norepinephrine concentrations.

Swim stress for 30 minutes decreased concentrations of norepinephrine in the hippocampus more than handling (control). Swim and restraint with swim decreased concentrations of norepinephrine in the hypothalamus compared to control and restraint alone. Norepinephrine modifies glutamatergic response in the hippocampus and plays a role in learning and memory (reviewed by Ferry et al. 1999). Our results corresponded to Sudo's study (1983), in which swim stress from 15 minutes to 4 hours decreased concentrations of norepinephrine in the hypothalamus, pons-medulla, and midbrain.

The HPA axis and noradrenergic systems may interact. For example, the presence of noradrenergic synapses on cells that contain corticotrophin releasing factor (CPF) in the paraventricular nucleus (PVN) suggests close anatomical and functional interactions between central noradrenergic activity and HPA function (Liposits et al. 1986). Stress also induces norepinephrine release in the PVN of the hypothalamus as measured by *in vivo* microdialysis (Pacak et al. 1995). Norepinephrine in the PVN stimulates release of corticotropin releasing factor, CRF (Alonso et al. 1986). Norepinephrine-CRF interaction may occur in the locus coeruleus or in the terminal projections of the forebrain norepinephrine systems in the PVN of the hypothalamus, the bed nucleus of the stria terminalis, and the central nucleus of the amygdala where norepinephrine stimulates CRF release (Koob 1999, Goldstein and Pacak 2001). Koob (1999) proposed that stress activates CRF release in the locus coeruleus, resulting in stimulation of norepinephrine

release in the terminal projection regions of forebrain. Norepinephrine in the forebrain in turn stimulates the release of CRF. Swim and restraint with swim in our study influenced the HPA axis by increasing plasma corticosterone and decreasing norepinephrine concentrations in the hippocampus and hypothalamus. Under- or overactivation of noradrenergic response may decrease coping behaviors because the central noradrenergic pathways are thought to influence arousal, specifically activities such as selective attention and vigilance (reviewed by Stanford 2001a), emotional impact such as anxiety (Stanford 2001c) and depression (Stanford 2001d). Our findings suggest that the HPA response to swim and restraint with swim is interconnected to the noradrenergic system and may lead to deficit in coping behavior. However, a further study is needed to examine how they are related.

Chlorpyrifos in our study decreased norepinephrine in the hippocampus when compared to control. Since cholinergic pathways have projections to the hippocampus (Webster 2001a), inhibition of acetylcholinesterase and the decrease of norepinephrine concentrations by chlorpyrifos may modulate the response of stress to norepinephrine. However, an interaction of chronic stress and chlorpyrifos in this study was not demonstrated.

Our stress models did not affect dopamine and DOPAC in the hippocampus or cerebral cortex. Dopaminergic activity in the prefrontal cortex is thought to be involved in stress responses (Mizoguchi et al. 2000). Concurrent exposure to restraint and water immersion impairs working memory by reducing dopamine transmission and increasing dopamine D_1 receptor density in the prefrontal cortex of rats undergoing this treatment for 4 weeks with a 10 day recovery period (Mizoguchi et al. 2000). Unavoidable stress by

chronic restraint and acute immobilization with tail shock reduces extracellular dopamine in the prefrontal cortex and nucleus accumbens of rats (Mangiavacchi et al. 2001). Interaction between dopamine and norepinephrine terminals within the prefrontal cortex may involve norepinephrine acting on noradrenergic receptors (Finlay et al. 1997). In contrast, our stressors did not alter dopamine and DOPAC in the cerebral cortex, suggesting that they were not sufficient to affect the dopaminergic system in the cerebral cortex. Chlorpyrifos at 160 mg/kg sc also did not alter concentrations of dopamine and DOPAC in the hippocampus and cerebral cortex. Furthermore, there was no interaction of stress and chlorpyrifos on dopamine and DOPAC in these brain parts. The results suggested that dopamine in the hippocampus and cerebral cortex was not involved in the response to stress and chlorpyrifos in our study.

Swim increased concentrations of dopamine in the hypothalamus more than handling and restraint alone. Swim and restraint with swim increased concentrations of DOPAC in the hypothalamus. The arcuate and paraventricular nuclei of the hypothalamus project dopaminergic pathways to the median eminence of the hypothalamus and the intermediate lobe of the pituitary gland (Goldstein and Pacak 2001). Lowry et al. (2003) demonstrated that acute restraint in rats for 30 minutes immediately increases dopamine concentrations in the hypothalamus. Our repeated stressors were similar to Lowry et al. (2003). Release of norepinephrine and dopamine may be mediated via presynaptic adrenergic receptors as demonstrated *ex vivo* in slices of rat hypothalamus using adrenergic agonists and antagonists (Ueda et al. 1983). However, we found that concentrations of norepinephrine in the hypothalamus of restrained and control rats were similar. This could be the result of acclimation to the stressor. The

swum rats appeared to be more stressed than restrained rats as they had higher plasma corticosterone and higher concentrations of dopamine but lower concentrations of norepinephrine in the hypothalamus. Although there were no effects of chlorpyrifos and no interaction of stress and chlorpyrifos on dopamine and DOPAC in the hypothalamus, there still is a suggestion that noradrenergic and dopaminergic pathways are interrelated with the function of HPA axis in the stress response. Forced swimming may activate motor activity, which is mostly regulated by dopaminergic neurons in the striatum that project pathways to the hypothalamus. Norepinephrine in the brain appears mainly to modify responsiveness to other synaptic inputs, rather than acting directly as a stimulator or inhibitor of neuronal function (Goldstein and Pacak 2001). Noradrenergic neurons may be less activated and then release less norepinephrine. Dopamine is a precursor of norepinephrine synthesis and dopamine is metabolized to DOPAC. The high levels of dopamine and DOPAC in the swum and restrained with swim rats indicated that dopaminergic system was activated, but these precursors were not efficiently transformed to norepinephrine.

Chlorpyrifos increased concentrations of serotonin in the cerebral cortex of rats that were handled and restrained but not of rats that were swum or restrained with swim. This demonstrated that handled and restrained rats responded to chlorpyrifos in a similar way, but differed from swum and restrained with swim rats. Chlorpyrifos had a statistical trend to decrease serotonin concentrations in the hypothalamus of rats that were subjected to restraint and restraint with swim, but not to handling or swim alone. The reason was not clear; however, chlorpyrifos inhibited acetylcholinesterase activity in the hippocampus and cerebral cortex (chapter 4). Cholinegic pathways innervate the cerebral

cortex, hypothalamus and raphe nuclei. Serotonergic pathways innervate the cerebral cortex and hypothalamus. Excess acetylcholine overactivates the cholinergic system and then may modulate serotonergic response and release of serotonin.

Serotonergic neurons in raphe nuclei receive afferents from different regions of the brain, including those that release CRF and glucocorticoids, suggesting that, during stress, serotonergic neurons are connected to or under the control of the HPA axis (Jacobs and Azmitia 1992, Azmitia 1999). Other neurotransmitters such as acetylcholine, norepinephrine, dopamine, and GABA are present in the extracellular of the raphe nuclei (reviewed by Azmitia 1999). There are nerve inputs from the cortex, hypothalamus, brainstem, and spinal cord to the raphe nuclei. Serotonergic efferents from the raphe nuclei project to fear- and anxiety-related regions such as the hypothalamus, hippocampus, periaqueductal grey (Jacobs and Azmitia 1992, Chaouloff 2000), and cerebral cortex (Stanford 2001b). The serotonergic system is involved in the manifestations of stress (Reuter and Jacobs 1996, Adell et al. 1997). Forced swim has been reported to decrease (Adell et al. 1997), increase (Reuter and Jacobs 1996), or have no affect (Kirby et al. 1997) on serotonin in the hippocampus. Restraint stress for 14 days has inhibitory effects on the regeneration of axons of serotonin-containing neurons, and the axons respond to neurotoxins (6-hydroxydopamine and 5,7-dihydroxytriptamine) earlier than noradrenergic axons (Liu et al. 2003).

Our findings demonstrated that the hypothalamus was vulnerable to the effects of concurrent exposure to stress and chlorpyrifos. The hypothalamus, especially the dorsalmedial region, relays information to neural pathways mediating neuroendocrine, autonomic, and behavioral responses to stress (Lowry et al. 2003). Stressors elevate

dorsalmedial hypothalamic concentrations of norepinephrine, dopamine, and serotonin (Lowry et al. 1996). For instance, restraint for 30 minutes immediately increases concentrations of norepinephrine, dopamine, serotonin and 5-HIAA in the dorsal hypothalamic area (Lowry et al. 2003). Serotonergic and dopaminergic systems modulate the activity of glutamic acid decarboxylase in the hypothalamus, probably contributing to the excitability of efferent hypothalamic pathways that regulate stress responses (DiMicco et al. 2002). There are mutual interactions between the noradrenergic and serotonergic systems and the HPA axis in patients with depression (reviewed by Stanford 2001d). HPA dysfunction could affect monoamines either through effects of CRF on monoamine release or through its effects on glucocorticoid secretion. Norepinephrine could diminish the release of serotonin by hyperresponsive α_2-adrenergic receptors or hyporesponsive α_1-adrenergic receptors. Hyporesponsive 5-HT_{1A} (serotonin) receptors would diminish the release of norepinephrine in the locus coeruleus (Stanford 2001d). Changes in monoaminergic activity within the dorsalmedial hypothalamus may lead to some clinical signs. Norepinephrine in the hypothalamus plays a role in regulation of heart rate, blood pressure, and respiratory rate.

In conclusion, swim decreased norepinephrine in the hippocampus, suggesting that swim stress may contribute to reduction in long-term potentiation, learning and memory. Swim and restraint with swim stress decreased concentrations of norepinephrine and increased concentrations of dopamine and DOPAC in the hypothalamus, suggesting that these stressors may contribute to less arousal, depression, and less higher motor activity. Our data indicated chlorpyrifos treatment of restrained rats increased serotonin concentrations in the cerebral cortex in similar way to handled rats but differed from

swum rats and restrained rats. A reduction of serotonin in the hypothalamus of restrained rats and restrained with swim rats that were injected with chlorpyrifos sc suggests that these rats may have neurochemical features associated with depression. An interaction of stress and chlorpyrifos on extracellular concentrations of serotonin in specific regions of the hypothalamus and on cholinergic and serotonergic receptors in the cerebral cortex and hypothalamus remains to be examined.

Acknowledgments

The authors would like to acknowledge the assistance of Kristel Fuhrman, Geraldine Magnin, Kristin Knight, Jonathan Hinckley, Daniel Ward, Sandy Hancock, Delbert Jones, Barbara Wise, Ellen Uri and Nikola Agatic. Appreciations are to the Faculty of Liberal Arts and Science, Kasetsart University and the Thai government for T. Pung's scholarship. This work was supported by DAMD 17-99-1-9489 from the U.S. Army Medical Research and by Virginia-Maryland Regional College of Veterinary Medicine.

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Table 1. Effects of repeated stress on concentrations (nmole/g tissue) of monoamines and their metabolites in the hippocampus, cerebral cortex, and hypothalamus on day 28

Brain part	Type of stress	Norepinephrine	Dopamine	DOPAC	Serotonin	5-HIAA
Hippocampus	Handling	1.34 ± 0.09 [a]	0.45 ± 0.20	0.62 ± 0.24	0.64 ± 0.07	1.76 ± 0.12
	Restraint	1.16 ± 0.10 [a,b]	0.47 ± 0.21	0.64 ± 0.25	0.51 ± 0.08	1.62 ± 0.12
	Swim	0.86 ± 0.09 [b]	0.16 ± 0.20	0.35 ± 0.24	0.68 ± 0.07	1.66 ± 0.12
	Restraint with swim	1.04 ± 0.10 [a,b]	0.61 ± 0.21	0.48 ± 0.25	0.80 ± 0.08	1.82 ± 0.12
Cerebral cortex	Handling	1.12 ± 0.07	0.20 ± 0.06	0.22 ± 0.04	0.48 ± 0.03	0.95 ± 0.05
	Restraint	1.07 ± 0.07	0.30 ± 0.07	0.30 ± 0.05	0.48 ± 0.03	1.10 ± 0.05
	Swim	1.03 ± 0.07	0.18 ± 0.06	0.23 ± 0.04	0.54 ± 0.03	1.02 ± 0.05
	Restraint with swim	0.90 ± 0.07	0.28 ± 0.06	0.31 ± 0.04	0.45 ± 0.03	0.95 ± 0.05
Hypothalamus	Handling	10.15 ± 0.31 [a]	1.36 ± 0.15 [a]	0.53 ± 0.12 [a]	1.66 ± 0.11 [a, b]	3.00 ± 0.10

	Restraint	9.64 ± 0.32 [a]	1.20 ± 0.16 [a]	0.44 ± 0.12 [a]	1.29 ± 0.11 [b]	3.22 ± 0.10
	Swim	7.90 ± 0.32 [b]	1.94 ± 0.16 [b]	1.22 ± 0.12 [b]	1.78 ± 0.11 [a]	3.08 ± 0.10
	Restraint with swim	7.53 ± 0.32 [b]	1.74 ± 0.16 [a,b]	1.21 ± 0.12 [b]	1.79 ± 0.11 [a]	3.15 ± 0.10

Data are presented as mean ± SEM (n= 16). [a,b] indicate that means with no letters in common were significantly different at $\alpha = 0.05$ according to Tukey's comparison.

Table 2. Effects of chlorpyrifos 160 mg/kg sc on concentrations (nmole/g tissue) of monoamines and their metabolites on day 28

Brain part	Type of injection	Norepinephrine	Dopamine	DOPAC	Serotonin	5-HIAA
Hippocampus	Corn oil	1.24 ± 0.07	0.54 ± 0.15	0.56 ± 0.18	0.74 ± 0.05	1.78 ± 0.09
	Chlorpyrifos	0.96 ± 0.07**	0.30 ± 0.15	0.49 ± 0.18	0.58 ± 0.05*	1.65 ± 0.09
Cerebral cortex	Corn oil	0.99 ± 0.05	0.20 ± 0.04	0.25 ± 0.03	0.45 ± 0.02	0.97 ± 0.03
	Chlorpyrifos	1.07 ± 0.05	0.28 ± 0.04	0.28 ± 0.03	0.53 ± 0.02*	1.04 ± 0.03
Hypothalamus	Corn oil	8.64 ± 0.23	1.50 ± 0.11	0.83 ± 0.09	1.77 ± 0.08	3.09 ± 0.07
	Chlorpyrifos	8.96 ± 0.22	1.63 ± 0.11	0.87 ± 0.08	1.49 ± 0.08*	3.13 ± 0.07

Data are presented as mean ± SEM (n= 28). * indicates significant difference of chlorpyrifos injection from corn oil injection ($p < 0.05$). ** indicates significant difference of chlorpyrifos injection from corn oil injection ($p < 0.01$).

Table 3. Effects of repeated stress, CPF, and interaction of stress and CPF on monoamine concentrations

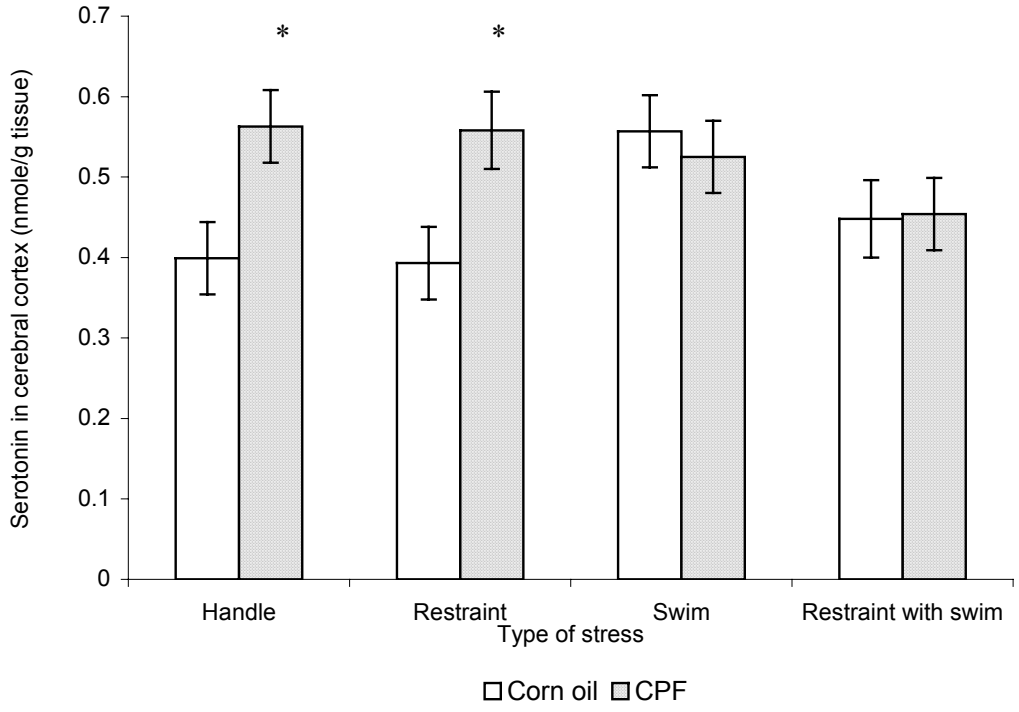
Treatment	Norepinephrine	Dopamine	DOPAC	Serotonin	5-HIAA
Stress					
Handling	No effect	No effect	No effect		No effect
Restraint	No effect	No effect	No effect		No effect
Swim	Decreased NE in the HP and HT	Increased DA in the HT	Increased DOPAC in the HT		No effect
Restraint with swim	Decreased NE in the HT	No effect	Increased DOPAC in the HT		No effect
CPF	Decreased NE in the HP	No effect	No effect		No effect
Stress*CPF					
Handling*CPF	No effect	No effect	No effect	CPF increased serotonin in the CC of handled rats.	No effect
Restraint*CPF	No effect	No effect	No effect	CPF increased serotonin in the CC. CPF decreased serotonin in the HT.	No effect
Swim*CPF	No effect	No effect	No effect	No effect	No effect
Restraint with swim *CPF	No effect	No effect	No effect	CPF decreased serotonin in the HT	No effect

HP = hippocampus, CC = cerebral cortex, HT = hypothalamus

Figure legends

Figure 1. Effects of repeated stress and chlorpyrifos on concentrations of serotonin in the cerebral cortex. Data are presented as mean ± SEM (n= 7). There was a trend toward an interaction of chronic stress and chlorpyrifos on the concentrations of serotonin in the cerebral cortex ($p = 0.06$). * indicates significant difference of sc injection of chlorpyrifos compared to corn oil in the control rats (rats that were handled) and rats that were restrained ($p < 0.05$).

Figure 2. Effects of repeated stress and chlorpyrifos on concentrations of serotonin in the hypothalamus. Data are presented as mean ± SEM (n=7). There was a trend toward an interaction of chronic stress and chlorpyrifos on the concentrations of serotonin in the hypothalamus ($p = 0.08$). * indicates significant difference of sc injection of chlorpyrifos compared to corn oil in rats that were restrained and restrained with swim ($p < 0.05$).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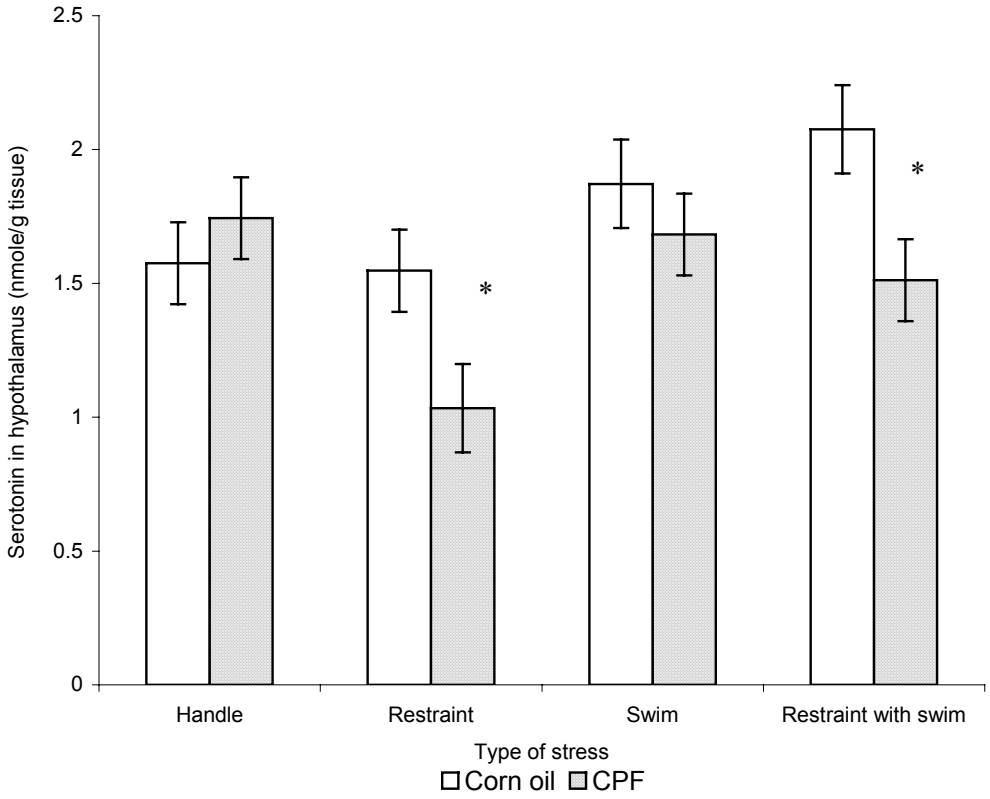
Serotonin in cerebral cortex (nmole/g tissue)
*
*
Handle
Restraint
Swim
Restraint with swim
Type of stress
Corn oil
CPF

Figure 1.


2.5
2
1.5
1
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Serotonin in hypothalamus (nmole/g tissue)
*
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Handle
Restraint
Swim
Restraint with swim
Type of stress
Corn oil
CPF

Figure 2.

PART V: DISCUSSION

CHAPTER 7

DISCUSSION AND CONCLUSIONS

DISCUSSION AND CONCLUSIONS

Stress has been reported to alter the glutamatergic and cholinergic systems in the hippocampus (McEwen 1999, Kaufer et al. 1999, Sunanda et al. 2000). Cholinesterase inhibitors (organophosphate insecticides) inhibit acetylcholinesterase activity resulting in increased acetylcholine in synapses with excess acetylcholine desensitizing cholinergic receptors. Cholinesterase inhibitors also alter the concentrations of catecholamines and glutamate (Lallement et al. 1991, El-Etri et al. 1992, Giacobini et al.1996, Ali et al. 1980). The interaction between concurrent exposure to repeated stress and a cholinesterase inhibitor on the cholinergic and glutamatergic systems has not been reported. These two systems are mainly distributed in the hippocampus, an important brain region for learning and memory (Bear et al. 2001) and cerebral cortex, a center of sensory and motor systems and cognition (Purves et al. 2001). The effects of stress-induced modulation of glutamatergic and cholinergic systems in the presence of cholinesterase inhibition are of concern and may contribute to illness seen in veterans of the 1991 Gulf War (Sapolsky 1998). Gulf War veterans were exposed to chronic stress and toxic agents such as nerve gases and organophosphate insecticides. Our hypothesis was concurrent exposure to repeated stress and chlorpyrifos (CPF) would have interactions on glutamatergic and cholinergic systems in the hippocampus, cerebral cortex, or hypothalamus.

Repeated stress affected the glutamatergic and cholinergic systems. CPF affected the cholinergic system. The interactions of repeated stress and CPF were few (Table 1).

Table 1. Effects of repeated stress, CPF, and interaction of stress and CPF on the glutamatergic and cholinergic systems

Treatment	Glutamatergic system			Cholinergic system			
	Glutamate	Aspartate	NMDA	AChE	Cbxy	ChAT	mAChR
Stress Handling Restraint Swim Restraint with swim	 No effect No effect No effect Had a trend to increase glutamate in the HP more than swim	 No effect No effect	 No effect Increased Kd of NMDA in the HP. No effect No effect	 No effect No effect No effect No effect	 No effect No effect No effect Inhibited Cbxy in the CC more than handling and restraint	 No effect No effect No effect No effect	 No effect No effect
CPF	No effect		No effect	Inhibited AChE in the HP and CC	Inhibited Cbxy in the HP and CC	No effect	
Stress *CPF Handling Restraint*CPF Swim*CPF	 No effect No effect No effect	 No effect No effect No effect	 No effect No effect No effect	 No effect No effect No effect	 No effect No effect No effect	 No effect No effect No effect	 No effect CPF had a trend to decrease the elevated Kd of mAChR of restrained rats. CPF had a trend to decrease the elevated

Restraint with swim*CPF	No effect	CPF attenuated the elevated aspartate in the HP of restraint with swim rats	No effect	No effect	No effect	No effect	Bmax and Kd of mAChR of swum rats. No effect

HP= hippocampus, CC= cerebral cortex, HT= hypothalamus

Some parts of our proposed model for the concurrent exposure to repeated stress and CPF on the glutamatergic and cholinergic systems have been elucidated based on our results (Figure 1).

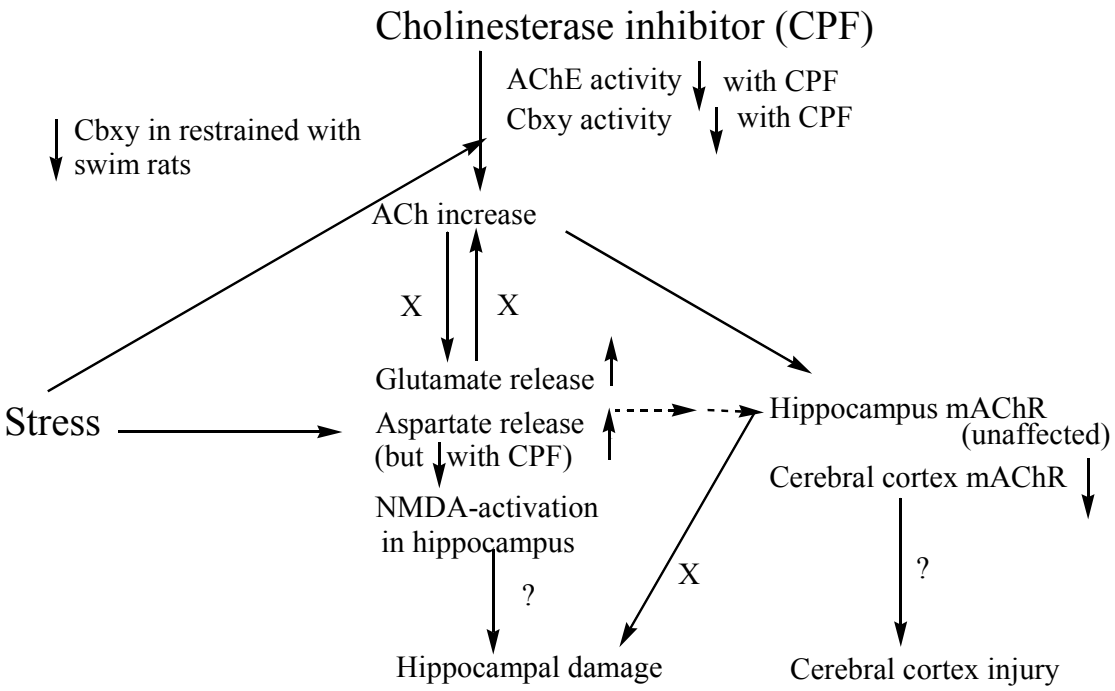

Figure 1. Model of the interaction of repeated stress and CPF on the glutamatergic and cholinergic systems.

As regards to stress effects on the glutamatergic system in our study, a statistical trend toward an increase in glutamate was seen in the hippocampus of restrained with swim rats compared to swum rats, suggesting that the response of these two stressors were different even though they elevated plasma corticosterone at similar levels. Disturbance of glutamate transmission is associated with long-term potentiation (Baudry and Lynch 2001), Alzheimer's disease (Francis et al. 1993), and epilepsy (Meldrum and Chapman 1999). Excess exogenous and endogenous glutamate could cause excitotoxic cell death (Francis 2003). Excitotoxicity is mediated by an excessive synaptic release of glutamate followed by overstimulation of glutamate receptors (Sattler and Tymianski 2001). Therefore, restraint with swim could contribute to neuronal cell death via excitotoxicity. This would need to be examined in a future study. Furthermore, Kd of NMDA receptors was elevated in the hippocampus of the restrained rats. Restraint, the psychological stress (Magarinos et al. 1997), elevated the binding affinity of NMDA receptors in the hippocampus. The higher affinity of NMDA receptors in the hippocampus may contribute to enhancement of memory and learning since NMDA receptors in the hippocampus function in those activities (Castellano et al. 1996). Therefore, it is possible that restrained rats may develop better memory and learning. While this would need to be verified in future experiments, we observed that these rats appeared to become familiar with the restraint tubes with time. These findings suggested that different types of repeated stress affected the glutamatergic system in the hippocampus in different ways.

Restraint with swim elevated aspartate when compared to control and CPF decreased these elevated concentrations of aspartate in the hippocampus of the restrained

with swim rats. This suggests there is an antagonism of restraint with swim and CPF on asparate concentrations in the hippocampus. Aspartate is an excitatory amino acid neurotransmitter in the central nervous system and it can activate NMDA receptors (Dingledine and McBain 1999). Restraint with swim stress resulted in a statistical trend toward an elevation of glutamate concentrations in the hippocampus. This stressor, however, significantly increased aspartate concentrations in the hippocampus. Our study demonstrated that restraint with swim stress (unpredictable stress) may damage the hippocampal structure via increased release of excitatory amino acids; however, histopathology of the hippocampus needs to be investigated.

Repeated stress did not affect acetylcholinesterase in the hippocampus and cerebral cortex, suggesting that repeated stress did not have direct effects on acetylcholinesterase activity. This supports other studies (Grauer et al. 2000, Song et al. 2002, Tian et al. 2002). As expected, CPF inhibited activity of acetylcholinesterse (AChE) in the hippocampus and cerebral cortex, but there was no interaction of repeated stress and CPF on acetylcholinesterase activity. Both restraint with swim stress and CPF decreased activity of carboxylesterase (Cbxy) in the cerebral cortex. Although no interaction of CPF and stress was found, CPF at 160 mg/kg sc alone caused very high inhibition of Cbxy (50%). Reduction of carboxylesterase could be expected to have an effect if exposure was to a cholinesterase inhibitor normally detoxified by Cbxy. CPF is detoxified by Cbxy (Chambers and Carr 1993, Karanth and Pope 2000). Excess acetylcholine in cholinergic synapses causes overstimulation of cholinergic receptors, resulting in cholinergic effects. Thus, restraint with swim may indirectly increase the availability of acetylcholine and probably facilitate cholinergic effects.

CPF at the dose used in our studies did not affect concentrations of glutamate in the hippocampus or cerebral cortex. As regards repeated stress and CPF on cholinergic enzyme activities and concentrations of excitatory amino acids, there was no direct interaction of acetylcholine and glutamate. Restraint with swim tended to increase glutamate in the hippocampus, but no interaction with CPF on glutamate was found. The antagonistic interaction observed was increased asparate concentrations in the hippocampus of restrained with swim rats.

CPF in our studies did not affect the Bmax and Kd of NMDA receptors in the hippocampus, cerebral cortex and hypothalamus. There were no interactions of repeated stress and CPF on NMDA receptors in these brain regions. Furthermore, rats did not have seizures during the experiments. Other cholinesterase inhibitors cause excess glutamate and stimulate NMDA receptors in the hippocampus during seizures (Lallement et al. 1991). These results demonstrated that the dose of CPF was insufficient to alter NMDA receptors in the hippocampus, cerebral cortex and hypothalamus.

Repeated stress, CPF or the interaction of stress and CPF did not affect total muscarinic receptors in the hippocampus. These results suggest that there is no role for effect on muscarinic receptors to contribute to possible hippocampal damage. However, this assumption needs to be verified by histopathological study.

CPF deceased the Bmax and Kd of total muscarinic receptors in the cerebral cortex but not the hippocampus of the restrained and swum rats. Overactivation of total muscarinic receptors may injure the cerebral cortex because the cholinergic system is an excitatory pathway in the central nervous system (Taylor and Brown 1999). The cerebral cortex functions in cognition and is the center of the sensory and motor systems,

suggesting that these functions could be altered in the CPF-treated restrained rats and the CPF-treated swum rats. However, further behavioral and pathological studies would be needed to verify these possibilities. Such future studies may have value because veterans in the 1991 Gulf War who were exposed to both chronic stress and cholinesterase inhibitors such as CPF reported effects on memory (National Academy of Sciences 2003). Also remaining to be investigated are subtypes of affected muscarinic receptors (such as M1 and M2 receptors) and histopathology of the hippocampus and cerebral cortex following exposure to repeated stress and CPF. Over down-regulation of total muscarinic receptors can desensitize muscarinic receptors and impair cholinergic transmission (Brown 1995).

Furthermore, swim stress and CPF each decreased norepinephrine in the hippocampus, but there was no interaction. Swim stress decreased norepinephrine but increased dopamine and DOPAC in the hypothalamus. These results suggested that swim was a competent stressor for modulation of monoamines in the hypothalamus. Forced swimming may activate the dopaminergic system in the central nervous system, which functions in regulation of motor activity. The increased dopamine that was released in the hypothalamus may modulate the hypothalamic-pituitary-adrenal axis. Dopamine is a precursor of norepinephrine and is metabolized to DOPAC. In the swum rats, higher concentrations of dopamine and DOPAC, and lower concentrations of norepinephrine may suggest that the activity of dopamine β-hydroxylase was low and resulted in decrease synthesis of norepinephrine. However, this assumption needs to be tested.

CPF had a statistical trend toward causing an increase in serotonin concentrations in the cerebral cortex of rats that were handled and restrained but not in swum and

restrained with swim rats. These results suggested that handled and restrained rats when exposed to CPF responded with serotonin effects in a similar way. From a toxicological aspect, there is probably no interaction of restraint and CPF on serotonin concentrations in the cerbral cortex. However, CPF may antagonize the effects on serotonin of swum and restrained with swim rats.

Administration of CPF resulted in a statistical trend toward reduction of the elevated concentrations of serotonin in the hypothalamus of rats that were subjected to restraint and restraint with swim stress. Serotonergic nuclei in the raphe nucleus innervate other brain regions including the hypothalamus and are thought to be involved in mood, anxiety, sleep, and appetite (Stanford 2001b). Modulation of serotonin can affect these emotions and activities. Therefore, swim and restraint with swim were potential stress models that altered noradrenergic, dopaminergic, and serotonergic responses in the hypothalamus. Restraint with swim decreased norepinephrine but increased DOPAC in the hypothalamus. These results suggest that the hypothalamus is a region of the brain that is involved in stress and has interactions with cholinesterase inhibitors such as CPF.

In conclusion, repeated stress affected both glutamatergic and cholinergic systems with response dependent on the types of stress. CPF inhibited degradatory enzyme activity of cholinergic system but did not affect glutamate. There was an interaction of stress and CPF on muscarinic receptor levels, as CPF decreased the elevated total muscarinic receptors in the cerebral cortex of the restrained rats and the swum rats. Restraint with swim tended to increase glutamate and aspartate and CPF attenuated the elevated aspartate concentrations of restrained with swim rats, suggesting that excitatory amino acids were affected. Repeated stress and CPF have effects on two interesting

systems (glutamatergic and cholinergic), but the interactions were few. Effects were present in all brain parts examined (cerebral cortex, hippocampus, and hypothalamus). The hypothalamus has not been part of previous studies on stress and organophosphates. Neurochemical effects could lead to behavioral and/or pathological changes that would require further studies.

PART VI: GENERAL REFERENCES

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PART VII: APPENDIX

CHAPTER 8

EFFECTS OF CORTICOSTERONE IN DRINKING WATER ON KINETICS OF A SINGLE DOSE OF CORTICOSTERONE

CORTICOSTERONE IN DRINKING WATER ALTERED KINETICS OF A SINGLE ORAL DOSE OF CORTICOSTERONE AND CONCENTRATIONS OF PLASMA SODIUM, ALBUMIN, GLOBULIN, AND TOTAL PROTEIN

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Running title: Corticosterone effects on corticosterone kinetics

Keywords: corticosterone, oral absorption, blood chemistry, brain catecholamines

Abstract

Effects of chronic exposure to corticosterone in drinking water on corticosterone kinetics, blood chemistry, and concentrations of catecholamines in parts of brain were studied in Long-Evans rats. Rats were randomly grouped into 3x2 treatments (n=4), which 3 treatments of drinking water (tap water, or 2.5% ethanol, or 400 μg/mL of corticosterone in 2.5% ethanol) for 28 days and 2 treatments of gavage with a single dose of either corn oil or corticosterone 20 mg/kg on day 28. Blood samples were collected at 0, 15, 30, 60, 120, 240, 480, and 720 minutes after dosing to determine plasma corticosterone concentrations. Blood samples were collected for clinical pathology on day 42. Hippocampus, cerebral cortex, caudate-putamen, and pons were examined to determine concentrations of catecholamines and activities of esterases. Concentrations of plasma corticosterone before gavage of the corticosterone-drinking rats (47.61 ± 1.13 ng/ml) were lower than the water (418.47 ± 1.13 ng/ml) or the ethanol rats (383.71 ± 1.13 ng/ml, $p < 0.0001$). Plasma corticosterone rose to peak concentrations by 15 minutes after gavage in all three groups of drinking rats. Corticosterone-drinking rats had concentrations of plasma corticosterone that returned to basal levels slower than water- and ethanol-drinking rats. Plasma sodium and chloride concentrations were lower in the corticosterone-drinking rats than the water-drinking rats ($p < 0.01$). Plasma albumin, globulin, and total protein were highest in the corticosterone-drinking rats when compared to the other groups of drinking rats ($p < 0.001$, $p < 0.05$, and $p < 0.001$, respectively). Corticosterone in drinking water did not affect activities of brain neurotoxic esterase, carboxylesterase, acetylcholinesterase, or concentrations of

monoamines and their metabolites. A single oral dose of corticosterone reduced neurotoxic esterase activity in the cerebral cortex ($p < 0.05$) and increased norepinephrine concentrations in the hippocampus ($p < 0.05$).

Introduction

Stress affects body functions, especially in the nervous, cardiovascular, and the immune systems (McEwen 2000). The physiological responses to acute stress are expressed immediately to prepare the organism for flight or fight, which in turn, facilitate adaptation by removing the threat (De Kloet et al. 1998). However, long-term exposure to stressful situations can cause physiological impairment. Chronic stress can cause hypertension, atherosclerosis, inflammation, and neuronal atrophy (McEwen 2001). The stress responses depend on past experiences and concentrations of stress hormones. Many veterans from the 1991 Gulf War have chronic stress-related symptoms such as fatigue, joint pain, memory problems, emotional change, and insomnia (Landrigan 1997, National Academy of Sciences, 2003). Anxiety disorders in veterans and others who have been exposed to long-term stress are the result of inappropriate stress responses to real or imagined novel stressors. Our experiments were designed to assess the effects of chronic stress upon the physiological responses to a novel stressor.

The major effects of stress are mediated through the functions of the hypothalamic-pituitary-adrenal axis (HPA) (De Kloet et al. 1998, Akil et al. 1999). Acute stress immediately stimulates the HPA, which affects secretion of its hormones. More corticotropin releasing factor (CRF) and adrenocorticotropin hormone (ACTH) are

released from the hypothalamus and anterior pituitary respectively and blood concentrations of cortisol in humans and corticosterone in rodents are increased. The major effect of corticosteroids is to increase blood glucose via stimulation of gluconeogenesis, mobilization of protein, and mobilization of fatty acids (Berne and Levy 1990, Guyton and Hall 1996). Concentrations of corticosteroids and ACTH also play an important role in regulating negative feedback to hypothalamus and pituitary, and to other regions of the brain such as the hippocampus and cerebral cortex (Akil et al. 1999), terminating the stress response and restoring homeostasis. The excess or deficiency of corticosteroid hormones, that may be produced by dysfunction of this negative feedback process, result in illnesses such as Cushing's disease and secreting adrenocortisol failure (Sapolsky 1986, Berne and Levy 1990, Guyton and Hall 1996). Chronic stress may interfere with the negative feedback system, resulting in dysfunction of normal corticosteroid levels and normal stress response. Therefore, studying the regulation of corticosteroids by chronic stress may provide further insight into controlling these disorders, as well as stress related problems of veterans.

As corticosterone is the downstream steroid stress hormone in rodents, exposure to exogenous corticosterone has been used as a stress model in rodents (O'Callaghan 1991). Therefore, this study uses corticosterone in drinking water as the chronic stress model. This model has been employed by other investigators and has been shown to induce stress-associated responses such as inhibition of ACTH release, regression of adrenal cortex with decrease in production of corticoids, including those regulating sodium retention, and atrophy of apical dendrites in CA3 of the hippocampus (Dunn and Canillo 1978, Berne and Levy 1990, Magarinos et al. 1998, Akil et al. 1999). We were

interested in the effects of chronic corticosterone administration in drinking water on the concentration of plasma corticosterone, a principal marker of HPA activity, and whether this model of chronic stress would alter the short term plasma corticosterone response to an acute stress (an intragastric dose of corticosterone). The effects of the chronic stress model on other indicators of adrenal function, such as plasma protein and plasma analytes (sodium, potassium, chloride, and carbonate) were also examined.

The HPA axis is activated under stressful conditions through neuronal input. Catecholamine input from the brainstem increases in response to stress and is thought to stimulate activity of the HPA axis (Akil et al. 1999). Acute and chronic exposures to restraint or immobilization stressors altered concentrations of monoamines and their metabolites in pons-medulla, hypothalamus, hippocampus and frontal cortex (Campmany et al. 1996, Helleriegel and Mello 1997, Sunanda et al. 2000). Although many reports indicate that brain monoamines and their metabolites are involved in stress response, none have examined these after exposure to corticosterone-containing drinking water as a model of chronic stress. Therefore, we examined how this chronic stress model affected those monoamines capable of activating components of the HPA axis.

Chronic restraint stress has also been shown to decrease acetylcholinesterase (AChE) activity in the hippocampus (Sunanda et al. 2000). Furthermore, two major cholinergic brainstem regions, the laterodorsal tegmental nucleus and pedunculopontine nucleus, have been demonstrated to carry visual and auditory inputs to the hypothalamus (Akil et al. 1999). Given this association of cholinergic activity with chronic stress and the potential activation of HPA-components by cholinergic inputs was of interest. Therefore, we also measured activities of acetylcholinesterase and carboxylesterase as

indicators of cholinergic activity. Activity of neurotoxic esterase was also determined because inhibition of this enzyme can predict organophosphate-induced delayed neuropathy. Concurrent exposure to chronic stress and some organophosphorus esters occurred, as some veterans of the 1999 Gulf War were exposed to both (National Academy of Sciences 2003).

As noted above, the first major objective of this study was to assess the effect of the drinking water model of chronic stress, in our laboratory, on plasma corticosterone and other plasma analyte markers of HPA activity. The second major objective was to determine the effect of the chronic drinking water stressor on the kinetics of the plasma corticosterone response to an acute novel stressor, intragastric corticosterone. The third major objective was to determine the effect of drinking water chronic stress model upon brain monoamines and esterases that have been shown to be altered by other chronic stress models. The monoamines and esterase variables are also associated with regulation of targets within the HPA axis.

Materials and Methods

A) Animals

Twenty-four adult male Long-Evans rats, 90-120 days old and weighing 424-529 g were obtained from Harlan Sprague Dawley (Indianapolis, IN) and shipped to Laboratory Animal Resources at Virginia Tech. Rats were allowed to acclimate to their surroundings for one week. They were housed individually, with food and water provided *ad libitum*. Room temperature was kept at 21-23 °C with a light cycle of 7 pm to 7 am.

All procedures involving animals were in accordance with the Virginia Polytechnic Institute and State University guidelines and approved by the Virginia Tech Animal Care Committee.

B) Experimental design

The experiment was a generalized, randomized, complete block design. Treatment structure was 3x2, which were 3 types of drinking water (water, 2.5% ethanol, 400 μg/ml corticosterone in 2.5% ethanol) and 2 types of gavage (corn oil, 20 mg/kg corticosterone). Four rats were randomly allocated into the 3x2 treatment design. Treatment and sacrifice were performed in two experimental blocks separated by 1 day. Sampling of body weight and plasma corticosterone were repeated measurement with body weight at 0, 2, 4, 7, 9, 14, 21, 25, 28, and 42 days and interim blood sampling to measure concentrations of plasma corticosterone. Plasma concentrations of sodium, potassium, chloride, bicarbonate, and anion GAP (K+), brain activities of acetycholinesterase, carboxylesterase, neurotoxic esterase, and brain concentrations of monoamines and their metabolizes were assessed with a single measurement at sacrifice. Significant differences were defined as $p < 0.05$.

Rats were allowed to drink water, 2.5% ethanol, or 400 μg/ml of corticosterone in 2.5% ethanol for 28 days. Each group was gavaged with a single dose of either corn oil or 20 mg/kg corticosterone on day 28, from 8:00 am to 10:30 am, with 3 minutes between rats. Blood samples were then collected from the orbital sinus, using isoflourene anesthesia, 30-45 minutes before gavage (time zero), and at 15, 30, 60, 120, 240, 480, and 720 minutes after the gavage to determine the concentrations of plasma

corticosterone. Rats were allowed to recover for fourteen days after gavage while continuing to receive the same type of drinking water. On day 42, the rats were weighed and sacrificed using carbon dioxide gas, and blood samples were taken by cardiac stick for clinical pathology analysis (sodium, potassium, chloride, bicarbonate, anion GAP (K+), total protein, albumin, globulin, and glucose). Brains were collected and dissected into hippocampus, cerebral cortex, caudate-putamen, and pons and each part of the brain was weighed. Brain parts were assayed for activities of acetycholinesterase, carboxylesterase, and neurotoxic esterase, as well as the concentrations of the monoamines norepinephrine, epinephrine, dopamine, and serotonin. The concentrations of the dopamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) and of the serotonin metabolite 5-hydroxyindole-3-acetic acid (5-HIAA) were also determined.

C) Test chemicals

Corticosterone used in drinking water (Steraloids, Inc., Newport, RI) was weighed and dissolved in absolute ethanol and the solution was diluted with tap water to give the final concentration of 400 μg/ml of corticosterone in 2.5% ethanol. The corticosterone in drinking water was prepared 2-3 times per week and changed at 9-11:30 am. Corticosterone used for a single intra-gastric dose (Sigma Chemical, St. Louis, MO) was suspended in corn oil at a concentration of 8 mg/ml because it did not completely dissolve. At a dose of 20 mg/kg the average intra-gastric dose volume was 1.3 ml for each rat and the suspension of corticosterone was shaken again before dosing.

D) Biochemical analysis

Corticosterone concentrations were determined using blood samples collected from the orbital sinus to heparinized microcentrifuge tubes and spun on a Beckman microfuge Lite® centrifuge at 3500 x g for 3 minutes. Plasma samples were kept in the freezer at -70 °C until assayed. Plasma corticosterone concentrations were measured using a ^{125}I radioimmunoassay kit for rats and mice (ICN Biomedicals, Inc., Costa Mesa, CA). In brief, plasma was diluted with a steroid diluent to 1:200. The diluted plasma (100 µl) was mixed with 200 µl of a solution of antiserum to corticosterone and incubated at room temperature for 2 hours. Then, 500 µl of a precipitating solution was added to stop the reaction, and the suspension was vigorously mixed. The precipitate was centrifuged at 1000 x g for 15 minutes. A COBRA II Auto-gamma was used for gamma counting of the precipitate.

The plasma analytes sodium, potassium, chloride, bicarbonate, anion GAP (K^+), total protein, albumin, globulin, and glucose were determined by using a combination of the specific electrode and spectrophotometric wet routine methodologies. Plasma analytes were assayed by the Clinical Pathology Laboratory, Virginia-Maryland Regional College of Veterinary Medicine Teaching Hospital, using an Olympus AU400 automated serum density analyzer.

Neurotoxic esterase and carboxylesterase activities in the brain tissues were determined using a spectrophotometric microassay (Correll and Ehrich 1991). Phenyl valerate was a substrate for both carboxylesterase and neurotoxic esterase. Phenyl valerate was hydrolyzed to phenol and valeric acid. Then, phenol reacted with potassium ferricyanide to give an orange color, with absorbance read at 510 nm. Neurotoxic esterase was determined by the difference in absorbance between incubates with paraoxon and

incubates with paraoxon + mipafox. Acetylcholinesterase activity was measured by spectrophotometry using acetylthiocholine iodide as a substrate (Ellman et al.1961, Correll and Ehrich 1991). Hydrolysis of the thiocholine substrate in the presence of enzyme and 5,5′-dithio-bis-2-nitrobenzoic acid gave a yellow color, which was read at an absorbance of 412 nm.

The concentrations of monoamines and their metabolites in the hippocampus, cerebral cortex, caudate-putamen, and pons were determined by using high performance liquid chromatography combined with electrochemical detection (HPLC-ECD) (Jussofie et al. 1993). Brain samples (0.02-0.05 g) were homogenized in 250 µl of mobile phase pH 4.7 and an internal standard (isoproterenol hydrochloride, Sigma, St. Louis, MO, with a final concentration of 1 µM) was added. Brain tissues were homogenized by using a Cell Disruptor Sonicator (Heat System-Ultrasonic, Farmingdale, NY) with speed at 6 cycles for 30 seconds. The homogenates were centrifuged by a Beckman Microfuge Lite® at 10,100 x g for 5 minutes and the supernatants were transferred and kept in HPLC-light protective vials at –20 °C until assayed. The supernatants were thawed on ice on the assay days and then filtered through an Acrodisc LC 13 mm syringe filter with 0.2 µm PVDF membrane into HPLC vials. The chromatographic instrument was a Hewlett Packard Series 1100 Quaternary Pump with a degasser and autosampler (Agilent Technologies, Wilmington, DE). The separation was performed on a reversed-phased C18 analytical column (Nucleosil 100 3µ, 250x4 mm, Macherey-Nagel, Easton, PA), preceded by a guard column (Nucleosil 100 C18 3µ 8x4 mm, Macherey-Nagel, Easton, PA). Electrochemical detection (Hewlett Packard, model 1049A) was used in the oxidation mode with +0.35 volt. Isocratic elution was performed at flow rate of 0.6

ml/min. The mobile phase was citric buffer pH 4.7 composed of 0.1 M sodium acetate, 25 mM citric acid, 134 μM ethylenediaminetetraacetic acid (EDTA), 230 μM octanesulfonic acid, and 6% of methanol (HPLC grade). The external standard solutions of norepinephrine bitartrate, epinephrine hydrochloride, dopamine hydrochloride, 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptamine oxalate (serotonin), 5-hydroxyindole-3-acetic acid (5-HIAA), and isoproterenol hydrochloride were purchased from Sigma used for quantity analysis.

E) Statistical analysis

The experimental design was a generalized, randomized, complete block design. Data were analyzed by two-way analysis of variance (ANOVA) using the SAS system (version 8.2, SAS Institute Inc., Cary, NC). To test the effect of corticosterone in drinking water on changes of body weight before gavage, changes of body weight from day 0 to day 28 were analyzed by mixed model ANOVA and post hoc comparisons were tests of the simple main effect of drinking water on separate days. To hold the experimentwise error rate to 0.05, the comparisonwise alpha level was Bonferroni-corrected. The interaction effects of corticosterone in drinking water and gavage on body weight on day 42 were tested by mixed effects ANOVA and comparison of means with Tukey's HSD test. Plasma corticosterone concentrations were transformed to the natural log of concentrations for statistical testing to stabilize variability. However, data are presented as back-transformed concentrations of corticosterone for more convenient interpretation. The effect of corticosterone in drinking water on plasma corticosterone concentration before gavage on day 28 was analyzed by mixed effects ANOVA and post hoc

comparisons were performed by Tukey's HSD. Concentrations of plasma corticosterone were analyzed by mixed effects ANOVA and post hoc comparisons were performed using tests of simple main effect of drinking, gavage and time of sampling. The effects of interaction of drinking, gavage and time after gavage were tested by comparisons of means of each drinking and gavage at each time after gavage. The effects of drinking of corticosterone on the response of plasma corticosterone to the oral dose were analyzed by comparisons of means within the group at times after gavage to time before gavage (t=0 minute). To hold the experimentwise error rate to 0.05, the comparisonwise alpha level was Bonferroni-corrected. The other results (plasma concentrations of sodium, potassium, chloride, bicarbonate, and anion GAP (K^+), brain activities of acetycholinesterase, carboxylesterase, neurotoxic esterase, and brain concentrations of monoamines and their metabolizes) were analyzed by mixed effects ANOVA with a single measurement and the post hoc comparisons were performed by Tukey's HSD test. Values of $p \leq 0.05$ were considered statistically significant.

Results

Prior to gavage, corticosterone in drinking water decreased body weight when compared to rats drinking tap water (df = 1, 147, $p < 0.05$) or rats drinking 2.5% ethanol (df = 1, 147, $p < 0.05$) from day 4 through day 28 but not on day 2 (Figure 1). Furthermore, before gavage on the morning of day 28, rats that drank corticosterone in drinking water (47.61 ± 1.24 ng/ml) had concentration of plasma corticosterone that was

lower than rats that drank tap water (418.47 ± 1.24 ng/ml, df = 1, 20, $p < 0.0001$) or ethanol (383.71 ± 1.24 ng/ml, df = 1, 20, $p < 0.0001$).

On day 28, gavage of corn oil did not change concentrations of plasma corticosterone from time 0 to the end of the 720 min observation period, within any of the drinking water conditions (water, ethanol, corticosterone; Figure 2a). However, after gavage of corn oil, plasma corticosterone of rats that drank corticosterone remained lower than rats that drank water or ethanol (df = 1, 126, $p < 0.05$, Figure 2a). The main effect of drinking, gavage and time of administration of corticosterone was significant ($p < 0.0001$). Plasma corticosterone rose to peak concentrations by 15 minutes in all three groups after gavage, with corticosterone increasing from baseline by 330%, 337%, and 2299%, respectively, in the water-, ethanol-, and corticosterone-drinking groups (Figure 2b). Mean values of plasma corticosterone for three types of drinking, from 15 to 120 minutes, were not significantly different (df = 1, 126, $p > 0.05$ according to Bonferroni's correction, Figure 2b). However, by 240 minutes, concentrations of plasma corticosterone of rats that drank corticosterone were lower than rats that drank water or ethanol (df = 1, 126, $p < 0.05$). Post hoc comparison of time after gavage to time before gavage demonstrated that concentrations of plasma corticosterone of rats that drank water or ethanol returned to their basal concentrations after 120 minutes (df = 1, 126, $p < 0.05$). Plasma corticosterone of rats that drank corticosterone returned to their basal concentrations by 720 minutes (df = 1, 126, $p < 0.05$).

On day 42, the weight of the corticosterone-drinking rats remained significantly lower than that of the water- (df = 1, 17, $p < 0.0001$) or ethanol-drinking (df = 1, 17, $p < 0.0001$) rats. This effect was not dependent upon whether the rats received corn oil or

corticosterone gavage. Effects of corticosterone in drinking water on plasma analytes measured on day 42 are shown in Table 1 and Figure 3. A significant main effect of drinking water was observed for the plasma analytes sodium, chloride, potassium, albumin, globulin, and total protein. For each of these analytes, there was no significant difference between the water- and ethanol-drinking groups. However, for the corticosterone-drinking group, chloride concentration was significantly lower than for both the water- (df = 1, 17, $p < 0.0001$) and ethanol- (df = 1, 17, $p < 0.0001$) drinking groups, while sodium concentration was significantly lower compared with the water-drinking group (df = 1, 17, $p = 0.001$). Also, for the corticosterone-drinking rats, potassium concentration was significantly higher than for the ethanol-drinking rats (df = 1, 17, $p = 0.007$), while albumin, globulin, and total protein concentrations were higher than both the water- (albumin, df = 1, 17, $p < 0.0001$; globulin, df = 1, 17, $p = 0.002$; total protein, df = 1, 17, $p < 0.0001$) and ethanol- (albumin, df = 1, 17, $p < 0.0001$; globulin, df = 1, 17, $p = 0.011$; total protein, df = 1, 17, $p < 0.0001$) drinking rats. However, plasma bicarbonate and glucose concentrations of all three drinking groups were not significantly different, while anion GAP (K+) concentration was lower in the ethanol-drinking rats (df = 1, 17, $p = 0.017$) compared with the water-drinking rats.

The neurotoxic esterase, carboxylesterase, and acetylcholinesterase activities in the hippocampus, cerebral cortex, caudate-putamen and pons on day 42 were not significantly different among the three groups of rats that received different drinking water treatment (Table 2). However, the neurotoxic esterase activity of the cerebral cortex was lower in the corticosterone gavaged group (6.80 ± 0.36 nmole phenyl valerate hydrolyzed/min/mg protein) than in the corn oil gavaged group (7.52 ± 0.36 nmole

phenyl valerate hydrolyzed /min/mg protein, df = 1, 17, p = 0.028), when data were collapsed over drinking water condition, as shown in Table 3.

Brain concentrations of norepinephrine, dopamine, serotonin, DOPAC and 5-HIAA were not significantly different among the rats in the three drinking water treatments on day 42 (data are not shown). However, the norepinephrine concentrations in the hippocampus were higher in the corticosterone-gavaged rats (23.7 ± 5.4 nmole/g tissue) than in the hippocampus of the corn oil gavaged rats (13.5 ± 5.5 nmole/g tissue) (df = 1, 16, p = 0.037, Figure 4).

Discussion

Corticosterone in drinking water at 400 μg/ml, for 28 days, caused body weights drop and then remain significantly lower than body weight for rats drinking plain water or 2.5% ethanol. This effect persisted while the corticosterone solution was administered through day 42. The present results are supported by Sousa et al. (1998), who reported that a daily injection of corticosterone 40 mg/kg sc for one month significantly reduced body weight. Since corticosterone is an adrenocortical hormone, an intake of corticosterone may affect the negative feedback of the hypothalamic-pituitary adrenal axis, resulting in the disruption of the hormone control. Therefore, administration of corticosterone may alter physiological response and the maintenance of static conditions in the internal environment following by reduction of body weight.

Consistent administration of corticosterone in drinking water for 28 days also significantly reduced basal plasma corticosterone concentrations compared with the two

vehicle groups, suggesting decreased basal HPA response. This result is consistent with studies in humans given synthetic corticosteroids (Berne and Levy 1990) and studies in rats, which demonstrated that subcutaneous implantation of a 50% corticosterone pellet decreased the basal plasma corticosterone and ACTH concentrations and caused atrophy of the adrenal gland (Lin and Singer 1990, Young 1995). In contrast, Magarinos et al. (1998) reported that administration of corticosterone in drinking water (400 μg/ml) for 21 days increased the plasma corticosterone in the morning but not in the evening when compared to ethanol treated rats on days 7, 14 and 21 of administration but still decreased the weight of the adrenal glands on day 21. Restraint stress (Magarinos et al. 1997) and corticosterone in drinking water (Magarinos et al. 1998) caused atrophy of apical dendrites in CA3 in the hippocampus. The morning increase in plasma corticosterone observed by Magarinos is puzzling given the decreased adrenal weight they reported and since our blood samples were also drawn in the morning. However, our rats were in their dark phase in the morning while Magarinos' rats were in the light phase, which could account for some of the discrepancy. Furthermore, 25% corticosterone pellets increased the morning corticosterone concentrations but did not alter the evening ACTH or corticosterone secretion whereas 50% corticosterone/cholesterol pellets inhibited the secretion of corticosterone and ACTH (Young et al. 1995). Our results and those of Young et al. (1995) demonstrated that corticosterone pretreatment affected the response to a second stressor, such as corticosterone gavage or restraint stress.

Chronic administration of corticosterone in drinking water altered kinetics when the rats were exposed to a high intragastric dose of this steroid. Such altered kinetics has not previously been reported but other studies have noted that long-term administration of

glucocorticoids by different regimens resulted in altered acute negative feedback responses within the HPA axis. Young (1995) reported that injection of cortisol at 500 μg/kg sc to rats after they were restrained for 2 minutes decreases plasma adrenocoticotropin hormone (ACTH) that released when the rats were restrained and injected with saline, at 15 and 30 minutes after injection. However, this acute restraint and cortisol injection did not alter the response of plasma corticosterone when compared to restraint and saline injection. Prior implantation of a 50% of corticosterone/cholesterol pellet at a dose of 50 mg/rat for 6 days elevates basal plasma corticosterone when compared to before implantation (Young 1995). Chronic stress as corticosterone implantation decreased the ACTH and corticosterone responses to acute restraint and cortisol at 15 and 30 minutes when compared to before implantation (Young 1995), suggesting that chronic administration of corticosterone by implantation modifies the response of HPA axis.

Other investigators have reported that acute stress by restraint cage and water immersion at the same time increased plasma corticosterone concentrations and the concentrations remained high for 5 hours (Mizoguchi et al. 2001). Chronic stress by using restraint and water immersion for four weeks increased the concentrations of plasma corticosterone after the last stress but rapidly decreased at 2 hours (Mizoguchi et al 2001). Our results showed that chronic administration of corticosterone reduced basal plasma corticosterone concentrations, increased corticosterone oral absorption and decreased corticosterone clearance. The effect of corticosterone in drinking water and gavage appear different from restraint or water immersion stress. The results indicated administration of corticosterone impaired the regulation of endogenous corticosterone,

suggesting that this deleterious effect would increase and/or prolong the response to the latter stress.

Corticosterone in drinking water was associated with decreased plasma sodium and chloride concentrations. In addition, plasma potassium concentrations were higher in the corticosterone-drinking rats. The results suggested that corticosterone in drinking water affected the function of the adrenal gland other than its corticosterone secretion. These electrolyte findings might best be explained by decreased mineralocorticoid secretion from the adrenal cortex in response to exogeneous corticosterone suppression of pituitary ACTH secretion leading to adrenal atrophy (Feldman and Nelson 1996). However, our experiments did not test this directly. Plasma albumin, globulin and total protein concentrations were higher in the corticosterone-drinking rats than those drinking water or ethanol, suggesting that animals experienced hypotonic dehydration as seen in a situation of mineralcorticoid deficiency where renal sodium concentration is impaired (Loeb and Quimby 1999). However, plasma glucose concentrations of all three drinking groups were not significantly different. Otherwise, factors other than corticosterone such as depletion of food intake or glycogen depletion from liver may have influenced plasma glucose.

Forty-two days of drinking water containing corticosterone did not alter activities of neurotoxic esterase, carboxylesterase and actylcholinesterase in the hippocampus, cerebral cortex, caudate-putamen, and pons. Stress has been reported to cause deleterious changes on acetylcholine and cholinesterase in the brain by some investigators (Kaufer et al. 1998), but not by others (Kant et al. 2001, Song et al. 2002). In our study, a single dose of corticosterone decreased neurotoxic esterase activity in the cerebral cortex. No

previous studies have examined the effects of corticosterone or stress on this endpoint. This result suggested the possibility that internal increase in corticosterone could facilitate interactions with neurotoxicants that are organophosphorus esters and also capable of inhibiting neurotoxic esterase. Such facilitated inhibition could have effects on the probability of developing organophosphate induced-delayed neuropathy (OPIDN).

Corticosterone in drinking water 400 μg/ml for 28 days did not alter the concentrations of norepinephrine, dopamine, serotonin, 3,4-dihydroxyphenylacetic (DOPAC) and 5-hydroxyindole-3-acetic acid (5-HIAA) in the hippocampus, cerebral cortex, caudate-putamen, and pons. Reports on the effects of stress on neurotransmitter concentrations in the brain are inconsistent and appear to depend on types and durations of stressors. For example, repeated restraint stress and repeated immobilization for 60 minutes/day for 27 days did not change the concentrations of norepinephrine, serotonin, 4-hydroxy-3-methoxyphenylglycol (MHPG) and 5-HIAA in the hippocampus, pons, and frontral cortex, however, norepinephrine concentration was increased in the hypothalamus by both chronic restraint and immobilization (Campmany et al. 1996). Previous chronic restraint increases MHPG concentrations in the hypothalamus that respond to the acute restraint stress and previous chronic immobilization also increases the MHPG concentrations in the hypothalamus and pons-medulla after acute immobilization (Campmany et al. 1996). Repeated intermittent foot shock 30 minutes per day for 14 days did not change basal norepinephrine concentrations in the prefrontal cortex or increase the response of norepinephrine following acute tail shock (Jedema et al. 1999). In contrast, restraint stress 6 hours per day for 21 days decreased the concentrations of norepinephrine, dopamine, and serotonin in the hippocampus (Sunanda

et al. 2000). From our data, it appears that 400 μg/ml corticosterone in drinking water for 28 days may not be a stressor that is strong enough to affect catcholamine concentrations in these four brain regions. However, norepinephrine concentrations in the hippocampus were elevated even 14 days after a single intragastric dose of 20 mg/kg of corticosterone. Glutamate receptors mediate excitatory synaptic transmission in the hippocampus and involve in long-term potentiation (Bear et al. 2001) and the adrenergic system appear to modify other synaptic inputs (Goldsein and Pacak 2001) and also innervates in the hippocampus (Stanford 2001). Therefore, elevated norepinephrine may modulate the glutamatergic system, which could be followed by in the alteration of long-term potentiation. Norepinephrine has been proposed to play a role in learning and memory and participate in regulating long-term memory as well (review by Ferry et al. 1999).

In summary, chronic exposure to a corticosterone stressor in the drinking water lowered basal concentrations of plasma corticosterone and altered the kinetics of the plasma corticosterone response to an acute, intragastric corticosterone stressor. This suggests that prior chronic stress may have important implications for the way organisms respond to discreet stressful events in their daily lives. Our data on plasma corticosterone concentrations combined with our findings on changes in plasma analytes further suggest that the chronic stressor may alter the integrity of an important component of the HPA axis: the adrenal gland. The effect of corticosteroid gavage upon cortical neurotoxic esterase and upon hippocampal norepinephrine suggests that increases in systemic corticosteroids may have important interactions with OPIDN-inducing compounds and may also be involved in long-term potentiation.

Acknowledgements

The authors would like to acknowledge the assistance of Kristel Fuhrman, David Moore, Sandy Hancock, Lisa Flory, Linda Correll, Delbert Jones, Geraldine Magnin Biesel, Barbara Wise, and Daniel Ward. Appreciations are to Faculty of Liberal Arts and Science, Kasetsart University and the Thai government for T. Pung's scholarship. This work was supported by DAMD 17-99-1-9489 from the U.S. Army Medical Research and Materiel Command and by Virginia-Maryland Regional College of Veterinary Medicine. This paper does not necessarily reflect the position or policy of the U.S. Government.

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Table 1. Plasma electrolyte concentrations in rats on different types of drinking water for 42 days.

Electrolytes	Water-drinking	Ethanol-drinking	Corticosterone-drinking
Sodium (mEq/L)	141.9 ± 0.8	139.6 ± 0.8	137.8 ± 0.8[a]
Potassium (mEq/L)	7.8 ± 0.4	7.2 ± 0.2	8.4 ± 0.2[b]
Chloride (mEq/L)	104.1 ± 0.5	103.5 ± 0.4	99.4 ± 0.5[a,b]
Bicarbonate (mEq/L)	22.5 ± 1.3	24.6 ± 0.4	24.8 ± 0.4
Anion GAP (K+)	23.1 ± 1.8	18.7 ± 0.9[c]	22.0 ± 0.5

Data are mean ± SEM, n=8.

[a] corticosterone group statistically different from water group.

[b] corticosterone group statistically different from ethanol group.

[c] ethanol group statistically different from water group.

Table 2. Brain neurotoxic esterase (NTE), carboxylesterase (Cbxy), and acetylcholinesterase (AChE) activites after the rats had been drinking corticosterone for 42 days.

Brain tissue	Enzyme activity	Water-drinking	Ethanol-drinking	Corticosterone-drinking
Hippocampus	NTE	7.43 ± 0.48	7.55 ± 0.42	7.54 ± 0.45
	Cbxy	47.35 ± 2.71	52.06 ± 3.35	52.82 ± 2.35
	AChE	44.82 ± 2.77	46.59 ± 2.53	39.93 ± 2.96
Cerebral cortex	NTE	6.90 ± 0.38	7.01 ± 0.25	7.57 ± 0.28
	Cbxy	49.33 ± 1.51	45.86 ± 0.78	47.86 ± 1.01
	AChE	37.82 ± 4.51	39.35 ± 3.49	35.15 ± 1.99
Caudate-putamen	NTE	7.66 ± 0.95	8.50 ± 1.42	7.56 ± 0.96
	Cbxy	46.25 ± 2.79	44.81 ± 2.67	42.76 ± 2.03
	AChE	225.50 ± 28.82	209.64 ± 20.11	204.10 ± 23.39
Pons	NTE	11.49 ± 0.79	10.90 ± 1.10	12.81 ± 1.26
	Cbxy	37.44 ± 1.32	32.08 ± 2.37	36.52 ± 2.72
	AChE	83.48 ± 2.66	94.70 ± 2.03	86.27 ± 5.33

There were no significant differences in NTE, Cbxy, and AChE activities in the hippocampus, cerebral cortex, caudate-putamen, and pons of rats on any of the drinking water treatments. The units of NTE and Cbxy are nmole phenyl valerate hydrolyzed

/min/mg protein. The units of AChE are nmole acetylthiocholine produced/min/mg protein. Data are represented as mean ± SEM, n=8.

Table 3. Brain neurotoxic esterase (NTE), carboxylesterase (Cbxy), and acetylcholinesterase (AChE) activities after the rats were gavaged with corticosterone.

Brain tissue	Enzyme activity	Corn oil gavage	Corticosterone gavage
Hippocampus	NTE	7.64 ± 0.32	7.39 ± 0.38
	Cbxy	49.75 ± 2.49	51.94 ± 2.24
	AChE	44.28 ± 2.17	43.23 ± 2.51
Cerebral cortex	NTE	7.52 ± 0.21	6.80 ± 0.26[a]
	Cbxy	47.61 ± 1.08	47.76 ± 0.93
	AChE	36.09 ± 3.33	38.79 ± 2.15
Caudate-putamen	NTE	8.65 ± 0.98	7.17 ± 0.78
	Cbxy	45.66 ± 1.84	43.55 ± 2.20
	AChE	207.24 ± 18.24	218.91 ± 20.79
Pons	NTE	11.57 ± 1.08	11.90 ± 0.63
	Cbxy	34.77 ± 2.20	35.93 ± 1.53
	AChE	88.14 ± 2.68	88.15 ± 3.71

An oral single dose of 20 mg/kg corticosterone decreased neurotoxic esterase in the cerebral cortex when determined 42 days after dosing. The units of NTE and Cbxy are nmole phenyl valerate hydrolyzed /min/mg protein. The units of AChE are nmole/min/mg protein. [a] indicates significant difference of corticosterone-gavaged rats from corn oil-gavaged rats ($p < 0.05$). Data are represented as mean ± SEM, n=12.

Figure legends

Figure 1. Change of body weight after the rats were given water, 2.5% ethanol, or corticosterone in drinking water for 42 days. They were gavaged with corn oil or corticosterone on day 28; however, the gavage did not affect body weight change on day 42. * indicates significant difference of body weight change of corticosterone-drinking rats from water- and ethanol-drinking rats ($p < 0.05$, according to Bonferroni's corrected multiplicity comparison). # indicates significant difference of body weight of ethanol-drinking rats from water-drinking rats ($p < 0.05$, according to Bonferroni's correction). Data represent mean ± SEM, n=8.

Figure 2. Plasma corticosterone concentrations after rats were given water, 2.5% ethanol, or 400 μg/ml of corticosterone and gavaged with corn oil (Figure 2a) or corticosterone 20 mg/kg (Figure 2b) on day 28. [a, b] indicate significant differences from each other at $\alpha < 0.05$ according to Bonferroni's corrected multiple comparison. [c] (lines underscoring the figure) indicates concentrations of plasma corticosterone at times within a drinking treatment that were significantly greater than those at time zero ($p < 0.05$ according to Bonferroni's correction). Plasma corticosterone rose to peak concentrations by 15 minutes in all three groups after gavage with corticosterone. Rats that drank corticosterone in drinking water had concentrations of plasma corticosterone returne to basal levels slower than the water- and ethanol- drinking rats. Data represent means of plasma corticosterone concentrations and n=4.

Figure 3. Plasma concentrations of albumin, globulin, total protein and glucose after rats were given either water, or 2.5% ethanol or 400 μg/ml of corticosterone in drinking water

for 42 days. Corticosterone at 400 μg/ml increased concentrations of plasma albumin, globulin, and total protein; however, it did not alter plasma glucose concentrations.

* Significant difference of corticosterone-drinking rats from water-and ethanol-drinking rats with $p < 0.05$. Data are expressed as mean ± SEM, n=8.

Figure 4. Norepinephrine concentrations in the hippocampus (HP), cerebral cortex (CC), caudate-putamen (CP), and pons on day 42 after rats were gavaged with corticosterone 20 mg/kg on day 28. Norepinephrine concentrations were higher in the hippocampus of corticosterone-gavaged rats than in the hippocampus of the corn oil-gavaged rats ($p < 0.05$). There were no significant differences of norepinephrine, DOPAC, dopamine, 5-HIAA, and serotonin concentrations. Data are expressed as mean ± SEM, n=12.

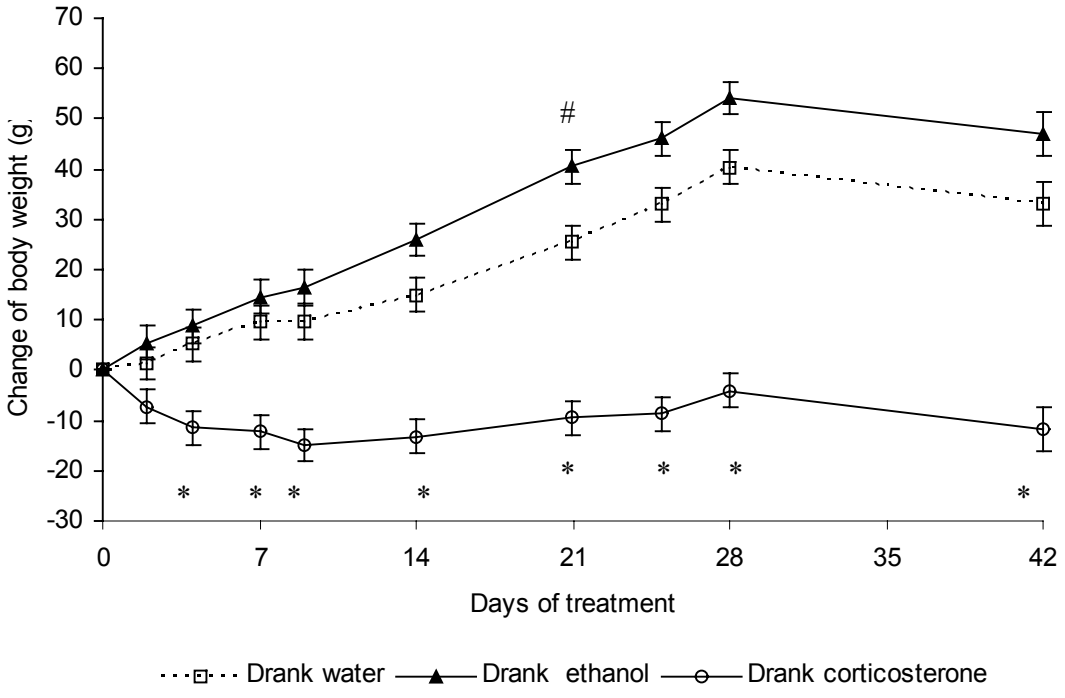

70
60
50
40
30
20
10
0
-10
-20
-30
Change of body weight (g)
#
*
*
*
*
*
*
*
0
7
14
21
28
35
42
Days of treatment
Drank water
Drank ethanol
Drank corticosterone

Figure 1.

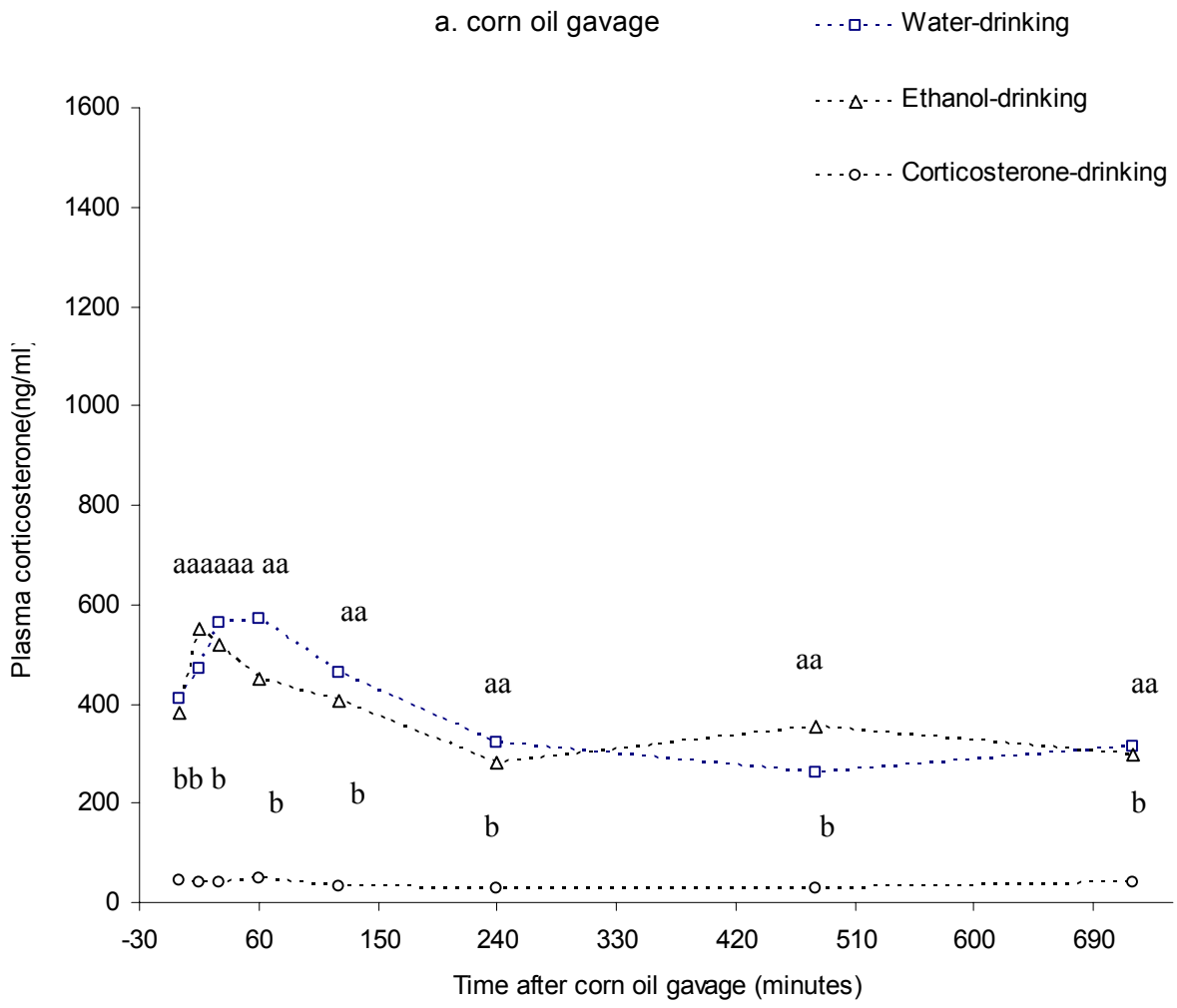

a. corn oil gavage
Water-drinking
Ethanol-drinking
Corticosterone-drinking
Plasma corticosterone(ng/ml)
1600
1400
1200
1000
800
600
400
200
0
-30
60
150
240
330
420
510
600
690
Time after corn oil gavage (minutes)
aaaaaa aa
aa
aa
aa
aa
bb b
b
b
b
b
b

Figure 2a.

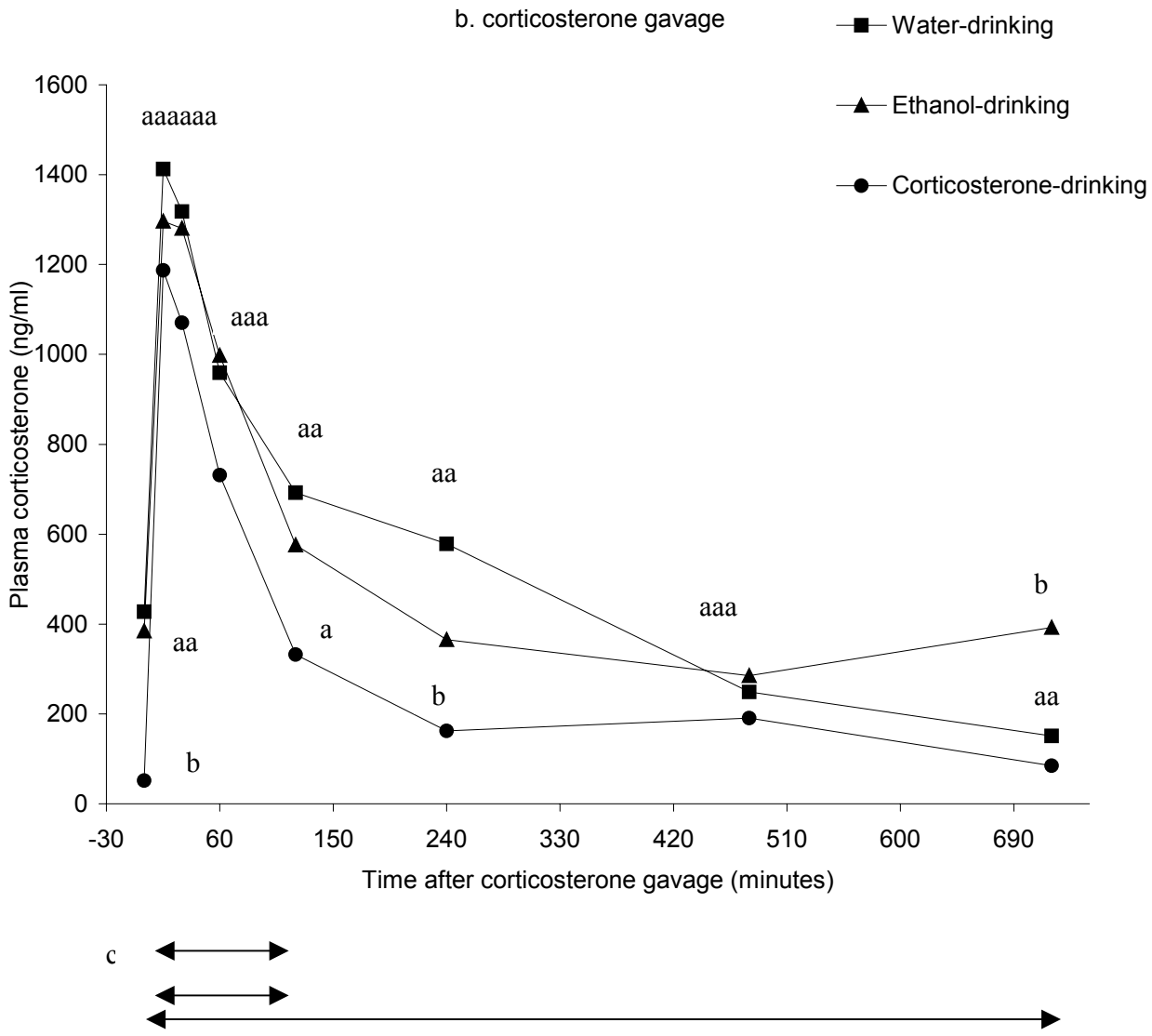
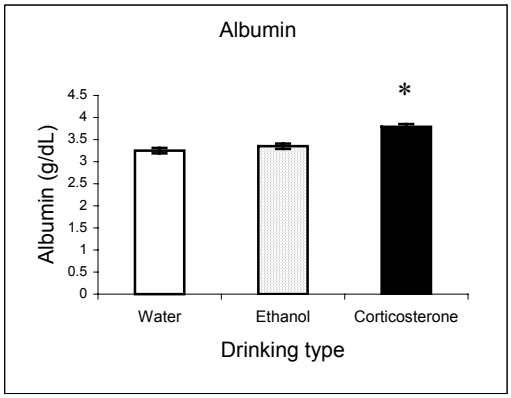
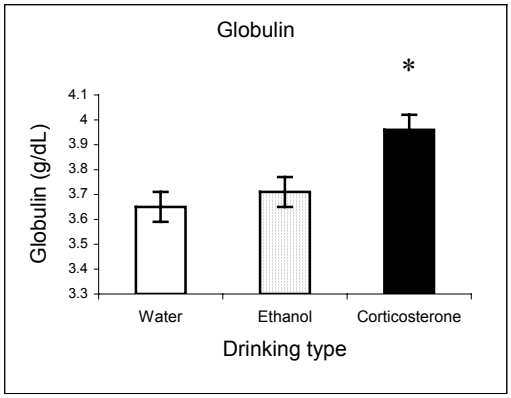

b. corticosterone gavage
Water-drinking
Ethanol-drinking
Corticosterone-drinking
Plasma corticosterone (ng/ml)
1600
1400
1200
1000
800
600
400
200
0
aaaaaa
aaa
aa
aa
aa
aaa
b
a
b
b
aa
aa
-30
60
150
240
330
420
510
600
690
Time after corticosterone gavage (minutes)
c

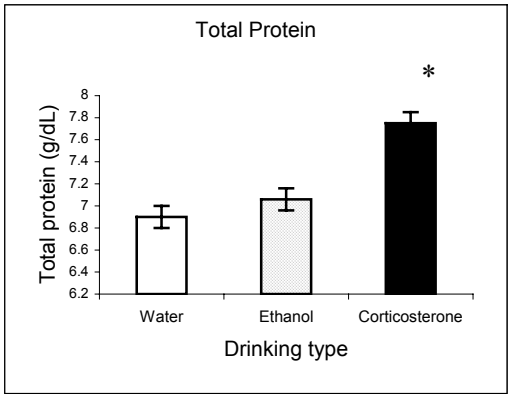
Figure 2b.



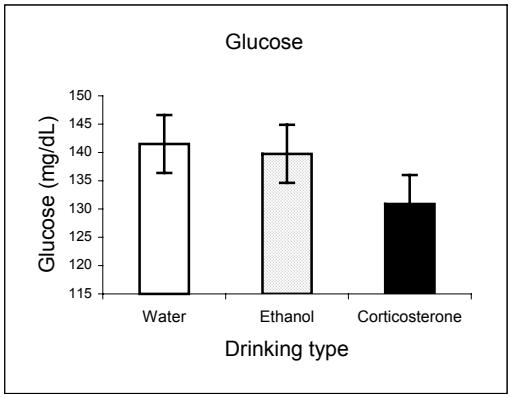
A: Plasma albumin



B: Plasma globulin



C: Plasma total protein



D: Plasma glucose

Figure 3.

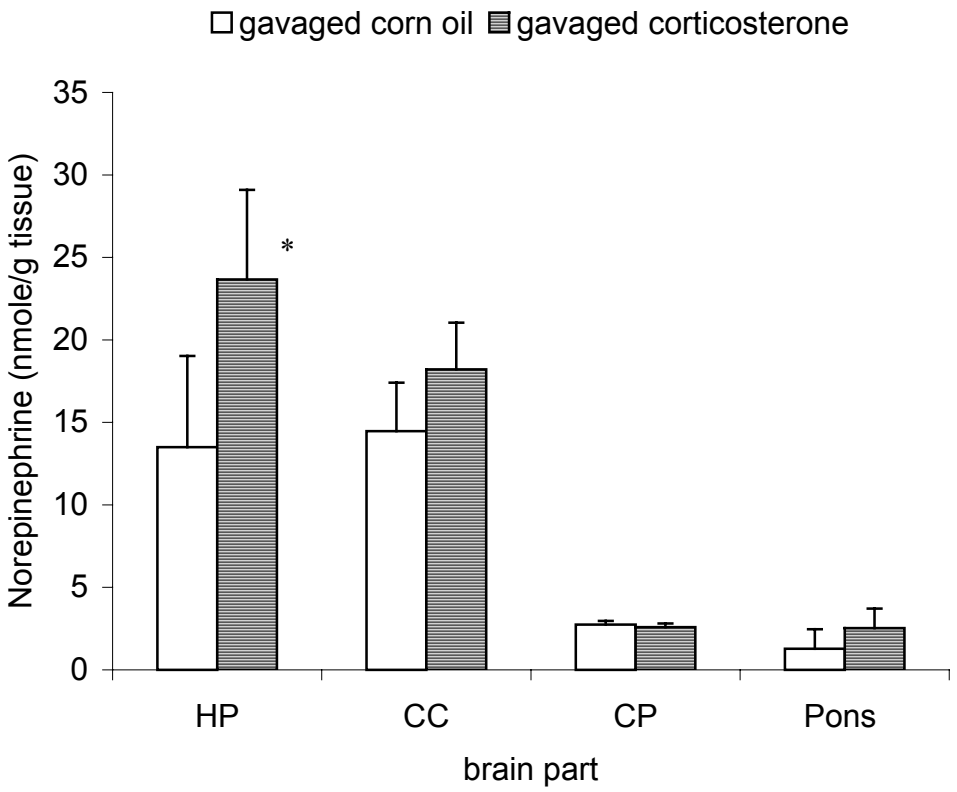

gavaged corn oil
gavaged corticosterone
Norepinephrine (nmole/g tissue)
35
30
25
20
15
10
5
0
*
HP
CC
CP
Pons
brain part

Figure 4.

PART VIII: VITA

VITA

Thitiya Pung was born in Samutprakan, Thailand. After finishing high school in Bangkok, she attended Chulalongkorn University in Bangkok where she received a Bachelor of Sciences in General Science in 1983. Thitiya then graduated with a Master of Sciences in Toxicology from Mahidol University in 1992. She has worked as a chemistry lecturer at the Faculty of Liberal Arts and Sciences, Kasetsart University, since 1994. As a faculty member, she was awarded a scholarship from the Royal Thai Government to study chemistry aboard. She graduated with a Master of Sciences in Chemistry from Virginia Tech in 2000. She continued her academic pursuit as a doctoral student under Dr. Marion Ehrich in the College of Veterinary Medicine, Department of Biomedical Sciences and Pathology. She will return to Thailand and Kasetsart University after completing her Ph.D.