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(CRF) in Rat Amygdala, Hypothalamus and Brain Stem

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ABSTRACT

Effects of Nicotine on Content of Corticotropin Releasing Factor
(CRF) in Rat Amygdala, Hypothalamus and Brain Stem

Sibonisiwe Ntini Masilela

Effects of 1.0 mg/kg and 0.4 mg/kg intraperitoneal nicotine on hypothalamic and extra hypothalamic levels of corticotropin releasing factor (CRF) were examined in 104 rats. CRF, a 41-amino acid peptide involved in responses to stress, fear and anxiety, influences the same neurotransmitter systems as nicotine, a psychoactive component of tobacco. Radioimmunoassays revealed that in macro-dissected hypothalami, 15 minutes treatment with 1.0 mg/kg nicotine significantly increased CRF content compared to no treatment. The 1.0 mg/kg dose did not elicit effects in macro-dissected amygdala and brain stem. With 0.4 mg/kg nicotine, no significant effects were observed in micro-dissected hypothalamic paraventricular nucleus, amygdaloid and brain stem nuclei. As expected, plasma corticosterone levels were significantly increased with both doses of nicotine. Overall, no clear cut effects of nicotine on CRF were observed. Micro-dissection studies on specific nuclei using varying doses and time points would help elucidate interactions of nicotine with CRF systems.

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CHAPTER I

THE PROBLEM

<u>Corticotropin releasing factor, amygdala and nicotine</u>

Corticotropin releasing factor (CRF) is a 41-amino acid peptide secreted mainly

by the paraventricular nucleus (PVN) of the hypothalamus (Owens and Nemeroff, 1991;

Vale, et al., 1981). CRF is the chemical messenger by which the central nervous system

(CNS) controls the activity of the hypothalamic-pituitary-adrenal (HPA) axis and

therefore, is responsible for mediating the neuroendocrine response to stress. CRF is also

released from other brain regions, particularly the amygdala, an almond shaped structure

located in the temporal lobe of the brain (Gray and Bingaman, 1996; Haines, 1991;

Owens and Nemeroff, 1991). The median eminence which is served by the

paraventricular nucleus (PVN) of the hypothalamus has the highest levels of CRF. The

PVN and central nucleus of the amygdala (CeA) also have considerably high levels

(Chappell, et al., 1986; Palkovits, et al., 1985; Skofitsch and Jacobowitz, 1985). This

suggests that the neuropeptide is also an important mediator for the emotional responses

to stress and anxiety, which are mediated through the amygdala (Gray and Bingaman,

1996; Owens and Nemeroff, 1991).

The amygdala is rich in CRF expressing cell bodies and terminals, receptors and

CRF binding protein immunoreactivity (Gray, 1993; Gray and Bingaman, 1996; Owens

and Nemeroff, 1991). Dense CRF cell bodies and terminals are located in the amygdaloid

central nucleus (CeA) while the basolateral nuclei have a high number of receptors (Gray

and Bingaman, 1996; Owens and Nemeroff, 1991; Sanakana, et al., 1986). The amygdala

has afferent and efferent neurons to and from structures including the hypothalamus, brain stem nuclei, bed nucleus of stria terminalis and substantia nigra (Gray and Bingaman, 1996; Swanson, et al., 1983; Uryu, et al., 1992). These reciprocal projections enable the amygdala to influence or be influenced by these brain regions through the action of their neurotransmitters including norepinephrine, epinephrine, dopamine and serotonin (Gray and Magnuson, 1987; Sanakana, et al., 1986; Van Bockstaele, et al., 1996). The amygdala also contains steroid receptors showing that it could be influenced by glucocorticoids or other steroids (Swanson, et al., 1983). Therefore, there are afferent and efferent amygdaloid CRFergic neurons that are strategically localized to mediate both autonomic and emotional responses to stress (Gray, 1992; Gray and Bingaman, 1996).

Animal studies have shown CRF to be a neuromodulator in functions associated with the amygdala. Behavioral models of anxiety including social interaction, elevated plus maze and fear potentiated startle, indicate that CRF is anxiogenic at increased levels (Dunn and Berridge, 1990; Koob, et al., 1992; Liang, et al., 1992a; Liang, et al., 1992b). Reactions seen in electrical stimulation or lesions of the amygdala parallel those seen in the injection or inhibition of CRF release, respectively. Imbalances in levels of CRF in the brain have been associated with psychiatric and affective disorders (DeSouza, 1995; Nemeroff, et al., 1991; Owens and Nemeroff, 1991; Owens, et al., 1991). Anti-anxiety drugs such as benzodiazepines have been reported to induce modifications in CRF in the hypothalamus, bed nucleus of stria terminalis (BNST), and septum. In addition, alprazolam and adinozolam decreased CRF immunoreactivity in the amygdala, piriform cortex and cingulate cortex (Owens and Nemeroff, 1991; Owens, et al., 1989). The

question is whether or not other CNS drugs including nicotine exert their effects through modulation of CRF levels in the brain.

Nicotine is a poisonous, volatile alkaloid derived from tobacco and is responsible for many of the tobacco effects (McDonough, 1994). Nicotine can be either a psychostimulant or a depressant at high and low dose respectively (Balfour and Fagerström, 1996; McDonough, 1994). Neuronal nicotinic receptors (nAChRs) are the primary receptors for the excitatory neurotransmitter acetylcholine and are located in sympathetic and parasympathetic ganglia, and the adrenal cortex (Mycek, et al., 1992). The receptors exist in various brain regions particularly the hippocampus, cortex and also in the amygdala and brain stem (Clarke and Pert, 1985; Fu, et al., 1998; Maren, 1996). The nAChRs consist of a family of heterogeneous receptor subtypes composed of α- and β- subunits encoded by 12 different genes (Wada, et al., 1989; Luetje and Patrick, 1991; Lukas, 1995). The $\alpha_4\beta_2$ receptor which is sensitive to nicotine and analogues is located in both the somatodendritic membrane and nerve terminal (Bylund and Yamamura, 1990; Flores, et al., 1997; Watson, et al., 1987). The receptor is a ligand gated ion channel permeable to Na^+, K^+, and Ca^{2+}, and mediates neurotransmission (McGehee and Role, 1995; McGehee and Role, 1996; Schwartz and Kandel, 1991).

Nicotine and its analogues seem to influence brain neural systems that are implicated in psychiatric and neural disorders; addictive mechanisms of nicotine have yet to be determined (Balfour, 1982; Balfour and Fagerström, 1996). In addition to the well-demonstrated stimulation of acetylcholine release in the brain and peripheral nerves (Armitage, et al., 1969; Balfour and Fagerström, 1996), animal studies have shown

nicotine to act on various brain regions to modulate the secretion of neurotransmitters,

including dopamine, epinephrine, norepinephrine and serotonin (Benwell, et al., 1988;

Benwell, et al., 1990; Fu, et al., 1997; Fu, et al., 1998; Mifsud, et al., 1989; Nisell, et al.,

1994; Sugita, et al., 1992). The rewarding properties of nicotine are believed to be

elicited through the interaction of neuronal nicotinic receptors with these neurotransmitter

systems (Benwell and Balfour, 1979; Dawe, et al., 1995; Rapier, et al., 1988; Rapier, et

al., 1990;). Nicotine is believed to exert differing responses in normal individuals

compared to depressed, and in stressed and unstressed animals (Balfour, et al., 1975;

Donnerer and Lembeck, 1990; Hall and Morrison, 1973). One of the most prominent

effects of nicotine is the elevation of circulating ACTH and corticosterone, the stress

response hormones. (Andersson, et al., 1983; Benwell and Balfour, 1982; Cam and

Bassett, 1983; Freund, et al., 1988).

<div align="center">Statement of problem</div>

Studies have been done on the effects of nicotine on neurotransmitters in the

hypothalamus and some extrahypothalamic regions including the locus coeruleus,

hippocampus, cortex and striatum, effects of nicotine on the corticotropin releasing factor

(CRF) have scarcely been documented. Whether or not nicotine exerts its effects in the

CNS by modulating CRF content is not known. A recent immunocytochemical study by

Matta, et al., 1997, reported a dose dependent increased *c-fos* expression in CRF-positive

neurons in limbic structures after low dose nicotine treatment. The proto-oncogene *c-fos*

is an early detector for neuronal activity. This suggests that nicotine stimulated neuronal

activity in CRF neurons of extrahypothalamic regions, including central nucleus of the amygdala, basal nucleus of stria terminalis, dorsal raphe, Barrington's nucleus and also in paraventricular nucleus of the hypothalamus. The present study was undertaken to examine the effects of nicotine on the content of CRF rich regions regions.

The objective of the study was achieved by examining effects of nicotine on CRF content in rat amygdala, hypothalamus and brain stem, using two doses of nicotine at different treatment time points.

Significance of the problem

The ultimate goal is to find effects of chronic nicotine on CRF content in hypothalamus and extrahypothalamic regions; this study will demonstrate whether or not acute nicotine has effects on CRF which would open a way for investigating the chronic effects. This study is a step towards the studies that will compare responses to nicotine in stressed and unstressed animals. An elucidation of effects of nicotine on CRF systems would also call for a study on the effects of nicotine withdrawal. This work is a lead to studies that will examine effects of prenatal exposure to nicotine, on CRF levels.

Hypotheses

1   There are significant increases in amygdaloid CRF levels for nicotine treated rats compared to untreated and saline treated rats, at a given dose and time point.

2   There are significant increases in hypothalamic CRF levels for nicotine treated rats compared to untreated and saline treated rats, at a given dose and time point.

3   There are significant decreases in brain stem CRF levels for nicotine treated rats compared to untreated and saline treated rats, at a given dose and time point.

4   There are significant differences in CRF levels per brain region at same dose of nicotine and different time points.

<u>Assumptions</u>

- The hypothesis of this study was based o the assumption that acute nicotine would evoke changes in CRF content due to one or more of the following: (I) increased release and utilization leading to decreased content, (II) increased synthesis, processing or reuptake leading to increased levels, (III) decreased utilization and degradation, leading to increased levels and (IV) inhibition of release leading to increase levels. The present study did not directly address these mechanisms but only measured CRF content.

- For untreated rats, CRF levels in a specific brain region would be similar at all time points, but would vary depending on method of dissection.

- For saline treated rats, CRF levels per brain region, would be similar at all time points, but would vary depending on method of dissection.

- Corticosterone levels for untreated rats would be similar at all time points.

Limitations of the study

(1)   Two different doses were used with two different methods of dissection. The cryostat-micro-punch method targeted selected brain nuclei, while the macro-dissection obtained the whole structure of the specific region. Results from these dissections can not necessarily be compared.

(2)   The specificity of nicotine was not confirmed by pretreatment with nicotinic receptor antagonist such as mecamylamine or dihydro-β-erythroidine.

(3)   Neither control nor test rats were handled prior to the test; handling all the rats would minimize non-specific stress.

CHAPTER II

REVIEW OF THE LITERATURE

<u>The amygdala</u>

The amygdala is located in the temporal lobe of the brain and is part of the limbic system,

enveloped by the cortex, hippocampus, striatum, hypothalamus and lateral ventricles

(Amaral, et al., 1992; Kupfermann, 1991). The amygdala is composed of heterogeneous

nuclei which are functionally distinct, making two subsystems which mediate responses

of anxiety and fear conditioning, that is, emotional memory storage and regulation of

memory storage in the hippocampus (Cahill and McGaugh, 1996; Cahill, et al., 1996;

McGaugh, et al., 1996). The nuclei consist of the lateral, basolateral and basomedial

nuclei (collectively, the basolateral nuclei) and the central nucleus (CeA) (Amaral, et al.,

1992; Kupfermann, 1991; Maren, 1996). The basolateral nuclei have a higher number of

CRF receptors compared to CeA and function as the primary sensory system of the

amygdala, with the CeA being the major output of the amygdala (with more CRF cell

bodies and terminals) mediating the anxiety and fear responses (De Souza, et al., 1985;

De Souza, 1995; Sanakana, et al., 1986; Swanson, et al., 1983). Therefore, lesions of

either or both nuclei result in deficits in acquisition and expression of conditioned fear.

Within the amygdala, therefore, the flow of information is from the basolateral nucleus to

the CeA (Maren, 1996). Output from the CeA of amygdala is through two fiber bundles,

the stria terminalis (which projects to the basal nucleus of the stria terminalis) and the

ventral amygdalofugal pathway (Amaral, et al., 1992; Haines, 1991; Kupfermann, 1991).

The amygdala has afferent and efferent neurons to and from the hypothalamus,

dorsal vagal complex, nucleus tractus solitarius (NTS), parabrachial nucleus,

mesencephalic nucleus, locus coeruleus and bed nucleus of stria terminalis (BNST) (Gray

and Bingaman, 1996; Haines, 1991; Swanson, et al., 1983; Uryu, et al., 1992). These

reciprocal projections enable the amygdala to influence or be influenced by these brain

regions through their neurotransmitters, including norepinephrine, epinephrine, dopamine

and serotonin (Gray and Bingaman, 1996; Gray and Magnuson, 1987; Sanakana, et al.,

1986; Sugita, et al., 1992). Even though the presence of efferents from the amygdala

have been established in the midbrain central gray, substantia nigra and ventromedial

medulla, afferents from these regions to the amygdala still need to be identified (Gray and

Bingaman, 1996).

Functions of the amygdala

       The amygdala, CRF, steroids and monoaminergic systems together play a role the

expression of behavioral, autonomic and neuroendocrine changes that occur in response

to stressful stimuli (De Souza, et al., 1985; Gray and Bingaman, 1996; Owens and

Nemeroff, 1991). The amygdala mediates the perception of emotions and is associated

with behaviors connected with emotional and arousal components related to stress, fear

and anxiety; that is, defensive/aggressive responses, and also reproductive and feeding

behaviors (Goldstein, et al., 1996; Gray and Bingaman, 1996; Owens and Nemeroff,

1991; Maren, 1996). The amygdala, therefore is believed to be influential in a variety of

psychopathological disorders such as depression, Tourette's syndrome, schizophrenia and

panic and anxiety disorders (De Souza, 1995; Gray and Bingaman, 1996; Jadresic, 1992;

Owens and Nemeroff, 1991). The amygdala is also linked to the development of

cardiovascular disease and gastric pathology (Gray and Bingaman, 1996; Junien and Gue,

1993; Tache, et al., 1993).

Direct outputs from the CeA to the hypothalamus and brain stem are believed to

mediate (through CRF) the symptoms of fear and anxiety which include, the arrest

reaction (cessation of behavior in anticipation of fight or flight), increased

arousal/vigilance and parasympathetic and sympathetic activation (increased heart rate,

sweating, decreased gastric motility (Davis, 1992; Gray and Bingaman, 1996; Owens and

Nemeroff, 1991). Similarly, electrical stimulation of the amygdala in animals results in

reactions of fear, aggression, and activation of the autonomic nervous system (Davis,

1992; Gray and Bingaman, 1996; Owens and Nemeroff, 1991). Bilateral lesions of the

amygdala produce disturbances of emotional behavior and block acquisition of fear

memory (as measured by fear potentiated acoustic startle responses); animals become

placid and display no reaction of fear, rage or aggression (Goldstein, et al., 1996;

Kupfermann, 1991; Roozendaal, et al., 1993). Lesions of the amygdala block CRF-

potentiated acoustic startle (Liang, et al., 1992a). Lesions of the CeA or bilateral

injection of benzodiazepines, gamma-aminobutyric acid (GABA; the major inhibitory

neurotransmitter in the brain) receptor agonists, to amygdala diminish the conditioned

fear and defensive freezing (Helmstetter and Bellgowan, 1993; Maren and Fanselow,

1996; Maren, et al., 1996; Owens and Nemeroff, 1991).

A number of studies have reported cellular mechanisms of fear conditioning.

Amygdaloid synaptic processes that mediate acquisition and expression of learned fear are

believed to be mediated by glutamatergic, GABAergic, noradrenergic and glucocorticoid

processes (Galvez, et al., 1996; Maren, 1996; Roozendaal, et al., 1996). It is believed that

long term potentiation (LTP) plays a pivotal role in cellular mechanisms of fear

conditioning (Cahill, et al., 1996; Maren, 1996). LTP is a "long term increase in synaptic

transmission induced by high frequency stimulation of excitatory afferents" (Kandel, 1991;

Maren, 1996; Morris, et al., 1986). Roozendaal, et al., (1996), studied adrenalectomized

rats with bilateral lesions into the basolateral, medial and central nucleus of the amygdala.

The researchers concluded that learning and memory storage was modulated through

glucocorticoid receptors in the basolateral nucleus. After studying changes in muscarinic

acetylcholine receptor immunoreactivity neurons in the amygdala, Roozendaal, et al.,

(1997), concluded that fear conditioning was mediated through the dynamic plasticity of

muscarinic acetylcholine receptors in the amygdala. Furthermore, Van der Zee, et al.,

(1997), in an immunocytochemical study, concluded that GABAergic and cholinoceptive

neural interactions in the CeA played an important role in fear induced responses.

Intra-amygdaloid infusions of norepinephrine or β-agonists, after training animals

for conditioned fear, indicated the amygdala plays a critical role in mediating the memory-

modulating effects of endogenously released stress hormones and the effects of drugs that

affect neuromodulator systems (Cahill and McGaugh, 1996; McGaugh, et al., 1996).

Stressful stimuli cause an activation of noradrenergic neurons and increased NE release

into the amygdala (Galvez, et al., 1996; Ida, et al., 1984; Tanaka, et al., 1991). Sugita, et

al., (1992), also reported that serotonin (a neurotransmitter that modulates mood states and

is implicated in affective disorders), functions as a neurotransmitter in the amygdala.

Neuronal transmission in the amygdala

Electrophysiological studies have demonstrated that afferent stimulation of the basolateral nuclei results in both excitatory and inhibitory synaptic transmission (Gean and Chang, 1991; Rainnie, et al., 1991a; Rainnie, et al., 1991b; Washburn and Moises, 1992). These findings have been confirmed by in vivo iontophoretic pharmacological studies on the basolateral nuclei complex which showed that excitatory responses were mediated by glutamate receptors, with GABA receptors mediating inhibitory responses (Li, et al., 1995; Maren, 1996; Maren and Fanselow, 1995; Mello, et al., 1992).

The amygdala has various glutamate receptor subtypes: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate and N-methyl-D-aspartate (NMDA), and also has glutamatergic cell bodies and neurons. Glutamate (major excitatory amino acid in the brain) is concentrated in the CeA (Farb and LeDoux, 1997; Farb, et al., 1992; McDonald, 1996). Glutamatergic terminals originate in the cerebral cortex and subcortical structures that transmit sensory information to the basolateral nuclei of the amygdala, the primary sensory region for anxiety and stress stimuli (Davis, 1992; Farb, et al., 1992).

In the 1996 review, Maren concluded from the studies that "glutamatergic projections synapse on both principal neurons, (class I) and inhibitory interneurons (Class II), and that inhibitory neurons, in turn, send feed forward information to GABAergic projections to principal neurons." Studies using an agonist of metabotropic glutamate receptors revealed a role for these glutamate receptors in inhibitory processes (Maren, et al., 1996; Rainnie, et al., 1994). The ionotropic receptors for AMPA and NMDA mediate

excitatory responses. This indicates that in the amygdala glutamate receptors generate

both excitatory and inhibitory responses (Campeau, et al., 1992; Maren, et al., 1996). The

degree of ionotropic and metabotropic activation determines the excitability of cells in the

amygdala. Researchers have reported that treatment of animals with glutamate receptor

antagonists diminish the acquisition and expression of fear conditioning (Fanselow and

Kim, 1994; Liang, et al., 1994).   As NMDA receptors and CRF receptors colocalize

(McDonald, 1996; Niehoff and Kuhar, 1983; Thomas, et al., 1985), it is likely that the two

neurotransmitters, CRF and glutamate, influence each other in amygdaloid processes.

In addition to glutamate and GABA, the main sources of amygdaloid

neurotransmission, other neurotransmitters in the amygdala have been reported to

contribute to inhibition or excitation of neuronal activity. These include CRF,

acetylcholine, endogenous opioids, norepinephrine and serotonin (Gray and Bingaman,

1996; Owens and Nemeroff, 1991; Sugita, et al., 1992). Researchers have observed

membrane hyperpolarization and inhibition of presynaptic transmission in the basolateral

complex after application of μ-opioid agonists, the cytokine, interleukin 1β and

anticholinesterase agent tetrahydro-9-aminoacridine (THA) (Sugita and North, 1993;

Wang, et al., 1995; Yu and Shinnick-Gallagher, 1994). Membrane depolarization and

excitability have been observed in the rat basolateral amygdala after treatment with

muscarinic and nicotinic acetylcholine receptor agonist carbachol and THA (Wang, et al.,

1995; Womble and Moises, 1993). Inhibitory effects have been observed after application

of CRF in both basolateral complex and CeA and this supports an autoregulatory role for

CRF in amygdaloid nuclei (Rainnie, et al., 1992). Noradrenergic receptor agonists like

isoproterenol have been shown to modulate cell excitability in the amygdala and also

enhance presynaptic calcium influx, thus facilitating neurotransmitter release (Gean and

Chang, 1992; Huang, et al., 1996).

Amygdaloid CRF and other neurotransmitters

Monoaminergic terminals are colocalized with CRF dendrites and cell bodies in

the amygdala (Gray and Bingaman, 1996; Fallon and Ciofi, 1992; Van Bockstaele, et al.,

1996). The amygdala has efferents and afferents to and from BNST, thalamus, lateral

hypothalamus, substantia nigra, central gray, nucleus raphe, parabrachial nucleus, locus

coeruleus, ventrolateral medulla and nucleus tractus solitarius (Fallon and Ciofi, 1992;

Moga and Gray, 1985; Van Bockstaele, et al., 1996). Norepinephrine originates from the

locus coeruleus and nucleus tractus solitarius and epinephrine from the ventrolateral

medulla (Fallon and Ciofi, 1992; Fu, et al., 1998). Dopamine originates from substantia

nigra and ventral tegmental area (Fallon and Ciofi, 1992; Haines, 1991). Serotonin

originates from the central gray and nucleus raphe and acetylcholine is from septum and

basal forebrain (Fallon and Ciofi, 1992; Haines, 1991; Sugita, et al., 1992). The

colocalization of CRF systems with these neurotransmitters systems suggests that

different neurotransmitters potentially play modulatory role in CRF expressing neurons in

the amygdala (Gray and Bingaman, 1996; Haines, 1991).

GABA containing cells which express enkephalin immunoreactivity are located in

close proximity with CRF containing cells in the CeA (McDonald, 1996; Niehoff and

Kuhar, 1983; Thomas, et al., 1985). The following peptidergic terminals are also

colocalized with CRF neurons; enkephalin, substance P, cholecystokinin, neurotensin, vasoactive intestinal peptide, neuropeptide Y and thyrotropin releasing factor (Gray and Magnuson, 1992; Heilig, et al., 1994; Larsen, et al., 1993; Shimada, et al., 1989b). Substance P and CGRP are colocalized and likely to be contained within the same terminals that synapse with CRF cells in the central nucleus (Larsen, et al., 1993; Shimada, et al., 1985).

To mediate the physiological and behavioral responses to stress, CRF also interacts with serotonergic and noradrenergic neurons (Liang and Lee, 1988; Sugita, et al., 1992;). Enhanced in vivo activity of tryptophan hydroxylase, (the rate limiting enzyme for 5-hydroxytryptamine (5-HT) synthesis) in the dorsal raphe is seen after intra-amygdaloid injections of CRF, actions that are blocked by CRF antagonist CRF_{9-41} (Beaulieu, et al., 1986; Swiergiel, et al., 1993). Stress increases norepinephrine (NE) levels in NE producing brain regions and also in the amygdala (Tanaka, et al., 1991; Valentino, et al., 1991). These increases may be centrally mediated by CRF because CRF injections intracerebroventricularly produced stress-like alterations in central noradrenergic activation in locus coeruleus and parabrachial nucleus, an effect that is abolished by CRF_{9-41} administration and bilateral lesions of CeA (Tanaka, et al., 1991).

An increase in noradrenergic cell firing in locus coeruleus, (LC) caused by CRF administration, has been reported to result in anxiogenic effects as seen in open field testing, and local release of CRF also triggered increased activation of NE release from LC (Valentino, et al., 1991; Valentino, et al., 1983). These and other studies point to increased CRF as being anxiogenic and suggest that CRF possibly acts both directly and

indirectly through other neurotransmitters to produce net anxiogenic effects (Liang, et al., 1992a; Liang, et al., 1992b; Stenzel-Poore, et al., 1994).

Manifestations of Amygdaloid CRF

The role of the amygdala and amygdaloid CRF in stress and anxiety has been studied in behavioral models of anxiety (Gray and Bingaman, 1996; Owens and Nemeroff, 1991). The amygdala is associated with behaviors involving emotional and arousal components, such as defensive/aggressive, reproductive and feeding (Gray and Bingaman, 1996; Goldstein, et al., 1996; Maren, 1996; Owens and Nemeroff, 1991). CRF injected intracerebroventricularly (ICV CRF) or into the amygdala mimics the behavioral and autonomic effects exerted by electrical stimulation of the amygdala (Davis, 1992; Dunn and Berridge, 1987). When injected into the amygdala, CRF has been shown to interfere with exploratory behavior while enhancing inhibitory avoidance/avoidance learning (Liang and Lee, 1988). The acoustic startle (conditioned fear) responses are potentiated when CRF is injected into the ventricles, but lesions of the amygdala block CRF potentiated acoustic startle (Liang, et al., 1992a; Liang, et al., 1992b; Liang and Lee, 1988). In elevated plus maze, ICV CRF decreased exploratory behavior (Koob and Bloom, 1985). In novel environment, ICV CRF increased defensive freezing and grooming, decreased locomotion and rearing, while potentiating acoustic startle responses (Dunn and Berridge, 1987; Dunn and Berridge, 1990). In addition, Owens, et al., (1989), showed that anxiolytics like adinozolam and alprazolam resulted in decreased CRF immunoreactivity in the amygdala, piriform cortex and cingulate cortex. These researchers also showed that

these benzodiazepine induced modifications in levels of CRF in the hypothalamus, bed nucleus of stria terminalis and septum. These studies indicate an activation of endogenous release of CRF in the amygdala during stress or anxiety like behaviors. These findings suggest that CRF is anxiogenic.

The anxiogenic effects of high levels of CRF levels are manifested by increased sensitivity to stressful stimuli, and are blocked by the CRF antagonist α-helical CRF$_{9-41}$ (Cratty, et al., 1995; Swiergiel, et al., 1992; Swiergiel, et al., 1993; Wiersma, et al., 1995). Injection of a small dose of α-helical CRF$_{9-41}$ into the amygdala reverses the stress induced suppression of behavior in plus maze test of anxiety (Heilig, et al., 1994). Tests on the elevated plus maze show that CRF antagonists injected into the CeA reversed the anxiogenic effects of alcohol withdrawal, reduced stress induced freezing and increased exploratory behavior (Rassnick, et al., 1993; Swiergiel, et al., 1992).

Amygdala and hypothalamo-pituitary-adrenal (HPA) axis

Hypothalamic mechanisms are reported to be modulated by limbic structures including the amygdala and activation of NE neuronal projections to the amygdala is believed to contribute to the stimulation of hypothalamus, in a process that involves α-adrenoceptors (Feldman and Weidenfeld, 1996a; Feldman and Weidenfeld, 1996b). The amygdala projects neurons directly to the PVN and innervates areas that contain neurons which regulate the release of adrenocorticotrophic hormone (ACTH) in the anterior pituitary (Feldman and Weidenfeld, 1996b; Gray, et al., 1989; Gray and Bingaman, 1996; Owens and Nemeroff, 1991). The PVN projects to the median eminence, which in turn

releases CRF into the portal vessels to transport CRF to the anterior pituitary (Rivier and

Plotsky, 1986; Sawchenko and Swanson, 1990; Swanson, et al., 1983). Hypothalamic

CRF triggers the release of ACTH from the anterior pituitary, causes increased

sympathetic outflow and also stimulates β-endorphin for analgesic effects (Brown, et al.,

1982; Rivier, et al., 1982). ACTH in turn triggers the release of glucocorticoids from the

adrenal cortex, which in turn can exert feedback inhibition on CRF (Feldman and

Weidenfeld, 1996b; Makino, et al., 1995; Owens and Nemeroff, 1991; Sapolsky, et al.,

1990).

The amygdala is also indirectly connected to the PVN through projections to the

bed nucleus of the stria terminalis, which in turn sends a dense plexus of axon terminals to

the PVN (Gray and Bingaman, 1996; Owens and Nemeroff, 1991). Other amygdaloid

connections via the brain stem nuclei also influence the PVN. After electrical stimulation

of the amygdala, immunoreactivity of the *c-fos* proto-oncogene is observed in the

parvocellular part of the PVN indicating activation of neurons (Gray, et al., 1989; Petrov,

et al., 1994). Lesions of the amygdala have been shown to attenuate ACTH release and

plasma corticosterone after the animal has been subjected to immobilization and

psychological stress, while electrical stimulation results in increased corticosterone levels

(Beaulieu, et al., 1986; Van de Kar, et al., 1991). Allen and Allen, (1974), reported that

hypersecretion of ACTH which normally occurs after adrenalectomy is attenuated if the

amygdala is destroyed. This indicates that the amygdala has a tonic excitatory effect on

the HPA axis in the absence of glucocorticoids.

<u>CRF release, receptors and CRF binding protein</u>

<u>CRF Release</u>

CRF synthesis occurs in the parvocellular cell of the paraventricular nucleus (PVN) (Vale, et al., 1981). The cells project to the median eminence (ME) where CRF is released into the portal circulation of the hypophysis to be transported to the anterior pituitary to trigger ACTH release (Lightman and Harbuz, 1993). Regulation of CRF release in the hypothalamus has been studied and both protein kinase A (PKA) and protein kinase C (PKC) play a role in mediating CRF release (Hu, et al., 1992). It has been reported by Furutani, et al., (1983) and Castro, et al., (1991) that CRF is synthesized as pre-proCRF, and after post-translational processing the active CRF is stored in vesicles at terminals. Gabr, et al., (1995), reported a depolarization-induced, calcium dependent release of CRF into the median eminence of stressed rats. In their study of the possible involvement of interleukin-2 and nitric oxide in CRF release, Raber, et al., (1995) also reported that both acetylcholine and norepinephrine induced the release of CRF from the hypothalamus and amygdala. The acetylcholine effect was antagonized by both atropine and mecamylamine indicating the involvement of both muscarinic and nicotinic receptors. The norepinephrine effect was also antagonized by phentolamine and propranolol suggesting that both α and β adrenergic receptors are involved in stimulation of CRF release in amygdala and hypothalamus. Plotsky, (1988) and Joanny, et al., (1989) however, found that norepinephrine has either stimulatory or inhibitory effects depending on predominant receptor subtype (α or β) localized on CRF neurons. GABA, ACTH and corticosterone have an inhibitory effect on CRF release (Calogero, et al., 1988; Lightman

and Harbuz, 1993).

For amygdaloid CRF, Cratty, et al., (1995), studied release of CRF from amygdala minces of prenatally stressed rats and found that these rats had increased levels of the peptide and released more CRF compared to rats from unstressed mothers. Furthermore, in a study on primary neuronal cultures, Cratty and Birkle, (1994), reported a depolarization induced release of corticotropin-releasing factor in the amygdala. Raber, et al., (1995) used rat amygdala slices and reported that interleukin-2, acetylcholine and norepinephrine play an important role in the induction of CRF release and also concluded that this is possibly mediated through nitric oxide signaling. In addition, immunocytochemical techniques by Kasckow, et al., (1997) demonstrated that interleukin-6 possibly acts to stimulate CRF release in the hypothalamus and amygdala.

CRF receptors

In situ hybridization has enabled the identification of the two subtypes of receptors CRF_1 and CRF_2 (De Souza, 1995; Dieterich, et al., 1997; Gray and Bingaman, 1996). CRF_1 is densely located in the basolateral nucleus and moderate in the cortical, medial, and lateral amygdaloid nuclei. $CRF_{2\alpha}$ is exclusive to the brain; the densest $CRF_{2\alpha}$ mRNA hybridization has been observed in the cortical and medial nucleus and moderate labeling in the basolateral nucleus (Chalmers, et al., 1996; Lovenberg, et al., 1995). $CRF_{2\beta}$ is mainly found in the peripheral muscles including heart, skeletal muscle, adrenal medulla, spleen, liver, kidney, prostrate and testes (Chalmers, et al., 1995; Lovenberg, et al., 1995).

Studies have shown that the CRF receptor is a seven member transmembrane

protein linked to G_s proteins, with the generation of cyclic AMP (Battaglia, et al., 1987;

De Souza, 1995; Olianas and Onali, 1995). Cyclic AMP generation results in ACTH

release in pituitary and is a substrate for potentiation by protein kinase C activators (Ca^{2+},

diacylglycerol and phosphatidyl serine) (Battaglia, et al., 1987; De Souza, 1995; Hu, et al.,

1992; Olianas and Onali, 1995). Owens and Nemeroff, (1991) reported that the presence

of CRF resulted in increased phosphoinositide turnover. CRF may also activate the

calcium-calmodulin system (Murakami, et al., 1985). This infers a possible cross-talk

between second messenger systems or synergistic effects between mechanism of ACTH

release and other neurotransmitters (Koch and Lutz-Bucher, 1991).

Autoradiographic methods and in situ hybridization have been used to study CRF

binding sites and showed that the anterior pituitary has the highest density of CRF binding

receptors (De Souza, 1987; DeSouza, 1995; Owens and Nemeroff, 1991). This is to be

expected since the pituitary is the site for ACTH release. A significant number of binding

sites is also found in the olfactory bulb, cerebellum, cerebral cortex and striatum (De

Souza, et al., 1985; De Souza, 1987). Autoradiographic methods have shown the highest

number CRF receptor binding in the basolateral nuclei of the amygdala compared to the

central nucleus which has the highest number of cell bodies and terminals instead

(Cummings, et al., 1983; De Souza, et al., 1985; De Souza, 1995; Meister, 1993; Swanson,

et al., 1983). A high number of CRF receptors is also found in regions of the brain that are

targeted by the CeA, including BNST, PBN, LC, PVN and solitary nucleus (De Souza,

1987). While CRF has an excitatory action at receptors in CeA targets, the neuropeptide

has both excitatory and inhibitory effects, and functions in autoregulation of CRF release

in the CeA and PVN (De Souza, et al., 1985; Rainnie, et al., 1992).

CRF binding protein

Deactivation of CRF occurs by degradation to smaller fragments by endopeptidases or through binding by CRF binding protein (Behan, et al., 1993a). A 37-kDa protein that binds CRF to block the HPA axis was identified in human plasma and has been termed CRF-binding protein (CRBP) (Behan, et al., 1993a). CRF-binding protein and its mRNA have been observed in rat brain, with large concentrations being found colocalized with CRF in the amygdala (Behan, et al., 1993). Increased levels of the CRBP mRNA have been found in the medial amygdala and CeA (Behan, et al., 1993a; Potter, et al., 1992). In addition, Potter, et al., (1992) used dual labeling experiments to demonstrate that in the central nucleus, both CRF immunoreactivity and CRBP are mainly expressed in the same neurons, but not colocalized in the same neurons in other amygdaloid nuclei. Although studies on the specific functions of the protein in the amygdala are still needed, studies done on hypothalamic CRBP indicate that the protein functions to limit CRF transport and release, thus regulating activity of CRF neurons (Behan, et al., 1993b; Gray and Bingaman, 1996; Potter, et al., 1992).

Brain stem nuclei

The CRF-containing brain stem nuclei include the locus coeruleus (LC), parabrachial nucleus (PBN), nucleus tractus solitarius, Barrington's nucleus and the raphe nuclei (Gray, 1993; Valentino, et al., 1991; Valentino, et al., 1994). The amygdalofugal

pathway is the major CeA output bundle to the brain stem nuclei. The putative role of brain stem nuclei is in the modulation of sympathetic and parasympathetic activation, the associated behavioral changes and also in nociceptive functions (Fu, et al., 1997; Gray and Bingaman, 1996; Owens and Nemeroff, 1991). CRF is found in regions that are involved in pain processing, that is, periaquiductal gray (PAG), raphe nucleus and spinal cord (Swanson, et al., 1983). The LC is located in the upper pons and is the center for norepinephrine containing neurons and projections. Injections of CRF antagonists into LC by Valentino, et al., (1991) demonstrated that this nucleus has a role in integrating CRF and noradrenergic mediation of the stress response. Koegler-Muly et al., (1993) showed that electrolytic lesions of the CeA caused a threefold decrease in CRF levels in the LC, thus implying a CRF pathway from CeA to locus coeruleus. In 1986, Chappell reported that stress caused an increase in content of CRF in the LC. CRF from the amygdala is believed to activate the locus coeruleus to trigger the production of catecholamines (Gray and Bingaman, 1996; Owens and Nemeroff, 1991).

CRF neurons are densely innervated by calcitonin gene related peptide (CGRP)-containing terminals that originate from the parabrachial nucleus and thalamus (Harrigan, et al., 1994; Shimada, et al., 1989a; Shimada, et al., 1992). These studies indicate that the interrelationships between amygdala and brain stem nuclei manifest a concerted role involving CRF and brain stem neurotransmitters in the expression of behavioral and autonomic components of anxiety, fear and defense responses.

Effects of acute and chronic stress on CRF levels

The fluctuations in CRF levels in hypothalamus and amygdala that are observed in acute and chronic stress and related pathologies may signify alterations in the synthesis, active releasable pool, release or degradation of the neuropeptide (Chappell, et al., 1986; Lightman, et al., 1993). Chappell, et al., (1986) demonstrated that in the rat, acute stress caused a two fold increase in CRF levels in the LC and a decrease in the median eminence/arcuate nucleus and median preoptic nucleus. Chronic stress maintained similar changes in the locus coeruleus and median eminence/arcuate nucleus with decreased levels in the dorsal vagal complex and increases in anterior hypothalamus and PVN (Chappell, et al., 1986). *In situ* hybridization techniques demonstrated that hypothalamic CRF mRNA was reduced by corticosterone, confirming inhibition of CRF synthesis (Makino, et al., 1995). A study by Herman, et al., (1992), demonstrated that a 15 minute reduction in the corticosterone negative feedback of hypothalamic CRF increases *c-fos* in the hypothalamus resulting in detectable changes of CRF mRNA pools which were observable in 60 minutes. Chronic stress also down regulates CRF receptors in the hypothalamus (Anderson, et al., 1993), which may be an adaptation to high levels of amygdaloid CRFergic input, in addition to corticosterone's feed back inhibition. Ratka, et al., (1989) studied the neuroendocrine regulation by Type I and Type II glucocorticoid receptors by using antimineralocorticoid RU28318 and antiglucocorticoid RU38486 respectively, and both antagonists prolonged the adrenocortical secretion following stress, indicating a blockade of receptors to the feedback inhibition of corticosteroids.

Immobilization stress affects both the amygdala and CRF systems, eliciting

adaptive changes in CeA (Henke, 1983; Honkaniemi, et al., 1992a; Honkaniemi, et al.,

1992b). Immobilization stress caused changes in the firing rate of amygdaloid neurons

(Henke, 1983) and also increased *c-fos* and corticosteroid receptor expression

(Honkaniemi, et al., 1992a; Honkaniemi, et al., 1992b). This change in *c-fos* was in

conjunction with increased CRF mRNA in the CeA and hypothalamus (Mamalaki, et al.,

1992). Kalin, et al., (1994) reported an increase in CRF mRNA content in PVN and

amygdala after restraint stress, suggesting that chronic stress elicits chronic elevations of

corticosterone, which manifests opposing effects to the putative inhibitory effects of

corticosterone on the PVN. A down regulation of CRF receptors in amygdala can also

occur after chronic CRF administration and in mild stress (De Souza, 1995; Hauger, et al.,

1988; Hauger, et al., 1993).

<u>CRF in affective and neurological disorders</u>

CRF plays a role in affective disorders and this is due to the persistent activation of

HPA axis, as has been observed in depressed patients (De Souza, et al., 1985; De Souza,

1995; Owens, et al., 1991). There is evidence that CRF is hypersecreted in depressed

patients, thus causing increased ACTH and adrenal glucocorticoid activation (De Souza,

1995; Owens, et al., 1991; Owens and Nemeroff, 1993). These researchers further

reported that CRF is increased in cerebrospinal fluid of depressed patients. This

hypersecretion of CRF would ultimately lead to down regulation of CRF receptors, as

reported by Nemeroff, et al., (1988), after studying the frontal cerebral cortex of suicide

victims. CRF imbalances have been implicated in other neurological diseases including

Alzheimer's, Parkinson's and Huntington's diseases (De Souza, 1995; De Souza, et al., 1986). Alzheimer's disease is manifested by a decrease in CRF content in the frontal, occipital, temporal lobes of the cerebral cortex, amygdala, caudate and cerebrospinal fluid while an up-regulation of CRF receptors is observed in some of these regions (De Souza, 1995; De Souza, et al., 1986). In Parkinson's disease CRF concentrations are decreased in the frontal, temporal, and occipital cortices and a significant decrease in caudate/putamen CRF is seen in Huntington's disease (De Souza, 1995). CRF is also implicated to play a role in satiation and weight control (Appel, et al., 1991; Le Feuvre, et al., 1991).

<u>Glucocorticoid receptors</u>

In vitro binding assays conducted by Reul and De Kloet, (1985) on microdissected brain regions were the initial studies to demonstrate the presence of glucocorticoid receptors in central nucleus, amygdala-hippocampal region and anterior amygdaloid area. Other studies using immunocytochemistry, autoradiography and in situ hybridization have also reported the presence of glucocorticoid receptors in the amygdala (Honkaniemi, et al., 1992a; Ahima and Harlan, 1990). The inference from these studies is that glucocorticoids can modulate CRF expressing neurons in the amygdala.

A majority of CRF neurons that contain glucocorticoid receptor immunoreactivity descend to the brain stem autonomic regions and thus influence catecholaminergic sectretion (Honkaniemi, et al., 1992a; Honkaniemi, et al., 1992b). Circulating glucocorticoids are believed to directly modulate production of CRF in the amygdala. Administration of increasing doses of corticosterone to adrenalectomized animals have

been shown to correspond to as much as five fold increase in CRF mRNA expression in

the CeA of the amygdala, while CRF mRNA in the hypothalamus decreased (Makino, et

al., 1994; Swanson and Simmons, 1989). In animals and humans, increased anxiety or

fear have been reported to increase circulating levels of CRF and glucocorticoids

(Nemeroff, et al., 1991; Owens, et al., 1991). Glucocorticoids therefore have direct effects

on the amygdaloid CRF expressing neurons, altering their activity during stress.

<u>Circulating corticosterone</u>

Corticosterone is the main glucocorticoid (equivalent of human cortisol) secreted

by the adrenal cortex of rats in response to stress and is a useful indicator of the

neuroendocrine response to stress in the laboratory animal (Shimizu, et al., 1983). ACTH

is released in the pituitary in response to CRF release from hypothalamus and ACTH

triggers adrenal secretion (Rattner, et al., 1980). Exposure to stressful stimuli including

drug injection, barometric shock, excessive handling, disease and abrupt changes in

temperature or environment, augment circulating corticosterone levels (Rattner, et al.,

1980; Shimizu, et al., 1983). Concentrations of the hormone vary according to handling

techniques, sample collection method and time of day. Generally, the baseline range for

rat corticosterone is between 50 and 400 nanograms/milliliter (ng/ml) (Shimizu, et al.,

1983). In this study, corticosterone was measured as a marker for the presence of nicotine

in the system. It was expected that nicotine would elevate corticosterone levels as reported

by other researchers (Benwell and Balfour, 1979; Cam and Bassett, 1983; Winders and

Grunberg, 1990). Notably, corticosterone has been reported to increase CRH mRNA in

the amygdala while not inducing it in the hypothalamus (Makino, et al., 1994; Makino, et al., 1995). The effects of nicotine induced corticosterone at chronic levels have not been documented.

<div align="center">Manifestations of nicotine</div>

Nicotine

Nicotine (1-methyl-2-(3-pyridyl) pyrolidine) is a poisonous, volatile alkaloid derived from tobacco and is responsible for many of the tobacco effects (McDonough, 1994). Nicotine has a pKa of 7.9 and at blood pH (7.4) is approximately 69% ionized and 31% unionized, making it more lipid soluble (Beckett, et al., 1972; Jones, 1987). Nicotine therefore crosses the blood brain barrier, and is a psychostimulant acting on neuronal nicotinic receptors located in various brain regions. In small doses, nicotine stimulates the CNS (Armitage, et al., 1969; Gray, et al., 1996; Nordberg, et al., 1989; Rowell, 1995), while large doses depress the nervous system at autonomic ganglia and myoneuronal junctions (Benwell and Balfour, 1982; Benwell and Balfour, 1985; Sharp, et al., 1993).

Nicotine Pharmacokinetics

Nicotine has been reported to accumulate rapidly in the brain after intravenous injections, within five minutes, and high levels last up to 50 minutes (Sastry, et al., 1995; Schmiterlöw, et al., 1967). In the 1967 review, Schmiterlow, et al., reported the fate of ^{14}C and ^{3}H labeled nicotine in rat, mouse and cat, on studies that used autoradiographic methods. Nicotine was reported to accumulate rapidly in the brain, within five minutes

peaking at about 15 minutes and decreasing by 30 minutes. (Sastry, et al., 1995) studied

the distribution and retention of nicotine and its metabolite, cotinine, in rat plasma and

brain homogenates after intravenous administration of a 1.0 mg/kg dose. Using high

performance liquid chromatography (HPLC) for nicotine measurements, the researchers

reported that nicotine peaked in plasma after 5-10 minutes to 2.2 nmol/ml. They observed

a 50% decrease (in plasma levels) by 20 minutes which remained constant up to 60

minutes. Within 20 minutes, plasma cotinine levels had peaked to 1.2 nmol/ml, with

levels remaining similar to nicotine levels for up to 60 minutes. In the brain, nicotine

peaked within five minutes of treatment, remaining constant for 20 minutes with a decline

by 60 minutes. The peak levels of nicotine in the brain were about half of plasma levels.

The brain retained more nicotine compared to other organs including the heart, liver,

kidney and plasma, this possibly due to the fact that the brain has a higher density of

nicotinic receptors which would bind nicotine with high affinity (Sastry, et al., 1995).

Isaac and Rand, (1972), collected blood samples in humans from 0.5-60 minutes

after a single cigarette. The researchers measured plasma levels of nicotine using gas-

liquid chromatography and reported that plasma levels increased within two minutes of

smoking and peaked to 50 ng/ml by five minutes, and then decreased to half of the peak by

20 minutes. Nicotine is metabolized by liver and kidney and its biological half life is

about 0.5, 0.6 and 0.96 hour in humans, dogs and rats respectively (Adir, et al., 1976;

Castro, 1979; Isaac and Rand, 1972; Monji and Benowitz, et al., 1988). The resultant

metabolite cotinine has a half life of 19-24 hours and remains constant in all the organs,

meaning that it has a longer retention time (Sastry, et al., 1995).

Neuronal Nicotinic receptors

The neuronal acetylcholine nicotinic receptor (nAChR) is a ligand-gated ion channel permeable to sodium (Na^+), potassium (K^+), and calcium (Ca^{2+}), and therefore participates in neurotransmission (McGehee and Role, 1995; McGehee and Role, 1996; Schwartz and Kandel, 1991). The neuronal nAChR, is the primary receptor for acetylcholine in neurons and is found in sympathetic and parasympathetic ganglia, and the adrenal cortex (Mycek, et al., 1992). Molecular biology, immunocytochemistry and *in situ* hybridization techniques have demonstrated that nAChRs consist of a family of heterogeneous receptor subtypes composed of α- and β- subunits encoded by 12 different genes (Luetje and Patrick, 1991; Lukas, 1995; Wada, et al., 1989). The formation of nicotinic receptors with heterogeneous subunit stoichemistry gives rise to multiple channel properties and therefore, receptor types (Balfour and Fagerström, 1996; Cooper, et al., 1991; Luetje and Patrick, 1991; Wada, et al., 1989;).

Eight alpha (α2-α9) and four beta (β2-β5) subunits have been identified in rat (Benwell and Balfour, 1982; McGehee and Role, 1995; Steinbach and Ifune, 1989; Vidal, 1996). The neuronal type nicotinic receptors include α2-5 and β2-4 (Fu, et al., 1997; Fu, et al., 1998). While the β2-β4 subunits are widely distributed in the brain, the α2, α3, and α4 subunits are expressed only in distinct brain areas (Shioda, et al., 1997; Wada, et al., 1989).   Molecular biology studies using Xenopus oocytes to express genes coding for the nicotinic receptors have shown that when the oocytes were injected with mRNA or cDNA of α2, α3, or α4 combined with β2 or β4, the receptors became functional and responded to acetylcholine or nicotine stimulation, by exhibiting depolarizations (Bertrand, et al.,

1990). The alpha subunit is the agonist binding site and the beta subunit influences the affinity and response of the receptor to the agonists (Luetje and Patrick, 1991; Lukas, 1995; Wada, et al., 1989).

In situ hybridization techniques have shown that different combinations of α-and β- subunits exist in various brain regions (Papke, et al., 1989; Wada, et al., 1989). The α4β2 receptor is widely distributed in the brain, while other subunit combinations are restricted to specific areas (Clarke, et al., 1984; Flores, et al., 1991; Flores, et al., 1997). For example, the α2β2 is found in the interpeduncular nucleus, α3β4 in the medial habenula (Wada, et al., 1989). The α7 subunit which is mainly expressed in muscular AChRs, is expressed only in the hippocampus and hypothalamus (Bylund and Yamamura, 1990; Clarke, et al., 1985; Seguela, et al., 1993). The α4β2 receptor is sensitive to nicotine, acetylcholine, and other agonists including lobeline, epibatidine, cytisine and ABT418, but not sensitive to α-bungarotoxin, the snake venom (Bylund and Yamamura, 1990; Flores, et al., 1997; Watson, et al., 1987). Alpha bungarotoxin selectively binds to the α7 subunit (Bylund and Yamamura, 1990; Watson, et al., 1987).

Nicotinic receptors are believed to exist in two inter convertible states; ground and refractory (Bhat, et al., 1994; Colquhoun, et al., 1987; McGehee and Role, 1996). The ground state of the receptor is activated by binding of agonists whereas the refractory is the desensitized state and is non functional (McGehee and Role, 1996; Bhat, et al., 1994). High or chronic doses of nicotine desensitize the receptors and an up-regulation of receptors ensues, to compensate for the non functional receptors (Flores, et al., 1997; Rowell and Li, 1997; Schwartz and Kellar, 1985; Ulrich, et al., 1997). Benwell, et al.,

(1988) reported an increase in density of nicotinic receptor binding sites in post mortem brains of smokers, suggesting that over-exposure to nicotine may have induced this increase. It also has been reported that the up-regulated receptors, like the desensitized, are not active and therefore can not be associated with behavioral effects of nicotine (Caggiula, et al., 1993; Collins, et al., 1990; Pauly, et al., 1992; Rowell and Li, 1997). In addition to exciting neurons in the brain, effects of nicotine are also enhanced by its metabolites mainly cotinine, which also remain active on the nAChRs (Crooks and Dwoskin, 1997; Sharp, et al., 1993).

Autoradiographic techniques and immunocytochemistry have demonstrated the existence of neuronal nicotinic receptors (nAChRs) in various brain regions, particularly in the cerebral cortex and hippocampus (Clarke, et al., 1984; Rubboli, et al., 1994). The presence of nicotinic receptors and choline acetyl transferase, the acetylcholine (ACh) synthetic enzyme, has been reported in the amygdala (Hellendall, et al., 1986; Van der Zee, et al., 1997; Yajeya, et al., 1997). Electrophysiological data suggest that nAChRs are located in both the somatodendritic membrane and in the nerve terminal (Clarke and Pert, 1985; Vidal, 1996). This makes the receptor mediate neural activity in two ways. At the nerve terminal, the nicotinic receptor influences neurotransmitter secretion on target neurons; at dendrites, the receptor mediates changes in rate and pattern of firing. Both mechanisms are believed to be present in the brain (Balfour and Fagerström, 1996; Vidal, 1996). In presynaptic terminals for example, low doses of nicotine activate nAChRs on synaptic terminals to evoke an influx of Ca^{2+} which in turn enhances exocytosis of a neurotransmitter, for example, glutamate (McGehee and Role, 1996).

Biochemical studies on synaptosome have demonstrated that nicotine administration triggers the release of neurotransmitters including ACh, GABA, norepinephrine, epinephrine, dopamine, serotonin, aspartate and glutamate, therefore confirming the presence of presynaptic nAChRs, (Armitage, et al., 1969; Balfour and Fagerström, 1996; Rapier, et al., 1988; Rowell and Hillebrand, 1994; Toth, et al., 1992; Valentine, et al., 1996). As discussed earlier, CRF neuronal systems are colocalized with those of other neurotransmitters, which are influenced by nicotine (Fallon and Ciofi, 1992; Okuda, et al., 1993; Shinohara, et al., 1988; Van Bockstaele, et al., 1996). Since nicotine influences neuronal systems that colocalize with CRF systems (Okuda, et al., 1993), this suggests that nicotine could also have an influence on the CRF.

<p align="center">Nicotine and HPA axis</p>

Nicotine administration into rats increases plasma ACTH and corticosterone levels (Benwell and Balfour, 1979; Cam and Bassett, 1983; Freund, et al., 1988). Catecholamines are reported to play a role in triggering CRF release in the PVN of hypothalamus for the ultimate release of ACTH in the anterior pituitary (Andersson, et al., 1983; Matta, et al., 1993b). Nicotine stimulates *c-fos* immunoreactivity in the CeA, PVN and brain stem catecholaminergic regions (Matta, et al., 1993a; Matta, et al., 1997; Salminen, et al., 1996). This suggests that nicotine is a potent activator of neuronal activity in catecholaminergic and CRF regions. It has been reported that CRF receptors are possibly colocalized with nAChRs in the median eminence (Okuda, et al., 1993) suggesting that nicotine may modulate CRF release at median eminence. Majewska, et al.,

(1985) reported that glucocorticoids also induced muscimol binding in the GABA receptors which are possibly colocalized with nicotinic and CRF receptors. Together this and other studies further confirm the inhibitory effects contributed by glucocorticoids on CRF receptors and possibly on nicotinic receptors (Johnson, et al., 1995; Sapolsky, et al., 1990).

<center>Nicotine and the amygdala</center>

The amygdala has considerable levels of CRF, cholinergic projections from basal forebrain (Heckers and Mesulam, 1994) and also expresses nicotinic receptor immunoreactivity, and choline acetyl transferase, the acetylcholine synthetic enzyme (Hellendall, et al., 1986). Cholinergic fibers from basal forebrain reach the amygdala through the stria terminalis and the amygdalofugal pathway (Heckers and Mesulam, 1994). Roozendaal, et al., (1997), reported that in amygdala, 90% of the cholinergic afferents expressed nicotinic receptor immunoreactivity, and as seen in the neocortex, muscarinic and nicotinic receptors are colocalized in amygdala (Van der Zee, et al., 1992; Van der Zee, et al., 1997). Electrophysiological techniques by Yajeya, et al., (1997), showed that most of the amygdaloid neurons responded to carbachol which is an agonist at both nicotinic and muscarinic receptors. A more specific nicotinic agonist would be necessary to help determine nicotinic receptor responses in the amygdala. Microdialysis techniques showed that administration of nicotine into the amygdala induced NE release in brain stem catecholaminergic regions (Fu, et al., 1997; Fu, et al., 1998). Raber, et al., (1995), studied primary cultures and reported that CRF release in the amygdala was

influenced by interleukin, ACh and norepinephrine. A 1997 study by Matta, et al.,

reported increased *c-fos* expression in CRF immunoreactive neurons after nicotine

administration.

<u>Nicotine and neurotransmitters</u>

<u>Acetylcholine secretion</u>

Acetylcholine (ACh) is an excitatory neurotransmitter, its secretion in the brain is

strongly associated with locomotor activity and cognitive stimulation (Armitage, et al.,

1969; Balfour and Fagerström, 1996; Warburton and Rusted, 1993). Nicotine has been

shown to modulate locomotor activity in animals and this is mediated through activation

of ACh secretion resulting in muscarinic activation (Armitage, et al., 1969; Levin, 1992).

There are variations in responses elicited by different dosing times, regimens and routes

of administration of nicotine. However, Armitage, et al., 1968 showed that high doses of

nicotine suppressed ACh release, and could be demonstrated by decreased locomotion,

due to desensitization of the nicotinic receptor (Armitage, et al., 1968; Armitage, et al.,

1969; Hakan and Ksir, 1991). This is to be expected because nicotine is a stimulant at

low doses and depressant at high doses (Benwell and Balfour, 1982; Gray, et al., 1996;

Sharp, et al., 1993).

<u>Role in nicotine rewarding properties.</u> It is believed that smokers use tobacco for

nicotine to modulate level of arousal, attention or sustained concentration and cognitive

performance (Acri, et al., 1994; Balfour and Fagerström, 1996; Warburton, 1992). The

need to satisfy this CNS effect is believed to modulate the desire to smoke. It has been

reported that one of the ways nicotine elicits these CNS effects is through stimulation of the ACh secretion in the brain, particularly the hippocampus (Levin, 1992; Armitage, et al., 1968; Armitage, et al., 1969).

Pharmacological disruption of cholinergic neurotransmission or neurodegeneration cause impairments in cognitive skills (Balfour and Fagerström, 1996; Levin, 1992; Levin, et al., 1992). For example, Alzheimer's and Parkinson's diseases are characterized by a loss of cortical nAChRs and patients show an improvement in memory and attention after nicotine treatment (Balfour and Fagerström, 1996; Kellar, et al., 1987; Levin, 1992; Vidal, 1996). Two reviews by Warburton, (1992) and Warburton and Rusted, (1993) surmised that tobacco smoking or administration of nicotine elicited cognitive-enhancing effects, more related to information processing, as observed in Alzheimer's patients treated with nicotine.   In monkeys, ABT418, an agonist at nAChRs, improved accuracy in learning and memory (Prendergast, et al., 1997).

<u>Norepinephrine secretion</u>

Nicotine has been reported to excite noradrenergic neurons in the brain stem (Fu, et al., 1997; Fu, et al., 1998; Sharp and Matta, 1993). Noradrenergic innervation arises from the locus coeruleus, nucleus tractus solitarius and neurons of the reticular formation (Gray, 1993; Haines, 1991). The noradrenergic system innervates mainly the cortical, subcortical and spino medullary regions and PVN (Haines, 1991). Norepinephrine plays a vital role in memory and learning, sleep-wake cycle and affective disorders (Bremner, et al., 1996). Svensson and Engberg, (1980) used single cell recording techniques and

demonstrated that noradrenergic neurons express nicotinic receptors on both

somatodendritic membranes in the LC and the nerve terminal membranes. Egan and

North, (1986), used LC slices to demonstrate that nicotine and ACh depolarize LC

neurons, indicating the presence of nAChRs. This and other studies suggest that nicotine

plays a part in exciting the NE neurons in brain stem (Benwell and Balfour, 1997; Fu, et

al., 1997; Fu, et al., 1998; Sharp and Matta, 1993).

In vivo microdialysis studies performed by Andersson, et al., (1981), and Mitchell,

(1993), showed that nicotine administration increased NE turnover in the rodent brain.

This was further confirmed in immunocytochemical studies (Valentine, et al., 1996) which

showed that an increase in *c-fos* immunonoreactivity in PVN correlated with *c-fos* in

nucleus tractus solitarius. Studies on hypothalamic synaptosomes revealed that low

concentrations of nicotine (5 x 10^{-6} M) caused an increase in NE release (Balfour, 1973;

Yoshida, et al., 1980). Acute administration of 100 μM nicotine increased NE in fetal LC

cell cultures (Gallardo and Leslie, 1998). Nicotine also induces NE release from nucleus

tractus solitarius and LC which project to the PVN and contribute to NE release for

ultimate stimulation of CRF release in PVN (Gray and Bingaman, 1996). A recent study

by Fu, et al., (1998) using microdialysis techniques showed nicotine to induce NE release

in amygdala and hippocampus. All these effects were inhibited by mecamylamine, a

centrally acting noncompetitive antagonist of nicotine (Morgan and Pfeil, 1979). A

decrease in NE in the amygdala has been associated with an increase in hypothalamic

activity (Goldstein, et al., 1996), suggesting a role of amygdala in activation of the

hypothalamus.

In addition to its brain stem effects, nicotine also modulates NE levels in other brain regions. However, brain regions including the hippocampus, cerebral cortex, and cerebellum, require high concentrations of nicotine to cause an increase in NE turnover (Smith, et al., 1991). Sershen, et al., (1997) studied hippocampal slices and reported that $\alpha_3\beta_2$ receptor controlled NE release in the slices. Three studies have reported that chronic nicotine injections seem to exert selective sensitization of hippocampal NE release and turnover probably due to increased synthesis of tyrosine hydroxylase, the rate limiting enzyme in the synthesis of NE (Joseph, et al., 1990; Mitchell, 1993; Smith, et al., 1991). However, subcutaneous minipump infusion of nicotine elicited contrasting results. Kirch, et al., (1987), reported that minipump infusions of nicotine lowered NE levels in the frontal cortex, possibly abolishing NE overflow in the hippocampus (Balfour and Fagerström, 1996). These findings suggest that NE secreting neurons located in the hippocampus originating from the LC get desensitized by chronic exposure to nicotine levels that are comparable to the 10-25 ng/ml found in habitual smokers (Armitage, et al., 1968; Balfour and Fagerström, 1996; Isaac and Rand, 1972). Therefore, nicotine influences both cholinergic and catecholaminergic systems that play a role in the interrelationships between hypothalamus, amygdala and brain stem (Fu, et al., 1997; Fu, et al., 1998; Levin, et al., 1992; Matta, et al., 1993b; Valentine, et al., 1996).

Role in nicotine dependence. The hippocampus plays a role in nicotine dependence (Markou, et al., 1998; Mitchell, 1993; Smith, et al., 1991). Chronic infusions of nicotine using minipumps desensitize nicotinic receptors and decrease NE release in neurons which innervate the hippocampus, originating from the LC (Balfour and

Fagerström, 1996; Smith, et al., 1991). This desensitization of receptors and reduction in

NE is believed to play a critical role in mediating the "calming" effects of tobacco (Gilbert,

et al., 1997; Pomerleau and Pomerleau, 1987). This therefore, suggests that smokers seek

to smoke in order to maintain desensitized nicotinic receptors and that an individual would

adjust the smoking behavior in order to achieve this effect (Benowitz and Jacob, III, 1997).

Dopamine release

Nicotine stimulates and sensitizes dopamine neurons to increase dopamine (DA)

secretion, and nicotine also inhibits DA reuptake by neurons (Benwell and Balfour, 1992;

Izenwasser, et al., 1991; Shoaib, et al., 1994). DA is abundant in the brain, mainly found

in the basal ganglia particularly the caudate nucleus (Carlsson and Waldeck, 1958). DA

secreting neurons originating from the ventral tegmental area (VTA) to the nucleus

accumbens (NAcc) and from substantia nigra (SN) to caudate/putamen (CP) have nicotinic

receptors on both the somatodendritic membranes and nerve terminal membranes (Clarke

and Pert, 1985; Corrigall, et al., 1992; Marshall, et al., 1997). Nicotine has been reported

to increase (DA) overflow in NAcc and CP, and dopamine release increased in striatal

synaptosomes (Grady, et al., 1994; Rapier, et al., 1988; Rapier, et al., 1990; Rowell and

Hillebrand, 1994). These researchers demonstrated that when synaptosomes derived from

caudate putamen (CP) area or nucleus accumbens were stimulated with nicotine an

increase in DA secretion was observed. The response was calcium dependent and

antagonized by mecamylamine, the nicotine noncompetitive antagonist. These

findings confirmed that responses to nicotine are elicited through presynaptic nicotinic receptors. Microdialysis probes into the NAcc also demonstrated increased DA overflow after nicotine treatment (Mifsud, et al., 1989; Nisell, et al., 1994). Microdialysis techniques have shown that DA overflow in the NAcc increases due to systemic nicotine injections and this overflow mediated mainly by nicotinic receptors located in the somatodendritic membranes in the VTA (Bassareo, et al., 1996; Benwell and Balfour, 1992; Marshall, et al., 1997).

Studies by Imperato, et al., (1986) and Mereu, et al., (1987), suggested that, compared to nigrostriatal system, mesolimbic neurons are more sensitive to nicotine. Garris and Wightman, (1994), used microdialysis and voltametric probes and which indicated that DA flow in the both CP and NAcc synaptic clefts is tightly controlled by a transport system which is more efficient in the CP, which would partly explain the differences in the sensitivity to nicotine. Unlike the desensitization effects observed in the ACh, NE and 5-HT systems, repeated injections of nicotine enhance DA overflow in the NAcc (Benwell and Balfour, 1992; Pietilä, et al., 1996), but this was contrasted by a study that demonstrated a decrease in dopamine release in synaptosomes after repeated nicotine doses (Grady, et al., 1994). Sensitization of the mesolimbic DA system is modulated through the nicotinic receptor since it is attenuated by prior administration of mecamylamine. It has also been reported that sensitization of these DA receptors depends on the co-stimulation of the NMDA receptor since this sensitization is diminished by prior treatment with NMDA receptor antagonists (Shoaib, et al., 1994; Shoaib, et al., 1997). This sensitization of DA neurons, reinforces the belief that DA systems mediate

behavioral responses to nicotine, particularly, locomotor stimulation and rewarding properties of the drug (Clarke and Pert, 1985; Corrigall and Coen, 1989; Corrigall, et al., 1992).

   Role in nicotine dependence. Existing studies on experimental models of depression (stress) indicate that behavioral activation is dependent on the enhanced secretion of dopamine (DA) in mesoaccumbens system, and the system is a critical component of the reward system that influences reinforcement (Benwell and Balfour, 1992; Gray and Bingaman, 1996).   Piccioto, et al., (1998) reported stimulation of dopamine release by nicotine in ventral striatum of wild type mice compared to $\beta2$ subunit mutant mice. These researchers concluded that striatal nAchRs containing the $\beta2$ subunit are involved in the reinforcing properties of nicotine. This as discussed earlier, suggests the involvement of the nicotine sensitive $\alpha4\beta2$ receptor (Flores, et al., 1991).

   Dopamine neurons are sensitized by high or chronic levels of nicotine to increase DA secretion, and nicotine also inhibits DA reuptake thus enhancing the availability of DA (Benwell and Balfour, 1992; Izenwasser, et al., 1991; Shoaib, et al., 1994;). This would suggest that in chronic doses, as nicotine receptors get desensitized, the capability to activate dopamine neurons is compromised thus eliciting a need for more nicotine, which eventually leads to negative effects. Researchers report that experimental animals need a "time out" from nicotine self administration because the presence of nicotine with receptors desensitized, becomes aversive. Time off reduces drug levels and minimizes receptor desensitization (Balfour and Fagerström, 1996). Possibly, smokers seek smoke to replenish nicotine levels, overcome desensititized receptors, and recruit non-

desensitized receptors to trigger neurotransmission and therefore increase dopamine

release, thus contributing to the "calming" effects of nicotine (Gilbert, et al., 1997;

Pomerleau and Pomerleau, 1987).

5-hydroxytryptamine (serotonin) secretion

Nicotine has been shown to decrease serotonin in selected regions of the

mammalian brain while decreasing the neurotransmitter in others (Balfour, et al., 1975;

Bannon, et al., 1998; Benwell and Balfour, 1979). The mammalian forebrain gets its

serotonergic innervation from the median and dorsal raphe nuclei located in the brain

stem (pons and medulla) (Haines, 1991). These nuclei express a significant amount of the

$\alpha 4$ subunit of the nicotinic receptor (Wada, et al., 1989). Other brain areas with

considerable amounts of 5-hydroxytryptamine (5-HT) include the hypothalamus, limbic

system and pituitary gland (Benwell and Balfour, 1979).

Serotonin is believed to modulate a number of behavioral aspects, including pain

perception, sleep, depression, anxiety, mood changes and sexual activity and acts through

different receptors including $5\text{-}HT_1$, $5\text{-}HT_2$, $5\text{-}HT_3$, $5\text{-}HT_4$ (Kidd, et al., 1993).

Antidepressants are used to modulate serotonin levels or antagonize receptors (Charney, et

al., 1990; Graeff, et al., 1996; Kandel, 1991; Kidd, et al., 1993). The $5\text{-}HT_{1a}$ receptor plays

a role in fear conditioning in the amygdala while $5\text{-}HT_2$ mediates mood states (Charney, et

al., 1990; Fernandes, et al., 1997; Graeff, et al., 1996). The $5\text{-}HT_3$ receptor is found in

high densities in the brain and is significantly localized in nerve terminals of amygdala

where it evokes fast excitatory transmission (Kidd, et al., 1993; Sugita, et al.,

1992). Kawahara et al., (1993), reported that psychological stress increases serotonin in the amygdala, and 5-HT$_3$ antagonists have been shown to be anxiolytic in animal models of behavior (Charney, et al., 1990; Graeff, et al., 1996).

Nicotine has been shown to decrease serotonin in selected regions of the mammalian brain, this may be possibly because of increased metabolism of the neurotransmitter caused by nicotine (Bannon, et al., 1998). Nicotine decreases serotonin in the hippocampus, but increases this neurotransmitter in the amygdala (Balfour, et al., 1975; Benwell and Balfour, 1979). Balfour, et al., (1975), demonstrated that acute injections of nicotine in normal rats cause a transitory decrease in hippocampal serotonin, decreases were observed within 45 minutes, with a rise occurring by 60 minutes. In stressed rats, acute nicotine caused a persistent reduction in serotonin for up to 75 minutes (Balfour, et al., 1975). Chronic infusion of 0.4 mg/kg for 40 days evoked a more stable, persistent, regionally selective decrease in hippocampal serotonin, resulting from selective reduction in the biosynthesis of serotonin (Benwell and Balfour, 1982). A regional reduction in serotonin and the metabolite 5-hydroxyindole acetic acid, and an up-regulation of serotonin receptors, like nAChRs, has been observed in postmortem tissue of habitual smokers (Benwell, et al., 1988; Benwell, et al., 1990). This supports the notion that this system may play a role in the psychopharmacological response to nicotine and its contribution to the habit of tobacco smoking (Balfour and Fagerström, 1996).

Role in nicotine dependence.   Nicotine withdrawal is strongly associated with symptoms of depression, while nicotine dependence has been associated with a history of depression (Breslau, et al., 1993; West and Hajek, 1997). Rasmussen, (1997) studied

effects of nicotine withdrawal on serotonergic neurons in rat nucleus raphe, using $5HT_{IA}$

receptor agonist 8-OH-DPAT. They reported that nicotine withdrawal increased

sensitivity to the auditory startle response, indicating sensitivity to external stimuli. In a

similar study, Rasmussen, et al., (1997) reported that $5HT_{IA}$ antagonists blocked the startle

response that is enhanced by nicotine withdrawal. These researchers concluded that the

serotonergic system is possibly associated with symptoms of nicotine withdrawal.   A

study by Hurt, et al, (1997), tested the antidepressant bupropion (which possibly functions

on both serotonergic and noradrenergic receptors) for smoking cessation in a study that

involved 615 subjects. The researchers reported that compared to placebo, more smokers

who received the sustained-release form of bupropion were able to stay smoke free for the

duration of the (one year) tested time. This suggested that the antidepressive effects of

bupropion enabled subjects to have a decreased need for nicotine. However, neural

mechanisms underlying the antidepressive properties of nicotine have yet to be elucidated.

<div style="text-align:center">Nicotine and the NMDA receptor</div>

The NMDA receptor is involved in responses to chronic nicotine (Shoaib and

Stolerman, 1992; Shoaib, et al., 1994; Shoaib, et al., 1997). The NMDA receptor plays an

important role in long term potentiation in the amygdala and nicotine has stimulatory

effects on glutamate release (McGehee and Role, 1996; Toth, et al., 1992). In pyramidal

cells of the prefrontal cortex, excitatory synaptic potentials mediated by glutamate are

potentiated by nicotine (Vidal, 1996; Vidal and Changeux, 1993). Since glutamate plays a

role in synaptic plasticity, the enhancement of glutamatergic terminals by nicotine in the

prefrontal cortex further explains the role of nicotine in learning and memory (Shoaib, et al., 1994; Shoaib, et al., 1997). These studies point to functional cooperation between nicotinic and glutamatergic systems in the cortex which would

represent a unified involvement of the two systems in cognitive processes (Toth, et al., 1992; Vidal, 1996).

<u>Positive reinforcing properties of nicotine</u>

The effects of nicotine on animal behavior are impacted by several factors including dose, route of administration, species and strain, prior exposure to the drug and nature of test used (Balfour and Fagerström, 1996; Donny, et al., 1998). Similar to psychostimulants like amphetamine and cocaine, the effects of acute nicotine on animals are manifested by locomotor stimulation (Hakan and Ksir, 1991; Mifsud, et al., 1989). Acute high doses or chronic low doses decrease locomotor stimulation due to receptor desensitization Ksir, et al., (1987) and also because high doses decrease ACh release (Armitage, et al., 1968). Positive reinforcing properties (rewarding properties) of addictive drugs are manifested in the animal's self administration of the drug as measured using the self administration paradigm (Balfour, 1982; Balfour and Fagerström, 1996; Donny, et al., 1998). With amphetamine and cocaine, both the acquisition and maintenance of drug usage behavior have been shown to depend on an intact DA system (Glick, et al., 1996), that is, DA projections to the NAcc, where a large increase in DA overflow is observed after drug treatment (Benwell and Balfour, 1992; Izenwasser, et al., 1991; Shoaib, et al., 1994).

Maintenance of nicotine self administration also involves DA neurons that project from the VTA, but the paradigm is not easy to demonstrate in animals, because nicotine has weaker reinforcing properties compared to cocaine and amphetamine (Corrigall and Coen, 1989; Corrigall, et al., 1992; Corrigall, et al., 1994). In a study by Dawe, et al., (1995), on humans who were initially treated with haloperidol, a DA receptor antagonist, individuals were found to increase their smoking to overcome this blockade and once achieved they maintained the new dose of smoke, with satisfaction. In the self administration paradigm, the response for nicotine is reported to be bell shaped, with higher doses possibly becoming aversive rather than rewarding (Balfour, 1982; Balfour and Fagerström, 1996; Hildebrand, et al., 1997; Rose and Corrigall, 1997; Valentine, et al., 1997). CRF has also been implicated to play a role in drug seeking behaviors in that intracerebroventricular injections of CRF to stressed animals induced a relapse to cocaine seeking, an effect that was attenuated by the presence of the antagonist, α-helical CRF and d-phe CRF (Heinrichs, et al., 1998).

<u>Nicotine and neurological diseases</u>

As discussed earlier, animal and human studies on nicotinic receptors suggest that neuronal nicotinic systems play a major role in cognitive functions and dysfunctions (Balfour and Fagerström, 1996; Clarke and Pert, 1985; Levin, 1992). Nicotine and analogues seem to influence brain neural systems that are implicated in psychiatric and neural disorders (Balfour and Fagerström, 1996; Roozendaal, et al., 1997; West and Hajek, 1997). Regionally selective losses of the nicotinic receptors that bind [^{3}H](-)-nicotine and

[^{3}H]-ACh are associated with cognitive impairments observed in diseases like Alzheimer's

(AD) which affects memory and Parkinson's (PD) which affects mobility (Flynn and

Mash, 1986; Jones, et al., 1992; Nordberg, et al., 1989). Nicotine therefore, could be used

therapeutically to treat these diseases (Levin, 1992). For example, Alzheimer's and

Parkinson's diseases are characterized by a loss of cortical nAChRs and patients show an

improvement in memory and attention after nicotine treatment (Kellar, et al., 1987; Levin,

1992; Vidal, 1996). CRF is also decreased in AD and PD, suggesting a possible

interaction between nicotine and CRF in the etiology of these pathologies

(De Souza, 1995; De Souza, et al., 1985).

Is nicotine anxiolytic?

Smokers report that after exposure to stressful stimuli smoking has a calming

effect (Benowitz and Jacob, III, 1997; Gilbert, et al., 1997; Pomerleau and Pomerleau,

1987). This is a paradox because nicotine elicits effects that resemble stressful stimuli,

for example, increased norepinephrine secretion and circulating ACTH and corticosterone

(Benwell and Balfour, 1982; Cam and Bassett, 1983; Jones, 1987). It should be noted that

having a cigarette in the hand, something to pay attention to, may contribute to the

alleviation of anxiety symptoms (Jones, 1987; West and Hajek, 1997). The role of

nicotine as an anxiolytic has been observed using animal models of anxiety, including

elevated plus maze, sensory motor gating and nicotine ingestion when exposed to stress

(Acri, et al., 1994; Brioni, et al., 1993; Brioni, et al., 1994). Rats treated with nicotine

(0.01 to 1.0 mg/kg) were reported to spend more active time in the illuminated area than

in the dark implying that animals were less fearful (Costall, et al., 1989). Neural

mechanisms in the reinforcing properties of nicotine are possibly different from those of

anxiolytic properties of nicotine in that they depend on desensitization of the nicotinic

receptors located on synapses of pathways that mediate the stress response, for example

CRF and NE (Balfour and Fagerström, 1996; Benwell and Balfour, 1997). Whether or not

the apparent anxiolytic effects of nicotine may be similar to those evoked by

antidepressants rather than anxiolytic drugs have yet to be investigated convincingly

(Balfour and Fagerström, 1996).

A few researchers have examined effects of nicotine withdrawal using animal

models of anxiety and it is still debatable whether or not nicotine or withdrawal from

nicotine increases anxiety (Helton, et al., 1993; Jones, 1987; Rasmussen, et al., 1997; West

and Hajek, 1997). Helton, et al., (1993), reported that nicotine withdrawal increased

sensory motor reactivity suggesting a manifestation of anxiogenic effects. Studies by

Costal, et al., (1989); Crooks and Dwoskin, (1997) used the light/dark box paradigm on

mice and found that nicotine withdrawal caused an increase in anxiety. This anxiety was

diminished by the antidepressant diazepam and by the 5-HT$_3$ receptor antagonist,

ondasteron, indicating an interrelationship between GABAergic and serotonergic with

cholinergic synapses (Costall, et al., 1989; Crooks and Dwoskin, 1997; Rasmussen, et al.,

1997).

Smoking or self administration of nicotine by animals may be a way to seek a

coping mechanism against aversive environmental stimuli (Balfour, 1982). A potent

nicotinic receptor agonist ABT 418 was studied by (Sajdyk and Shekhar, 1997). The

researchers found that 0.062- 30 μmol/kg of ABT 418 caused anxiolytic effects in rats, as shown by increased activity in the elevated plus maze, effects that were also produced by nicotine. Kumari, et al., (1996), reported a decrease in prepulse inhibition of the acoustic startle suggesting an increase in the ability to screen irrelevant sensory stimuli, that is, increased attention. Anhedonic properties of aversive environmental stimuli can be depressive or anxiogenic and for individuals who have been exposed to smoking, this triggers the desire to smoke (Balfour, 1982; Jones, 1987). To further support the antidepressive properties of nicotine, a smoking cessation study by Hurt, et al, (1997), reported that compared to placebo, more smokers who received the sustained-release form of bupropion were able to stay smoke free for the duration of the (one year) tested time. These findings could suggest that in humans, the ability of nicotine to alleviate stress is a quality that enables this drug to reinforce its self-administration (Balfour and Fagerström, 1996; Jones, 1987). If nicotine is anxiolytic or antidepressive, we should expect that both doses 0.4 mg/kg and 1.0 mg/kg which give rat levels equivalent to 10-40 ng/ml) maintained by regular smokers (Balfour and Fagerström, 1996; Isaac and Rand, 1972) would decrease CRF content in amygdala and hypothalamus.

<u>Is nicotine anxiogenic</u>?

Anxiogenic responses can be measured using animal models of anxiety, including mazes and/or the acoustic startle responses. As discussed earlier, CRF has been reported to induce defensive withdrawal symptoms in mazes and potentiate acoustic startle responses. Acri, et al., (1993) and (1994), used very high doses (6 and 12 mg/kg) of

nicotine and the acoustic startle paradigm. The 6 mg/kg/day dose gives rat levels

equivalent to (40-80 ng/ml) those of a human smoking 3 packs of 20 cigarettes per day

(Isaac and Rand, 1972). The researchers found that nicotine treated animals had increased

acoustic startle responses compared to untreated. The studies indicated that nicotine

treated rats had increased attention processes, as expected, but this also suggested

anxiogenic effects of nicotine.

The researchers (Acri, 1994; Acri, et al., 1994; Kumari, et al., 1996) further

reported that nicotine elevated sensory gating as measured by prepulse inhibition of the

acoustic startle. Like CRF, nicotine manifests anxiogenic and stress symptoms. The

inference from these studies is that nicotine can be anxiolytic or anxiogenic, depending on

the dose and whether treatment is acute or chronic. For the present study, if nicotine is

anxiogenic, we should expect that acute doses of 0.4 mg/kg or 1.0 mg/kg which give rat

levels equivalent to the 10-40 ng/ml maintained by regular smokers (Balfour and

Fagerström, 1996; Isaac and Rand, 1972), would elavate CRF content in the amygdala and

hypothalamus.

Nicotine and corticosterone

Acute and chronic nicotine have been shown induce increased plasma ACTH and

corticosterone levels (Benwell and Balfour, 1979; Benwell and Balfour, 1982; Cam and

Bassett, 1983; Freund, et al., 1988). The increase is dose dependent and is observed

within 15 minutes and persists for up to 60 minutes after acute treatment and up to two

weeks after chronic treatment (Benwell and Balfour, 1979; Benwell and Balfour, 1982;

Cam and Bassett, 1983; Freund, et al., 1988; Pauly, et al., 1990). Corticosterone and its

metabolites have been reported to decrease excitability in the nervous system and this

effect is through the modulation of the GABA receptor (Harrison and Simmonds, 1984;

Kendall, et al., 1982; Majewska, et al., 1985) and nicotine has been reported to modulate

glutamate activity on the NMDA receptor (Vidal and Changeux, 1993). Caggiula, et al.,

(1993), reported increased tail flick latencies in nicotine treated rats with intact adrenals

compared to adrenalectomized and stressed rats, thus confirming the nociceptive effects of

nicotine and possibly enhancement by corticosterone. The observations led Caggiula, et

al., (1993); Caggiula, et al., (1998) and Pauly, et al., (1988) to conclude that the increase

in corticosterone after nicotine treatment is a way for neuronal systems to cope with the

effects of nicotine.

Nicotine also manifests an increase in corticosterone through the its peripheral

effects on the adrenal cortex, an effect that was reported by (Bugaski, et al., 1998). In

conjunction with studying effects of indomethacin (a cyclooxygynase inhibitor) regarding

nicotine-induced effects on ACTH and corticosterone levels, the researchers confirmed

inhibitory effects of hexamethonium, a peripheral nicotinic receptor inhibitor, on

corticosterone synthesis.

The increase in corticosterone by nicotine administration reflects an increase in the

activation of the HPA axis, as seen in acute and chronic stress. Pretreatment with

corticosterone reduces responsiveness to nicotine and effects of nicotine are manifested

differently in stressed and unstressed rats because stressed rats already have increased

levels of corticosterone (Bullock, et al., 1997; Caggiula, et al., 1993). Balfour, et al.,

(1975); Hall and Morrison, (1973) tested effects of nicotine on stressed and unstressed rats and found that acute nicotine increased corticosterone in unstressed rats but had no effects on the feedback inhibition exerted by high corticosterone levels in stressed rats. These effects were confirmed by the antagonistic effects of metyrapone on adrenal corticosterone synthesis (Balfour, et al., 1975). The findings further confirm that corticosterone has a role in modulating the animal's response to nicotine.

Chronic nicotine causes an up-regulation of nicotinic receptors in the brain (Benwell, et al., 1988; Rowell and Li, 1997; Schwartz and Keller, 1983; Ulrich, et al., 1997) and corticosterone also causes an up-regulation of nicotinic receptors (Pauly, et al., 1990). Corticosterone and nicotine possibly have a synegystic effect in the up-regulation of nicotinic receptors. The upregulated nAChRs are believed to be nonfunctional, possibly because they are maintained at a refractory state, and thus contribute to the tolerance to nicotine (McGehee and Role, 1996). Behavioral and receptor binding tests on mice, by Pauly, et al., (1992) confirmed that chronic levels of corticosterone induce tolerance to nicotine. These studies suggest that this tolerance may also be mediated through corticosterone's up-regulation of nicotinic receptors (Johnson, et al., 1995; Pauly, et al., 1990; Sapolsky, et al., 1990). Conditioned tolerance occurs to some behavioral and endocrine effects of nicotine (Marks, et al., 1983; Sharp and Matta, 1993). Nicotine administration induced a dose dependent tolerance to nicotine's effects on heart rate, respiration rate, locomotor activity, acoustic startle responses, body temperature and seizure threshold (Marks, et al., 1983; Marks, et al., 1985; Hakan and Ksir, 1991). Pauly, et al., (1988) ran behavioral tests including the Y-maze and startle response, and

monitored heart rate and body temperature of normal and adrenalectomized rats. The researchers found that adrenalectomized rats, (with deficient corticosterone) exhibited increased sensitivity to nicotine compared to non-adrenalectomized. The researchers concluded as mentioned above, that the increase in corticosterone is a way of coping with nicotine's effects.

High concentrations of corticosterone in rats were reported to decrease CRF receptor (CRH-R) mRNA in the PVN and BLA confirming the negative feedback inhibition of CRF by corticosterone (Makino, et al., 1995). In this study, Makino, et al., (1995), further demonstrated that chronic stress up-regulated CRF-R mRNA, indicating that the stress input can override the feedback inhibitory effects of corticosterone to up-regulate CRH-R mRNA in the PVN.

<u>Nicotine versus CRF</u>

The discussed literature suggests that nicotine and CRF could influence each other in neuronal systems either directly or indirectly through other neurotransmitters. In their study of possible involvement of interleukin-2 and nitric oxide in CRF release, Raber, et al., (1995), also reported that both acetylcholine and norepinephrine induced the release of CRF from the hypothalamus and amygdala. The acetylcholine effect was antagonized by both atropine and mecamylamine indicating the involvement of both muscarinic and nicotinic receptors. The norepinephrine effect was also antagonized by phentolamine and propranolol suggesting that both α and β adrenergic receptors are involved in stimulation of CRF release in amygdala and hypothalamus.

It is believed that affective disorders are influenced by changes in biogenic amine transmission (Schwartz and Kandel, 1991). Both nicotine and CRF influence neurotransmitters such as norepinephrine and serotonin (5-hydroxytryptamine), suggesting that nicotine exerts its CNS effects by influencing these systems (Calogero, et al., 1989; Fu, et al., 1998). Nicotine craving or nicotine withdrawal are reported to be associated with affective disorders (Balfour and Fagerström, 1996; Fergusson, et al., 1996; Frederick, et al., 1998) while CRF is implicated in affective disorders (De Souza, 1995; Nemeroff, et al., 1991; Owens and Nemeroff, 1993; Owens, et al., 1991). Indeed, behavioral models of anxiety including social interaction, elevated plus maze and fear potentiated startle indicate that CRF is anxiogenic (Dunn and Berridge, 1990; Liang, et al., 1992a; Liang, et al., 1992b; Koob and Bloom, 1985). Some of these anxiogenic reactions can also be observed after nicotine administration (Acri, 1994; Acri, et al., 1994; Kumari, et al., 1996).

Diseases like Alzheimer's and Parkinson's are manifested by a decrease in CRF levels and an up-regulation of CRF receptors, while hypersecretion of CRF results in down regulation of receptors (De Souza, 1995; De Souza, et al., 1985; De Souza, et al., 1986; Nemeroff, et al., 1988). Nicotinic receptors are significantly decreased in the cerebral cortex of patients with Alzheimer's and Parkinson's diseases (Jones, et al., 1992; Kellar, et al., 1987; Vidal, 1996). These observations, would suggest a relationship between nicotinic receptor activation and CRF metabolism, and possibly in the etiology of these diseases (Christensen, et al., 1992). It is conceivable that nicotine used therapeutically for these diseases (Jones, et al., 1992), would also improve CRF levels, but this possibility still needs to be studied.

Like nicotine, CRF administration induces metabolism of brain stem catecholamines. CRF stimulates NE release from LC (Rivier, et al., 1984; Valentino, et al., 1991) but nicotine also increases NE release in hypothalamus and brain stem catecholaminergic regions (Andersson, et al., 1981; Fu, et al., 1997; Fu, et al., 1998; Yoshida, et al., 1980). Nicotine influences NE and has cardiovascular effects, (Grunberg, et al., 1988; Pauly, et al., 1988), and the amygdala which is influenced by CRF and NE also has effects on the cardiovascular system (Davis, 1992; Gray and Bingaman, 1996). With CRF and NE neuronal systems colocalized (Gray and Bingaman, 1996; McDonald, 1996; Van Bockstaele, et al., 1996), the question is whether or not nicotine also modulates CRF, which in turn modulates NE or possibly nicotine influences these neurotransmitters simultaneously. Since NMDA and CRF receptors colocalize, CRF and glutamate influence each other, while nicotine influences glutaminergic systems, and possibly CRF (Gray and Bingaman, 1996).

Both nicotine and CRF exert anorexic effects, and CRF has thermogenic effects (Balfour and Fagerström, 1996; Le Feuvre, et al., 1991; Owens and Nemeroff, 1991; Winders and Grunberg, 1990). The anorexic effects of nicotine have been reported in humans, with smokers reporting weight gain after smoking cessation (Jones, 1987). It has been documented that CRF suppresses food consumption and modulates gastric motility in rats, and CRF levels have been reported to be increased in anorexic patients (De Souza, 1995; Monnikes, et al., 1992; Owens and Nemeroff, 1991). High levels of CRF trigger sympathetic outflow which results in hyperglycemia as a result of activated gluconeogenic pathways, this leads to increased energy utilization and decreased body weight (Brown, et

al., 1982; Heinrichs, et al., 1996). The role of CRF in body weight is consistent with the

fact that the hypothalamus has a vital role in body thermoregulation and food intake (Le

Feuvre, et al., 1991; Leal and Moreira, 1996). Animal studies have shown that nicotine

diminished feeding, increased energy utilization and therefore caused a decrease in body

weight (Grunberg, et al., 1984; Grunberg, et al., 1988; Winders and Grunberg, 1990).

Nicotine has been shown to modulate a number of neurotransmitter systems that

also interact with CRF in the rat brain, including among others: norepinephrine, dopamine

and serotonin. Whether or not nicotine influences CRF in the amygdala has not been

extensively documented except for recent study by (Matta, et al., 1997). It is yet to be

documented whether or not nicotine also modulates CRF levels to achieve its CNS effects.

The effects of nicotine on CRF would certainly depend on dose and the duration of the

treatment and nicotine withdrawal would possibly have a different outcome. From the

discussed literature, it can be assumed that if nicotine is anxiogenic, acute doses would

transiently increase CRF in rat amygdala and hypothalamus or decrease the content if it is

anxiolytic, while causing a decrease in the brain stem. Chronic nicotine on the other hand

would cause increased content of CRF in the hypothalamus and extrahypothalamic

regions. No change would occur if the CNS effects of nicotine do not involve CRF.

CHAPTER III

METHODS

Sample

The sample consisted of 104 male Sprague Dawley rats (Hill Top, Hill Top, PA) aged 2-4 months and weighing 250-450 grams. Rats were kept at animal quarters of WVU Health Sciences Center, with 12 hour dark/light hours, food and water freely available. Controls included untreated rats and saline (vehicle) treated to control for the stress of injection.

Drug Solutions

Nicotine hemisulfate salt ($C_{10} H_{14} N_2 . \frac{1}{2} H_2$, Molecular weight $= 213.3$ grams, Fisher Scientific) approximately 40% weight/volume (w/v) was kindly provided by Drs. W.W. Fleming, and D.A. Taylor, (Pharmacology & Toxicology, WVU Health Sciences Center). Nicotine was prepared in 0.9% saline to 4.7 mM and 2.0 mM solutions and stored in the refrigerator. These nicotine solutions were equivalent to 1.0 mg/ml and 0.4 mg/ml respectively and were injected at 1.0 ml/kg body weight to give doses of 1.0 mg/kg and 0.4 mg/kg.

It has been reported that 2-3 packs/day of 20 cigarettes per pack give a smoker plasma levels of 40-80 ng/ml nicotine and in rat these are equivalent to 3-6 mg/kg/day (Balfour and Fagerström, 1996; Isaac and Rand, 1972). This makes a 20 cigarettes pack equivalent to 1.5 mg/kg in rat. In this study, the 1.0 mg/kg dose was chosen because it has been commonly used by other researchers (Andersson, et al., 1981; Sastry, et al., 1995;

Sharp, et al., 1993; Ulrich, et al., 1997). For example, Sastry, et al., (1995) measured

nicotine in the brain after 1.0 mg/kg and reported that nicotine can be detected in the brain

from 5-50 after intravenous injection. The 0.4 mg/kg dose has also been frequently used

by other researchers (Benwell and Balfour, 1985; Johnson, et al., 1995; Sharp, et al.,

1993), and is half of the 0.8 mg/kg dose which is equivalent to plasma concentrations of

nicotine elicited by smoking one 10 pack of cigarettes, that is, 10-25 ng/ml (Benowitz, et

al., 1988; Isaac and Rand, 1972).

Animal treatment procedure

Rats were treated and sacrificed between the hours of 9.00 am to 12.00 noon to

capitalize on the time before changes influenced by diurnal rhythms (Leal and Moreira,

1996; Owens, et al., 1990). Two doses of nicotine were used for acute treatments; 0.4

mg/kg intraperitoneal (ip) nicotine at 15 and 30 minute treatment, and 1.0 mg/kg (ip) at 15,

30 and 60 minute treatments. Each time treatment was an individual experiment with 12-

24 rats. As indicated on Tables 1 and 2, six animals received 0.4 mg/kg nicotine for 15 or

30 minutes and six to eight animals received 1.0 mg/kg nicotine for 15, 30, or 60 minutes,

respetively.

Animals were weighed and a nicotine or saline solution of 0 .25-0.45 ml was

injected to rats according to their weights. A 5-10 minute interval was allowed to

interspace the injections and facilitate the treatment procedure. Rats were kept at 3-4 per

cage during the procedure. The behavior of nicotine treated rats was observed during the

duration of the treatment. After the duration of a given time point, animals were

Table 1

<u>Sample and dosing for micro-punch dissections</u>

Experiment	n	Time (minutes)	*Nicotine dose (mg/kg)
1	12	15	0.4
2	20	30	0.4

| Total | 32 | | |

----

*Four to six animals received nicotine per time point

Table 2

Sample and dosing for macro-dissections

Experiment	n	Time (minutes)	*Nicotine dose (mg/kg)
3	24	15	1.0
4	24	30	1.0
5	24	60	1.0
Total	72		

----

*Eight animals received nicotine per time point

decapitated and brains carefully removed from cranium, placed in vial with temporal lobe

facing away from the surface, vial capped and immediately frozen in liquid nitrogen. The

brains were kept in dry ice box and later stored at -70°C before dissections. For each rat,

trunk blood was collected into labeled microcentrifuge tubes (immediately after

decapitation) for corticosterone assays. These were kept in ice and later centrifuged at

3000 rpm for 30 minutes using a cold microcentrifuge. Plasma was separated (using a

Pasteur pipette) into a new set of labeled microcentrifuge tubes and then stored at -20°C

until corticosterone radioimmunoassay.

Cryostat frozen sections of the brain

The cryostat-micro-punch procedure was the initial method in this study and was

used only on animals treated with 0.4 mg/kg (ip) nicotine at 15 and 30 minutes. The

cryostat, (Micron, GmbH, HM 505E, Germany, Walldorf.) was provided by Dr. W.T.

Stauber, Physiology Department, WVU and Dr. J. Flores, Biology Department, WVU. A

12 centimeter blade (Leica Instruments) was used for sectioning, with cryostat temperature

maintained at -6°C to-10°C. For each brain, coronal sections of 300 microns were made

(Palma and Brownstein, 1985). The sections were placed onto 10-15 labeled glass slides

and kept frozen in slide box placed in closed dry ice container and later stored at -20°C,

until the micro-punch procedure.

Micro-punch dissections of brain nuclei

The micro-punch dissection method of (Palma and Brownstein, 1985), was used to obtain nuclei from cryostat coronal sections. The amygdaloid nuclei, paraventricular nucleus of the hypothalamus and locus coeruleus/parabrachial nucleus (LCPB) were obtained separately to give three samples for each animal. Micro-punches were accomplished by using a 1 mm or 2 mm curette with slide placed on a glass petri dish placed on dry ice. The rat brain atlas (Paxinos and Watson, 1986) was used to decipher the landmarks; the amygdala distributed on plates 25-32, PVN plates 20-25 and LCPB plates 52-59. Tissue were immediately placed in labeled tubes containing 2.0 ml ice-cold 0.5N HCl solution containing 1.0 mg/ml ascorbic acid and 1.0% Triton X-100, for extraction. Homogenization of samples was accomplished with ultrasonic disruption (sonication) at setting 3 (Branson Sonic Power, Branson instruments Inc. Melville, L.I. NY). Samples were heated at 70°C for 10 minutes to denature enzymes and then stored at -70°C until lyophilizations and protein assays. In this procedure, it is assumed that CRF is spared from denaturation because it is a low molecular weight peptide. The cryostat-micro-punch method had both advantages and disadvantages.

Advantages

1. Good sections are obtained and different nuclei in a region could be accessed with different size curettes.

2. All the three regions per animal could be punched out in one sitting, the sequential order of sections allowed for an ordered access to the specific nuclei.

3. Curettes allowed for a more accurate access to nuclei and less extraneous tissue
    was obtained.

Disadvantages

1. The method is labor intensive, have to give identification numbers and use about
    10 or more glass slides to obtain three to four coronal sections per slide. Only four
    to five brains could be punched out per day because of setting up and micro-
    punching out the three regions which were distributed in four or more slides.
2. If the brain separated from the chuck amidst sectioning, some sections were likely
    lost in the process of readjusting to the appropriate width.
3. If the integrity of sections was compromised, nuclei were not easily accessed,
    therefore loss of CRF could have occurred and thus contribute to the variability in
    the results.
4. Caution needed to be taken as to avoid the formation of mist from the dry ice as
    this turned cloudy and camouflaged the tissue when the mist settled on the glass
    slide.
5. With this method, it took about five weeks or more to obtain the final results
    depending on the number of animals used.

Macro-dissections of brain regions

This procedure of macro-dissection was used on brains of animals treated with 1.0
mg/kg (ip) nicotine at 15, 30 and 60 minutes. This followed the dissection method for

slice experiments, already established in the laboratory. For the amygdala, the temporal lobe was targeted, the whole of the hypothalamus and median eminence are obtained together and the whole of the brain stem was accessed. Dissections were accomplished by placing brain on glass petri dish mounted on ice and using a scalpel. Tissue were immediately placed in labeled tubes containing 3.0 ml ice cold 0.5N HCl solution containing 1.0 mg/ml ascorbic acid and 1.0% Triton X-100, for extraction. Homogenization of samples was accomplished with ultrasonic disruption (sonication) at setting 3 (Branson Sonic Power, Branson instruments Inc. Melville, L.I. NY). Samples were heated at 70°C for 10 minutes to denature enzymes and then stored at -70°C until lyophilizations and protein assays (Clarke and Gillies, 1988; Smith, et al., 1986). The method has advantages and disadvantages.

Advantages

1. The method was fast, 12 brains (three regions each) could be dissected, and homogenized in one day.

2. The sequential removal of unwanted tissue resulted in easy access to the regions of interest.

3. There was little likelihood of living any nuclei out.

Disadvantages

1. The method allowed for extraneous tissue to be obtained which made samples need to be more dilute (3.0 ml instead of 2.0 ml used with nuclei).

2.   For the amygdala, the whole of the temporal lobe was accessed, and CRF levels

would reflect levels from neighboring areas including piriform cortex .

3.   The median eminence was obtained and this would augment hypothalamic CRF to

about 10 times the levels in PVN (Chappell, et al., 1986).

4.   Assaying the whole brain stem did not only involve the PBN and LC but also the

other nuclei including, nucleus tractus solitarius, raphe nuclei, dorsal vagal

complex and Barrington's nucleus, nuclei which also contain CRF.

5.   With brain stem, the increased amount of tissue in some samples reduced the

available supernatant after micro-centrifugation. Extreme care was needed to

minimize agitation of supernatant.

6.   Overall, results from the two methods; micro- and macro-dissections could not be

compared, because two variables were different, the dose and dissection method.

Lyophilization of acidic extracts

Each animal had three brain regions to be assayed for both radioimmunoassays

(RIA) and Lowry protein assays. The two different dissection methods produced two sets

of samples brain region and nuclei (Table 3). For each animal and per brain region, a 500

μl HCl extract was microcentrifuged using the Eppendorf table-top microcentrifuge at

14,000 rpm for 30 minutes. Parallel to samples, 500 μl of 0.5N HCl was also

microcentrifuged to be used in RIA buffer matrix. A 300 μl aliquot of supernatant was

transferred to another labeled microcentrifuge tube. The 300 μl samples and HCL were

lyophilized (freeze-dried in vacuum) using Savant Speed Vac concentrator, for three to

Table 3

Assayed brain regions and nuclei

Micro-punch dissections (nuclei)	Macro-dissections (brain region)
Amygdaloid nuclei	Amygdala
Paraventricular nucleus	Hypothalamus
Locus coeruleus/parabrachial nucleus	Brain stem

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four hours with heat or overnight without heat. The lyophilate was stored at - 70°C until

radioimmunoassay for CRF. For the assay, acidic lyophilized extracts were resuspended

in 1.5 ml RIA buffer (30 mM phosphate buffer with 25 mM EDTA, containing 0.1 ml

Triton X-100 and 0.02 mg/ml aprotinin). A 300 μl aliquot of this solution was used for

the radioimmunoassay of CRF.

Radioimmunoassays

Radioimmunoassay (RIA) is the measurement of concentration of antigen (e.g.

hormone) by determining the extent to which it combines with antibody. A limited

amount of antibody is reacted with the corresponding hormone (CRF, corticosterone)

labeled with a radioisotope. Upon addition of an increasing amount of hormone, a

corresponding decrease in amount of labeled hormone is bound to the antibody. The

secondary antibody recognizes and binds the antibody bound CRF or corticosterone. After

separation of bound and unbound labeled hormone, the amount of radioactivity in the

bound fraction is calculated and used to construct a standard curve against which

concentrations of unknown samples are measured. Non specific binding (NSB) is

determined in the absence of antibody.

Corticotropin releasing factor (CRF) radioimmunoassays

The method of Vale, et al., (1983), was used for the RIA for CRF. RIA on

lyophilized 300 μl of CRF extracts (per brain region) were done with a C-terminal

directed rabbit anti-human/rat CRF antibody, RC 70, (provided by Dr. Wylie Vale, Salk

Institute, San Diego, CA) and ^{125}I-iodohistidyl-CRF (Amersham, Arlington Heights, IL).

After treatment with secondary antibody, (anti-rabbit gamma globulin), the antibody-CRF

complex was precipitated using 2% polyethylene glycol (Sigma, St Louis, MO). Amount

of radioactivity in the complex (pellet) was counted for two minutes on the gamma counter

(Wallac-1470 Wizard automatic gamma counter, Wallac Inc, Gaithersburg MD).

Procedure

Day 1: RIA buffer (30 mM phosphate buffer with 25 mM EDTA, containing 0.1

ml Triton X-100 and 0.02 mg/ml aprotinin). A lyophilized CRF standard of 200,000

pg/tube stored at -70°C freezer was reconstituted fresh on day of use, with 1 ml of RIA

buffer to make tube #A_1. Primary antibody was rabbit anti-rat CRF, 50 μl aliquots were

stored in Fisherbrand microcentrifuge tubes at -70°C. The 50 μl aliquot of antibody

(1:250) was combined with 450 μl RIA buffer. Rabbit serum was stored in -20°C freezer

in 300 μl aliquots and primary antibody (Ab) cocktail consisted of RIA buffer, rabbit

serum and the antibody, at a final concentration of 1:500,000. Buffer matrix was made of

lyophilized HCL resuspended in 1.5 ml RIA buffer and pooled together.

Standards were prepared in labeled Sarstedt polypropylene (75 x 12 mm) tubes by

serial dilution of tube #A_1 with RIA buffer and concentrations of standard ranged from

0-25 pg/μl. A volume of 20 μl of the standard was injected into appropriate tubes. The

zero CRF standard was done in quintuplicate and the rest were done in triplicates. An

aliquot of 300 μl of buffer matrix was mixed with an appropriate standard (20 μl) in a

standard polypropylene tubes. Two nonspecific binding tubes were prepared, in which the

standards and primary antibody were replaced by an equivalent amount of RIA buffer.

The lyophilized acidic samples and HCL extracts were thawed, reconstituted with 1.5 ml RIA buffer, vortexed and then microcentrifuged for approximately two minutes. A 300 μl aliquot of diluted lyophilate was injected into three labeled tubes (for triplicates) and 20 μl sample matrix was added to maintain a volume equal to standards. A volume of 100 μl of primary antibody cocktail final concentration, 1:500,000 was added to standards and samples (except nonspecific binding tubes). The tubes were shaken gently to mix, covered with parafilm and then incubated at 4°C for at least 24 hours.

Day 2. The lyophilized label (^{125}Iodohistidyl-CRF, Amersham) was diluted (on day of arrival) with 200 μl RIA buffer containing 0.1% acetic acid and stored at -70°C in 10 μl aliquots (0.5 μCi). ^{125}I-CRF (10 μl) was reconstituted with RIA buffer in plastic beaker for 100 μl/tube (on day of use). The activity of ^{125}I-CRF solution was measured on the gamma counter before addition to assay. When fresh, a 10μl aliquot diluted to 12 ml RIA buffer yielded approximately 10,000-12,000 cpm/100 μl (counts per minute). Adjustments were made to buffer volumes according to age of label. 100 μl of ^{125}I-CRF solution (>10,000 cpm/tube) was added to all the tubes, after which the tubes were shaken gently, covered with parafilm and further incubated for at least 20-24 hours at 4°C (refrigerator).

Day 3. Precipitating solution was composed of polyethylene glycol (PEG, (1.45 g/10 ml buffer) secondary antibody and made fresh on day of use. Secondary antibody (sheep anti-rabbit IgG) stored in 2.0 ml aliquots in -20°C freezer was diluted 1:4 with RIA buffer. A volume of 100 μl of secondary antibody was added to each tube, after which

100 μl of PEG solution (final concentration 2%) was added and the contents vortexed.

The tubes were covered and incubated for 20 minutes at room temperature for

precipitation, and then centrifuged at 4000 g for 20 min (5,000 rpm, on Sorvall centrifuge).

The supernatant which contained unbound radioactive CRF was aspirated and

radioactivity of pellets counted for two minutes on the gamma counter (Wallac-1470

Wizard automatic gamma counter, Wallac Inc, Gaithersburg MD)

<div align="center">Corticosterone radioimmunoassays</div>

The ImmuChem^{TM} double antibody ^{125}I corticosterone RIA kit for rat and mice was

used to assay plasma samples for all tested rats. The kit was stored in a 4°C refrigerator

until day of use. The contents of the kit were as follows:

a. Steroid diluent, a phosphate gelatin buffer (ph 7.0 ± 0.1) containing rabbit gamma
    globulin.

b   Anticorticosterone (corticosterone-3-carboxymethyloxamine) developed using bovine
    serum albumin (BSA) as the antigen.

c. Six corticosterone calibrators (standards) with concentrations ranging from 25-1000
    ng/ml.

d. Precipitating solution was a mixture of polyethylene glycol (PEG) and goat anti rabbit
    gamma globulins contained in TRIS buffer.

e. The corticosterone ^{125}I derivative, a 0.2 ml aliquot of this radioactive derivative would
    provide approximately 50,000 cpm at 75% counter efficiency, on day of shipment.

f.   Prediluted lyophilized form of rat corticosterone controls (0.1 ml each). The high

control had corticosterone levels of 517-777 ng/ml and the low control had 78-112

ng/ml.

Procedure

All contents of the kit were brought to room temperature before use.   Rat plasma

sample was diluted 1:200, by adding 10 μl plasma to 2.0 ml steroid diluent, the contents

were mixed using a vortex mixer and 100 μl was assayed. For assay, glass test tubes of

size 12 x 75 mm were used and samples were assayed in triplicates. The procedure was

followed as directed in the kit. The tubes were mixed thoroughly and centrifuged in

Sorval centrifuge, at 2300 to 2500 rpm (1000 g) for 15 minutes. The supernatant

containing unbound radioactivity, was aspirated and the precipitate was counted in the

Gamma counter (Wallac-1470 Wizard automatic gamma counter, Wallac Inc,

Gaithersburg MD) for two minutes.

<div align="center">Protein determination on acidic extracts</div>

The Lowry protein assay method was employed to measure protein levels in all six

sets of acidic samples (Table 3); for use in the determination of CRF content relative to the

protein obtained from macro-dissections and micro-punches. The Lowry protein assay is

based on interaction of proteins with the phenol reagent and copper under alkaline

conditions. The blue color reaction arises from a copper catalyzed oxidation of aromatic

amino acids and other groups by the heteropolyphosphate reagent (Lowry, et al., 1951;

Pomeranz and Meloan, 1987). The intensity of the blue color is not strictly proportional to

the amount of protein in the sample, and the assay has a narrow linear range of between 10 to 70 μg protein. The method was appropriate for this study though, because samples were of similar type, from same brain regions and were highly dilute. Assuming that most tissue is about 95% water and 5% protein, levels would be approximately 5μg protein per milligram tissue weight. The brain would contain lower levels of protein because of its high lipid content.

Procedure

An aliquot of the HCl homogenate of brain region samples was placed in 12 x 75 glass tube and then combined with 1N NaOH to make a total volume of 500 μl. The tubes were sealed and refrigerated until the protein assay. On day of assay, Bovine serum albumin (BSA) standard (1.0 mg/ml) was prepared using distilled water. Standard solutions of 0, 5, 10, 20, 30, 50, 60, 80 and 100 μl (0-100μg) were aliquoted into 13 x 100 mm glass tubes. The volume was made up to 100 μl using 1N (NaOH). Standards and samples were run in triplicates. For assay, a 50 μl or 100 μl aliquot of alkaline sample was pipetted into 13 x 100 mm glass tube and the volume made up to 100 μl with 1N NaOH.

The Lowry reagents were 2% sodium carbonate (Na_2CO_3), 1 % copper sulfate ($CuSO_4$), 2% sodium tartrate and 2N Folin phenol reagent. The blue color was read on spectrophotometer at 750 nanometers, with distilled water used as reference blank.

CHAPTER IV

DATA AND ANALYSIS

<u>Treatment of data</u>

Radioimmunoassay results (gamma counts) for both CRF and corticosterone were

coded into a Microsoft Excel spreadsheet. Since all standards and samples were run in

triplicates, counts from the blank were subtracted from all standard or sample counts and

an average of triplicates was obtained. A standard curve was constructed and regression

analysis used to calculate concentrations. Percent binding was related to counts per

sample, divided by total counts added and at low amounts of CRF, there was high binding

of radioligand, that is, more counts, and vice versa. The ratio of binding per sample to the

zero level (B/B_0) was obtained and a plot against the log of standard CRF concentrations

gives sigmoidal curve. The curve was converted to a straight line by plotting logit (natural

log) $B/B_0/1-B/B_0$ against the log of standard CRF concentrations, after which the

concentration of the standard and samples were obtained. To obtain CRF content in brain

regions, all dilutions employed, from dissections to radioimmunoassays, were accounted

for. Taking into consideration that 2.0 ml or 3.0 ml HCL was the original dilution, with a

300 μl aliquot lyophilized, resuspended in 1.5 ml RIA buffer and 300 μl assayed, the final

dilution factor was 35 or 50 respectively.

Protein assays were run on all the brain regions for both doses and treatment time

points.   Absorbance values were coded into a Microsoft Excel spreadsheet. Results from

the blank were subtracted from all standard or sample counts and an average of triplicates

was obtained. A standard curve was constructed and regression analysis used to calculate

concentrations. To obtain the original amount of protein in each sample, the protein in the assayed 50 μl or 100 μl was converted to the 1:500 aliquot and finally to content in 3.0 ml or 2.0 ml of the acidic sample. For each brain region and treatment, protein levels were grouped together and average protein obtained per brain region/nucleus was calculated, for all time points, to indicate the average amount of protein obtained with dissections. However, individual protein values per sample were used to calculate concentration in picograms of CRF per milligram of protein (pg CRF/mg protein) for the corresponding sample. For example, rat number one after treatment for fifteen minutes with 0.4 mg/kg nicotine had three samples; amygdaloid nuclei, paraventricular nucleus and brain stem nuclei. The protein value from the each brain-region-sample was used to calculated CRF content, that is, amygdaloid samples (CRF and protein) for rat #1 in the 0.4 mg/kg group were used correspondingly.

An overall mean protein concentration (pg CRF/mg) of the untreated groups per brain region was used for comparisons with means of nicotine and saline treated rats. A factor was calculated to normalize individual experiments to an overall mean of the untreated group. This was achieved by grouping together pg CRF/mg protein for untreated rats, per brain region, at all treatment time points, after which gross mean was calculated. For example, CRF levels for amygdaloid nuclei of untreated rats at 15 and 30 minutes time points were grouped and their gross mean calculated. After calculating mean CRF levels of untreated rats per treatment time point, the gross mean was divided by the mean for untreated rats for each time point to get the factor for normalizing individual experiments. This factor was used to multiply individual values of pg CRF/mg protein.

Final concentration values were grouped according to treatments and then used for

statistical comparisons. The outlier test was used for individual experiments to discard

any value $\leq$ x -2SD or $\geq$ x +2SD.

Statistics

Data was analyzed using the Microsoft Excel spreadsheet and GB-Stat statistical package.

Descriptive statistics (mean, SD and SE), two way (time, treatment) analysis of variance

(ANOVA) and post-hoc comparison by Tukey's t-tests were used to test for statistical

significance. Hypotheses were tested using the critical probability values of alpha at $\underline{p} \leq$

0.05 as significant.

CHAPTER V

RESULTS

Sources of error

Errors could have arisen from a number of stages in the procedure of this study including: storage, sectioning, dissections, pipetting and individuality of animals. On the cryostat, technical errors led to minor losses due to deformities in some sections and micro-punch dissections depended mainly on the integrity of the sections. Macro-dissections were possibly not completely uniform and this could have led to an unevenness in amount of tissue obtained thus causing a variation in amount of CRF relative to protein. Pipetting errors in all various stages of the experiment including lyophilization and radioimmunoassays could introduce variability in results. Over-aspiration of radioactive supernatant could have led to minute losses of pellet with loss of radioactive counts, and under-aspiration could leave droplets of radioactive material, leading to an over-estimation of counts. Another source of error could arise from the protein assay where pipetting could introduce errors that could over- or under-estimate protein levels. In corticosterone assays, errors could have arisen mainly from pipetting and aspiration of the radioactive material. Control rats, being not under the influence of the stress of injection could have had enhanced variability due to the individuality of the animals.

Animal observations

Nicotine treated rats exhibited tremors, fast breathing and mild convulsions a minute after the injection. They also showed paralysis of the hind limbs and decreased

locomotion which agrees with previous observations that high doses of nicotine decrease locomotion in drug naive rats (Armitage, et al., 1969; Hakan and Ksir, 1991). On the separation of plasma, it was noted that some blood samples had lysed. Lysis does not affect the corticosterone levels, and use of either plasma or serum exhibits no variation in corticosterone levels. Generally, for micro-punch dissections (brain nuclei) CRF levels for untreated groups were comparable at all time points. Similarly, saline treated rats showed no significant differences at the two treatment time points. The same pattern was observed in macro-dissections for 15 and 30 minutes time points, but for the brain stem, the 60 minute time point had considerable high levels of CRF compared to 15 and 30 minutes and this may have been due to inconsistences in decapitations and dissections.

Expected results

Studies on nicotine pharmacokinetics indicated that nicotine accumulates in the rat brain within five minutes after intravenous injection (Sastry, et al., 1995; Schmiterlöw, et al., 1967). Nicotine peaks at about 20 minutes, with levels remaining high for up to 50 minutes but declined by 60 minutes. In this study, it was assumed that the response of CRF after 15 minutes treatment would correspond to the peaking of nicotine in the brain while the 60 minutes response would correspond to the relatively low levels. For the present study, if nicotine is anxiogenic, we should expect that acute doses of 0.4 mg/kg or 1.0 mg/kg would elavate CRF content in the amygdala and hypothalamus, while decreasing CRF, if nicotine is anxiolytic or antidepressive. Corticosterone levels were utilized as positive control for nicotine effects. Results are presented such that

corticosterone is addressed first. Even though microdissections were the initial method used, whole regions: hypothalamus, amygdala and brain stem are addressed before their nuclei.

<u>Plasma corticosterone</u>

Figure 1 shows plasma corticosterone levels after 15 and 30 minutes treatment with 0.4 mg/kg nicotine. Results are expressed in nanograms/milliliter (ng/ml), mean ± S.E.M per treatment. After 15 minutes treatment, mean corticosterone levels were 368 ± 32, 291 ± 79 and 178 ± 71 for nicotine, saline and untreated respectively. The thirty minute time point exhibited values of 473± 39, 174 ± 39 and 199 ± 53 for nicotine, saline and untreated rats respectively. Fifteen minutes treatment with nicotine produced significant effects compared to no treatment and 30 minutes nicotine produced significant effects compared to both saline treated and untreated rats.

Shown on Figure 2 are serum corticosterone levels 15, 30 and 60 min after treatment of rats with 1.0 mg/kg nicotine. Results are expressed in ng/ml, mean ± S.E.M per treatment. After 15 minutes treatment, mean corticosterone levels were 364 ± 45, 220 ± 36 and 111 ± 24 for nicotine, saline and untreated respectively. The 30 minute time point exhibited values of 216 ± 28, 134 ± 26 and 111 ± 24 for nicotine, saline and untreated rats respectively. After 60 minutes treatment, mean corticosterone values of 306 ± 65, 83 ± 31 and 111 ± 24 for nicotine, saline and untreated rats respectively were observed. Significant effects of nicotine were observed at 15, 30 and 60 minutes versus untreated rats and at 15 and 60 minutes compared to saline treated rats.
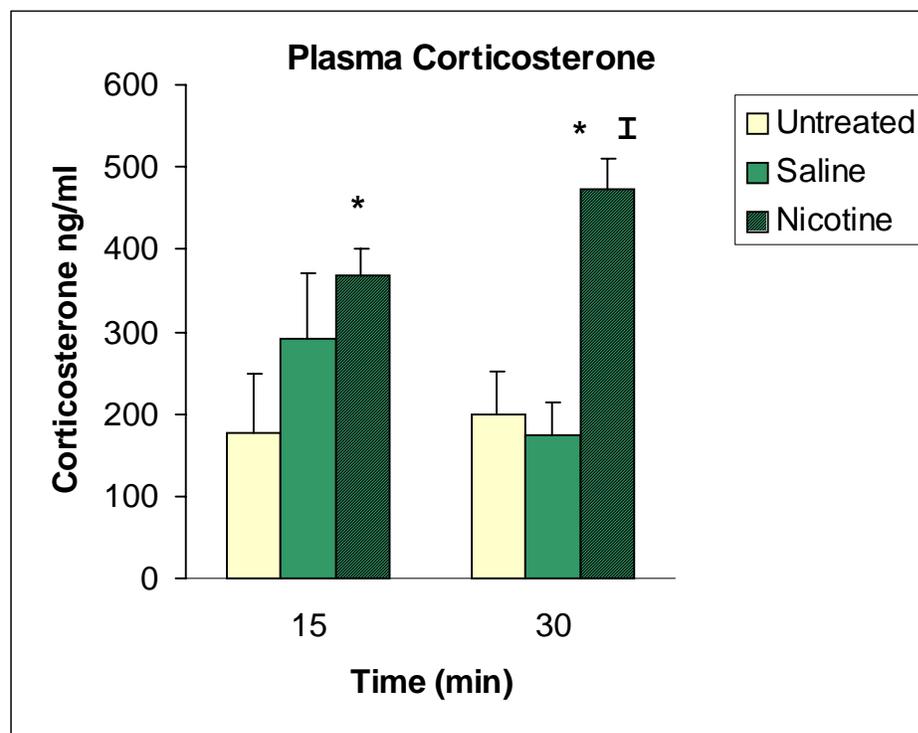
Figure 1. Plasma corticosterone levels after 15 and 30 minutes treatment with nicotine

0.4 mg/kg, 0.9% saline (vehicle 1.0 ml/kg) or no treatment: 15 minutes nicotine (n = 4),

saline (n = 4) and untreated (n =4), 30 minutes nicotine (n = 8), saline (n =8) and untreated

(n = 8). Values are expressed in ng/ml, mean ± S.E.M per treatment. *denotes significant

effect of nicotine versus sham and ‡ denotes significant effects of nicotine versus saline, P

≤ 0.05, ANOVA and Tukey's t test. Overall treatment effect F = 10.8 (2,34) p = 0.0003.
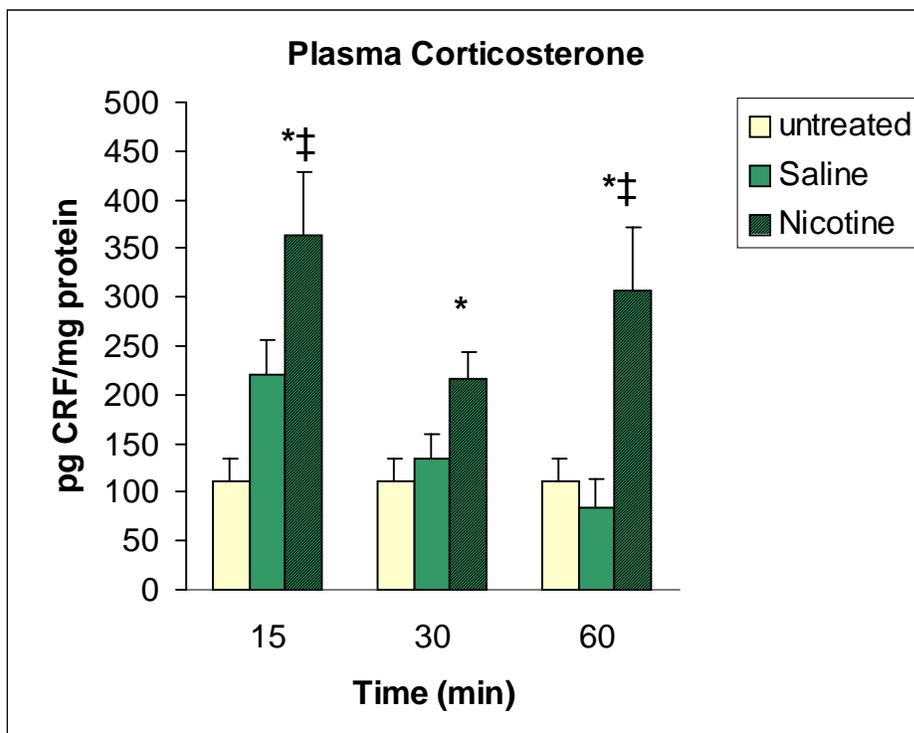
Figure 2. Plasma corticosterone levels after 15, 30 and 60 minutes treatment with nicotine 1.0 mg/kg, 0.9% saline (vehicle, 1.0 ml/kg) or no treatment: 15 minutes nicotine (n = 6), saline (n = 8) and untreated (n = 6), 30 minutes nicotine (n = 6), saline (n = 6) and untreated (n = 7), and 60 minutes nicotine (n = 4), saline (n = 4) and untreated (n = 7). Values are expressed in ng/ml, mean ± S.E.M per treatment. *denotes significant effect of nicotine versus sham and ‡ significant effects of nicotine versus saline, $P \leq 0.05$, ANOVA and Tukey's t test. Overall treatment effect at 1.0 mg/kg nicotine $F = 8.6$ $(2,53)$ $p = 0.0007$. Overall time effect at 1.0 mg/kg nicotine $F = 3.27$ $(2,53)$ $p = 0.05$.

<u>Summary of protein levels for all experimental rats</u>

Lowry protein assay results for each dissection and all time points were summarized to indicate the average of amount of protein obtained in dissections. Individual protein values were used to convert corresponding values of CRF to picograms CRF per milligram protein. Values are expressed in milligrams (mg), mean $\pm$ S.E.M.   A summary of protein levels from micro-punch dissections on animals treated with 0.4 mg/kg nicotine for 15 and 30 minutes indicated that in amygdaloid nuclei, mean levels of 3.16 ± 0.16 mg protein were obtained. The paraventricular nucleus and locus coeruleus/parabrachial nucleus had mean protein levels of 1.53 ± 0.08 mg and 1.89 ± 0.14 mg respectively. For macro-dissections on animals treated with 1.0 mg/kg nicotine for 15, 30 and 60 minutes, brain stem samples contained more protein (mean $= 61.12 \pm 2.08$ mg) compared to hypothalamus and amygdala with means levels of 21.10 ± 0.59 mg and 36.27 ± 0.92 mg respectively.

<u>CRF content in the rat brain after nicotine treatment</u>

<u>CRF content in untreated rats</u>

Figure 3 is a summary of CRF content in brain regions of untreated rats for both micro- and macro-dissections at all tested time points. Values are expressed in pg CRF/mg protein (mean $\pm$ S.E.M). CRF levels were higher in micro-punch dissections compared to macro-dissections and this was to be expected, because micro-punches were more precise at targeting areas where CRF is more concentrated. Comparison of mean pg CRF/mg protein in micro-punches of all the three brain nuclei revealed that in micro-

CRF content in untreated rats
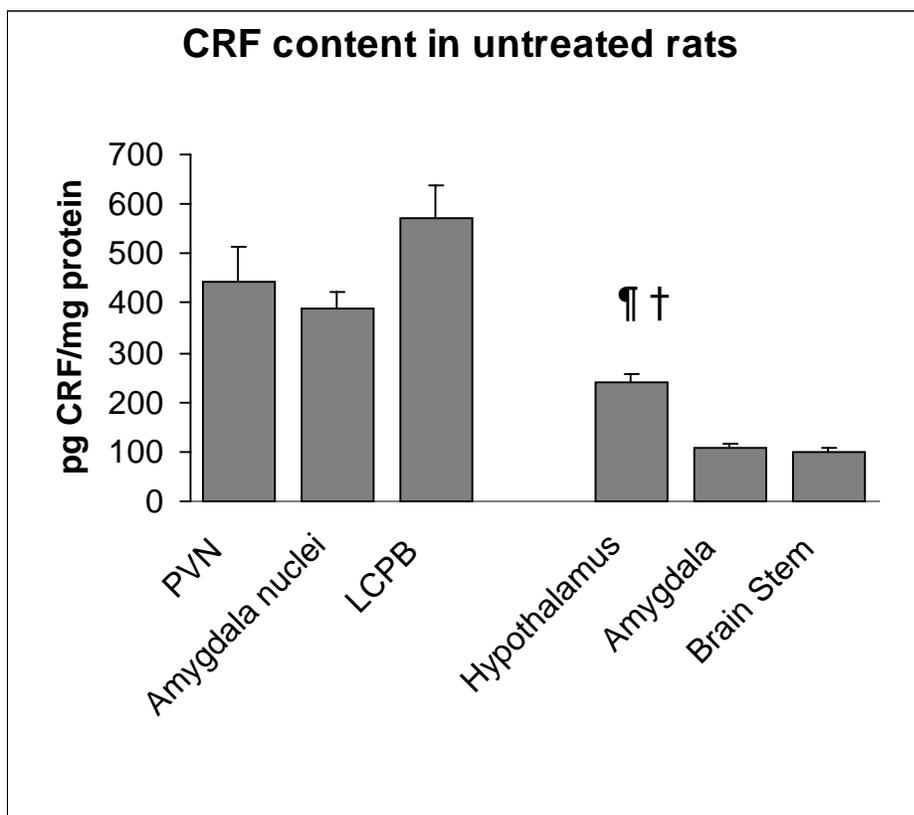
Figure 3. CRF content (pg CRF/mg protein) in untreated rats. Comparison of mean CRF content in: (1) micro-punches (0.4 mg/kg treatments) of all the three brain nuclei, paraventricular nucleus (PVN), amygdaloid nuclei and locus coeruleus/parabrachial nucleus (LCPB) and (2) macro-dissections (1.0 mg/kg treatments) of three brain regions, hypothalamus, amygdala and brain stem of untreated rats. Values are expressed in pg CRF/mg protein, mean ± S.E.M per treatment. ¶ Denotes significant differences between hypothalamus versus amygdala and † denotes significant differences between hypothalamus versus brain stem.

punches, the locus coeruleus/parabrachial nucleus region had generally higher levels of CRF compared to PVN and amygdaloid nuclei, although this was not statistically significant. These results are agreeable with those reported by Chappell, et al., (1986), in that study, controls had CRF levels of approximately 500 pg/mg, 600 pg/mg and 800 pg/mg for PVN, amygdala and brain stem respectively. In macro-dissections, the hypothalamus had significantly increased levels compared to amygdala and brain stem. This is consistent with the fact that the median eminence contains the highest levels of the neuropeptide (Chappell, et al., 1986; Palkovits, et al., 1985).

CRF content in hypothalamus and paraventricular nucleus (PVN)

It was hypothesized that there would be significant increases in hypothalamic CRF levels in nicotine treated rats compared to untreated and saline treated rats, at a given dose and time point. Figure 4 shows CRF content in macro-dissections of rat hypothalamus after 15, 30, and 60 treatment with 1.0 mg/kg nicotine. Values are expressed in pg CRF/mg protein, mean ± S.E.M per treatment. Mean CRF content for untreated rats from the three time points was 240 ±16 and this was compared to means of nicotine and saline treatments. At the 15 minute time point, mean CRF levels were 338 ± 50 and 290 ± 20 for nicotine and saline rats respectively. After 30 minutes, mean CRF levels were 231 ± 30 and 236 ± 21 for nicotine and saline rats respectively. Sixty minutes of treatment exhibited mean CRF levels of 252 ± 24 and 289 ± 32 for nicotine and saline respectively. CRF levels were significantly higher in rats treated with nicotine for 15 minutes compared to untreated.
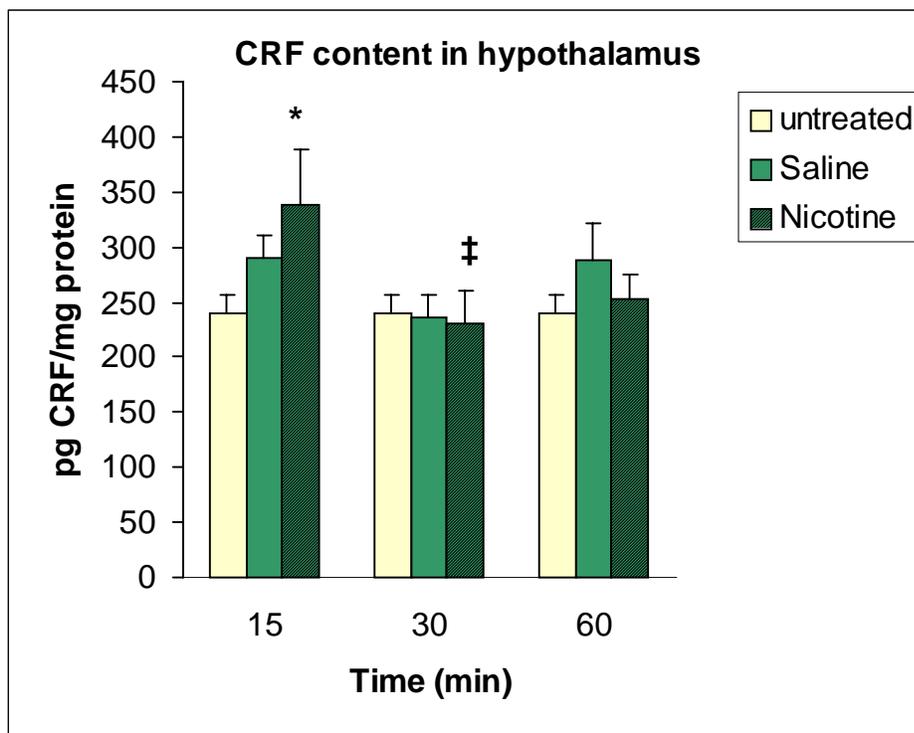
Figure 4. CRF content in macro-dissections of hypothalamus after 15, 30 and 60 minutes

treatment with nicotine 1.0 mg/kg, 0.9% saline (vehicle, 1.0 ml/kg) or no treatment: 15

minutes nicotine (n = 8), saline (n = 8), 30 minutes nicotine (n = 8), saline (n = 8), 60

minutes nicotine (n = 7), saline (n = 7) and total untreated (n = 17). Values are expressed

in pg CRF/mg protein, mean ± S.E.M per treatment. *Denotes significant effects of

nicotine versus untreated rats at 15 minutes treatment, and ‡ significant time effects for

nicotine 15 versus 30 minutes, P ≤ 0.05 ANOVA and Tukey's t-test. The overall

treatment effects at 1.0 mg/kg nicotine were F = (2, 96) p = 0.039.

No significant effects were observed between saline and nicotine treated rats. Significant time effects were seen between 15 minutes and 30 minutes nicotine treated groups.

A summary of CRF content in micro-punches of PVN after 15 and 30 min treatment with 0.4 mg/kg nicotine is shown on Figure 5. Values are expressed in pg CRF/mg protein, mean ± S.E.M per treatment. Mean CRF content for untreated rats from both time points was 445 ± 70 and this was compared to means of nicotine and saline treatments. After 15 minutes treatment, mean CRF levels were 447 ± 51 and 495 ±26 for nicotine and saline respectively. The thirty minute time point exhibited values of 531 ± 72 for nicotine and 568 ± 69 for saline treated rats. No significant effects were observed between nicotine versus saline versus untreated group and there were no significant time effects.

CRF content in amygdala and amygdaloid nuclei

The hypothesis was that there would be significant increases in amygdaloid CRF levels in nicotine treated rats compared to untreated and saline treated rats, at a given dose and time point. Figure 6 summarizes the CRF content in macro-dissections of rat amygdala after 15, 30, and 60 treatment with 1.0 mg/kg nicotine. Values are expressed in pg CRF/mg protein, mean ± S.E.M per treatment. As the figure shows, mean CRF content for untreated rats from the three time points was 106 ± 10. At the 15 minute time point, mean CRF levels were 128 ± 15 and 115 ± 9 for nicotine and saline rats respectively. After 30 minutes, mean CRF levels were 133 ± 16 and 97 ± 4 for nicotine and saline rats respectively.
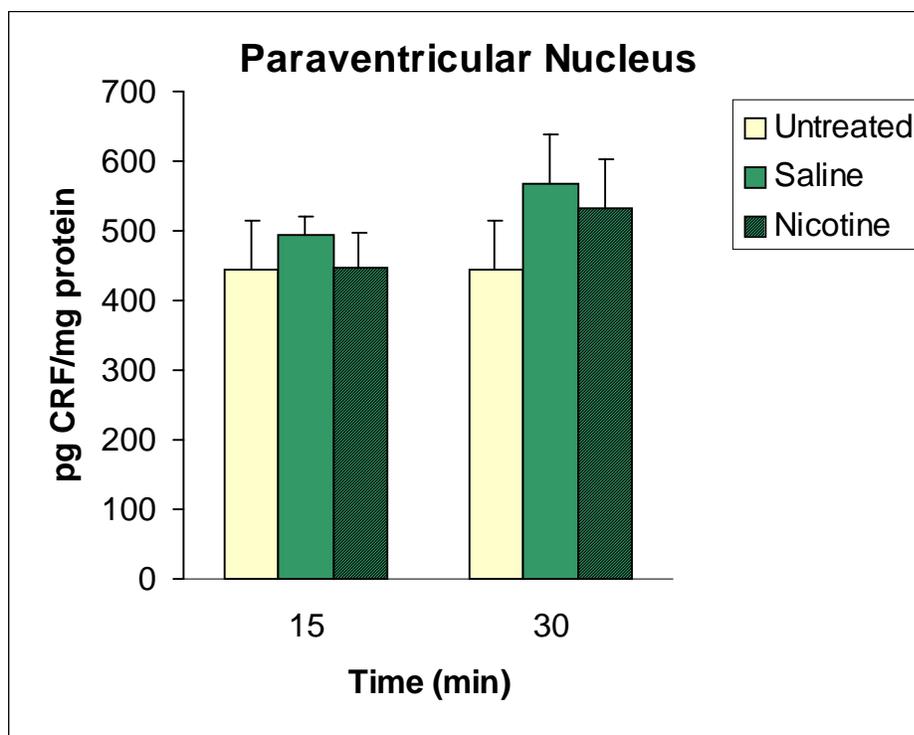
Figure 5. CRF content in micro-punches of paraventricular nucleus of the hypothalamus

after 15 and 30 minutes treatment with nicotine 0.4 mg/kg, 0.9% saline (vehicle 1.0 ml/kg)

or untreated: 15 minutes nicotine (n = 4), saline (n = 4), 30 minutes nicotine (n = 8), saline

(n = 7) and total untreated group (n = 8). Values are expressed in pg CRF/mg protein,

mean ± S.E.M per treatment.
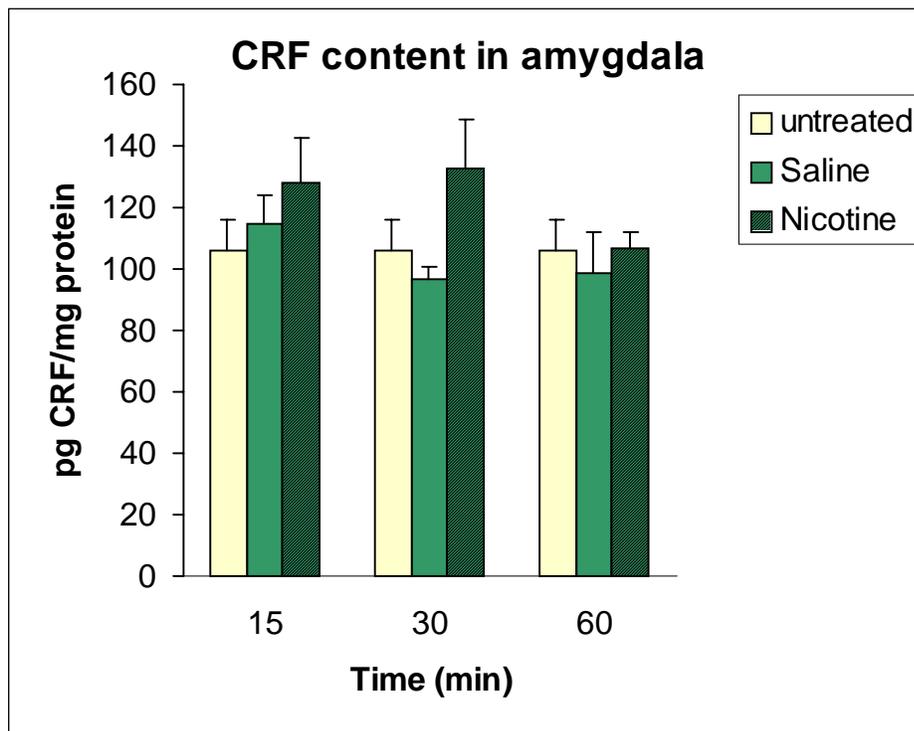
Figure 6. CRF content in macro-dissections of amygdala after 15, 30 and 60 minutes treatment with nicotine 1.0 mg/kg, 0.9% saline (vehicle, 1.0 ml/kg) or no treatment: 15 minutes nicotine (n = 8), saline (n = 8), 30 minutes nicotine (n = 8), saline (n =7), 60 minutes nicotine (n = 7), saline (n = 7) and total untreated (n = 23). Values are expressed in pg CRF/mg protein, mean ± S.E.M per treatment.

Sixty minutes of treatment exhibited mean CRF levels of 106 ± 10 and 99 ± 13 for

nicotine and saline respectively. No significant treatment and time effects were observed

between nicotine versus saline versus untreated group at all time points.

Shown on Figure 7 is CRF content in micro-punches of amygdaloid nuclei after 15

and 30 min treatment with 0.4 mg/kg nicotine. Values are expressed in pg CRF/mg

protein, mean $\pm$ S.E.M per treatment. Untreated groups from both time points had a mean

of 390 ± 32. After 15 minutes treatment mean CRF levels were 471 ± 80 and 417 ± 68

for nicotine and saline respectively. The thirty minute time point exhibited values of 331

± 25 for nicotine and 362 ± 29 for saline treated rats. No significant treatment effects were

observed between nicotine versus saline versus untreated group, at all time points.

However, the Tukey's t-test indicated that significant time effects occurred in the nicotine

treated group in that, when compared to the 15 minutes group, the 30 minutes nicotine

treated rats had significantly decreased levels of CRF in the amygdala.

CRF content in brain stem and the locus coeruleus/parabrachial nucleus

It was hypothesized that there would be significant decreases in brain stem CRF

levels in nicotine treated rats compared to untreated and saline treated rats, at a given dose

and time point. Figure 8 shows a summary of CRF content in macro-dissections of rat

brain stem after 15, 30, and 60 treatment with 1.0 mg/kg nicotine. Values are expressed in

pg CRF/mg protein, mean $\pm$ S.E.M per treatment. Mean CRF levels of untreated rats from

both time points were 101 ± 7. After 15 minutes treatment, mean CRF levels were $100 \pm$

10 and 117 ± 5 for nicotine and saline treated rats respectively.
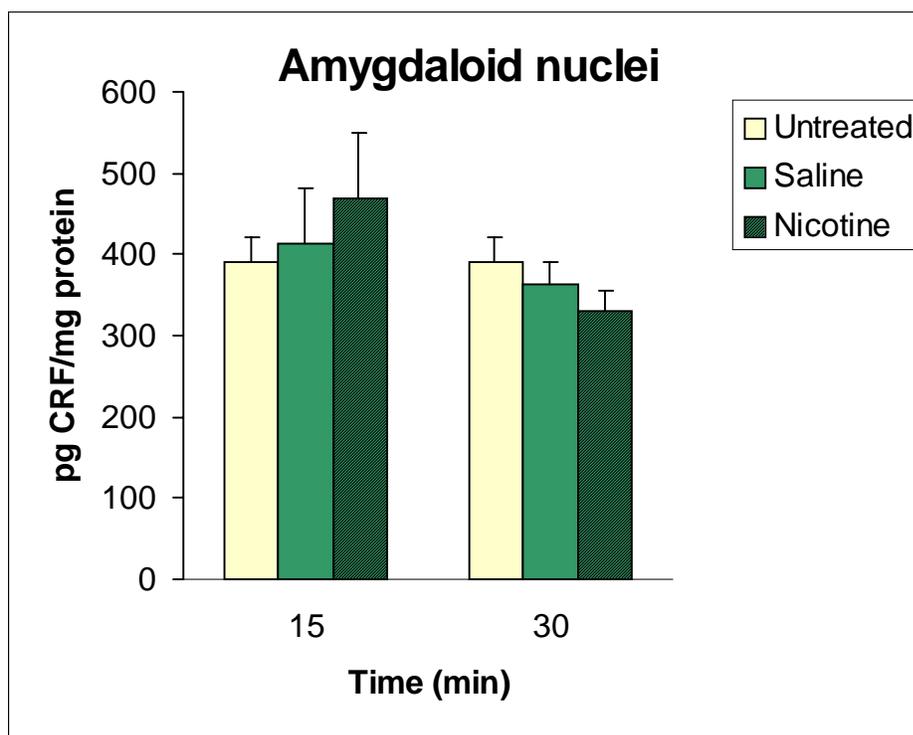
Figure 7. CRF content in micro-punches of amygdaloid nuclei after 15 and 30 minutes

treatment with nicotine 0.4 mg/kg, 0.9% saline (vehicle 1.0 ml/kg) or no treatment: 15

minutes nicotine (n = 4), saline (n =48), 30 minutes nicotine (n = 8), saline (n = 7) and

total untreated group (n = 8). Values are expressed in pg CRF/mg protein, mean ± S.E.M

per treatment. *Denotes significant effects of treatment time for 15 minutes nicotine

versus 30 minutes, $p \leq 0.05$ ANOVA and Tukey's t-test. Overall treatment effects at 0.4

mg/kg F = 3.6 (1,36) p = 0.069.
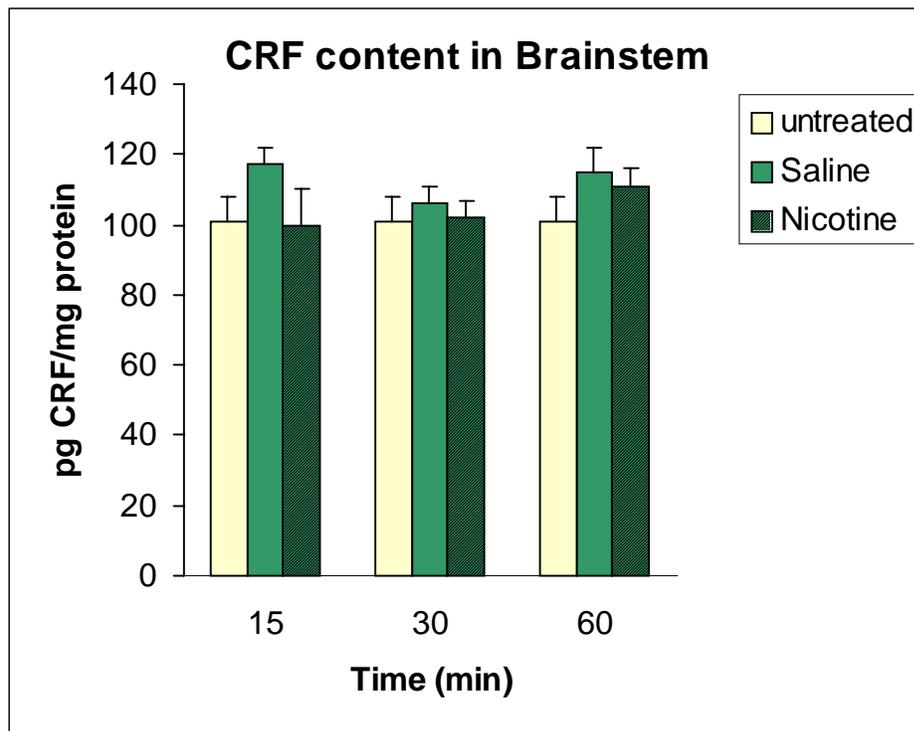
Figure 8. CRF content in macro-dissections of brain stem after 15, 30 and 60 minutes

treatment with nicotine 1.0 mg/kg, 0.9% saline (vehicle, 1.0 ml/kg) or no treatment: 15

minutes nicotine (n = 8), saline (n = 8), 30 minutes nicotine (n = 7), saline (n = 8), 60

minutes nicotine (n = 6), saline (n = 8) and total untreated (n = 24). Values are expressed

in pg CRF/mg protein, mean ± S.E.M per treatment.

After 30 minutes, mean CRF levels were 102 ± 5 and 106 ± 5 for nicotine and saline treated rats respectively. Sixty minutes of treatment exhibited mean CRF levels of 111 ± 5 and 115 ± 7 for nicotine and saline respectively. No significant treatment and time effects were observed between nicotine versus saline versus untreated group, at all time points.

Figure 9 summarizes CRF content in micro-punches of locus coeruleus and parabrachial nucleus (LCPB) after 15 and 30 minutes treatment with 0.4 mg/kg nicotine. Values are expressed in pg CRF/mg protein, mean $\pm$ S.E.M per treatment. The figure shows that mean CRF content in untreated rats from both time points was 570 ± 66. After 15 minutes treatment, mean CRF levels were 433 ± 79 and 579 ± 79 for nicotine and saline respectively. The thirty minute time point exhibited values of 512 ± 43 for nicotine and 475 ± 14 for saline treated rats. No significant treatment and time effects were observed in brain stem nuclei.
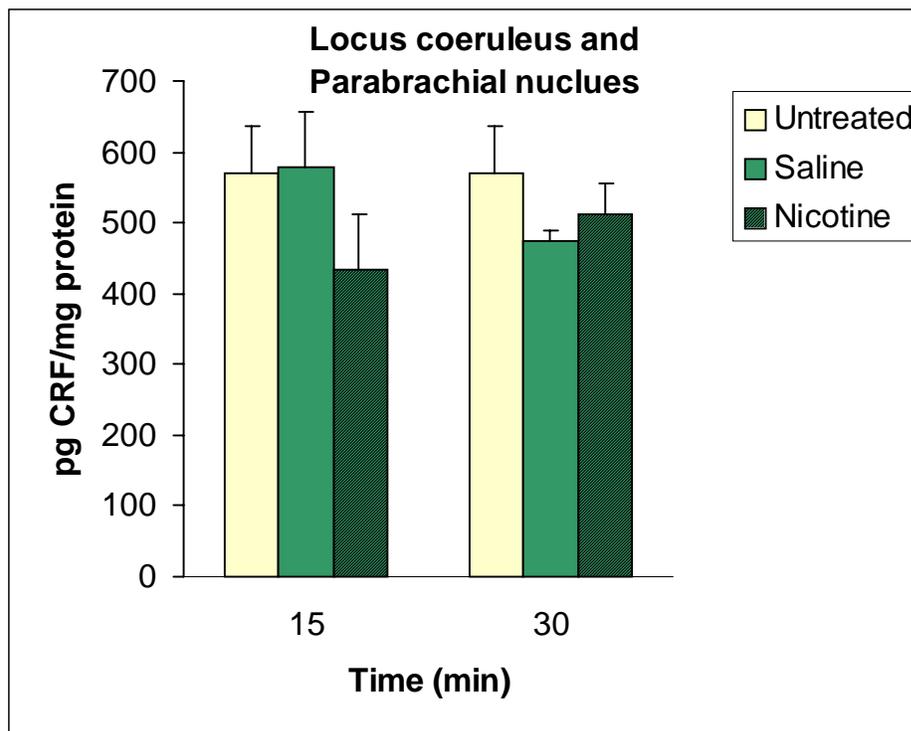
Figure 9. CRF content in micro-punches of locus coeruleus and parabrachial nucleus

(LCPB) after 15 and 30 minutes treatment with nicotine 0.4 mg/kg, 0.9% saline (vehicle

1.0 ml/kg) or no treatment: 15 minutes nicotine (n = 4), saline (n = 4), 30 minutes nicotine

(n = 8), saline (n = 8) and total untreated group (n = 8). Values are expressed in pg

CRF/mg protein, mean ± S.E.M per treatment.

CHAPTER VI

DISCUSSION AND FUTURE DIRECTIONS

<u>Discussion</u>

Effects of nicotine on hypothalamic and extrahypothalamic CRF have not been extensively reported. A recent study by (Matta, et al., 1997), reported increased c-fos expression in CRF positive neurons after low dose nicotine treatment. The proto-oncogene c-fos is an early indicator of neuronal activity, and this suggested that nicotine induced neuronal activity in CRF expressing neurons. The present study is the first to assess effects of nicotine on the content of CRF in the whole hypothalamus, amygdala and brain stem, and selected nuclei in these brain regions, at different time points. The study utilized two different doses of nicotine, 0.4 mg/kg and 1.0 mg/kg. The 1.0 mg/kg dose was examined for 15, 30 and 60 minutes whereas the 0.4 dose was examined for 15 and 30 minutes only.

In this study, it was assumed that the response of CRF after 15 minutes treatment would respond to the peaking of nicotine in the brain which occurs about 20 minutes after intravenous administration while the 60 minute response would correspond to relatively low levels of nicotine (Sastry, et al., 1995; Schmiterlöw, et al., 1967). The assumption was that acute nicotine would evoke changes in CRF content due to one or more of the following: (I) increased release and utilization leading to decreased content, (II) increased synthesis, processing or reuptake leading to increased levels, (III) decreased utilization and degradation, leading to increased levels and (IV) inhibition of release leading to increase levels. The present study did not directly address these mechanisms but only

measured CRF content. Electrophysiological, immunocytochemical, microdialysis and molecular biological techniques would be necessary to further elucidate the mechanisms of nicotine-induced changes in CRF content. Corticosterone levels were utilized as positive control for the presence of nicotine.

Plasma Corticosterone after 0.4 mg/kg treatment

Rats treated with nicotine 0.4 mg/kg for 15 minutes had significantly increased levels of corticosterone compared to untreated rats. Thirty minutes treatment with nicotine produced a strongly significant increase in levels of plasma corticosterone compared to both saline treated and untreated. These results confirm the results reported by other researchers, that nicotine injection augments plasma corticosterone. Corticosterone has been reported to peak at 10 to 30 minutes after injection and persist for up to 60 minutes (Benwell and Balfour, 1982; Cam and Bassett, 1983; Freund, et al., 1988).

Plasma Corticosterone after 1.0 mg/kg treatment

Rats treated with nicotine 1.0 mg/kg at either 15, 30 or 60 minutes exhibited a strongly significant increase in levels of plasma corticosterone compared to saline treated and untreated. There were no significant differences between 15, 30 and 60 minutes treatment with nicotine. These results also confirm the results by other researchers, that nicotine injection increases plasma corticosterone peaking at 10 to 30 minutes, and these increases persist for up to 60 minutes after nicotine injection (Benwell and Balfour, 1982; Freund, et al., 1988; Cam and Bassett, 1983). The present results also confirm that

nicotine activates the release of hypothalamic CRF causing increases in plasma corticosterone. How long this release of CRF continues is not clear; possibly the persistently high corticosterone levels reflect the continued release of CRF until the usable pool is depleted. The strongly significant increases in corticosterone are agreeable with the fact that nicotine administration augments plasma corticosterone which in turn quells down CNS effects of nicotine (Caggiula, et al., 1993, Caggiula, et al., 1998). This effect may also be due to the contribution of peripheral effects of nicotine directly on the adrenal cortex (Bugaski, et al., 1998; Malin, et al., 1997). Although levels were lower in all treatment groups at the 60 minutes time point compared to 15 and 30, nicotine treated rats still showed corticosterone levels significantly higher than untreated. The results further suggest that the stress of handling animals is minimal as shown by the lack of corticosterone response to saline injection.

<u>CRF content in brain regions</u>

Two doses of nicotine 0.4 mg/kg and 1.0 mg/kg were used to study effects on CRF content in hypothalamus, amygdala and brain stem. As expected, the hypothalamus had higher levels of CRF compared to amygdala and brain stem. Overall, there were no clear cut treatment effects of nicotine in all tested brain regions after 15, 30, and 60 minutes. The use of two different doses and methods may have confounded the results. Employing the cryostat-microdissection method at different doses would be more useful.

Hypothalamus

In macro-dissections of the hypothalamus there were significant treatment and time effects. Rats treated with 1.0 mg/kg nicotine showed a strongly significant increase in levels of CRF in the hypothalamus after 15 minutes treatment. Thirty minutes of treatment did not produce differences between treatments but the time effects of nicotine were prominent. Compared to the 15 minutes treatment time point, CRF was significantly decreased in the hypothalamus of nicotine treated rats after 30 minutes. This decrease persisted even though non-significantly, to 60 minutes. This decrease signified that CRF had been released from the median eminence and transported to the pituitary to trigger the release of ACTH thus causing the increased release of corticosterone from the adrenal cortex. The initial increase which was paralleled by increased corticosterone was probably due to the nicotine-induced mobilization of pre-pro CRF from PVN resulting in increased levels in whole hypothalamus, with this dose (1.0 mg/kg). Increased utilization of CRF could possibly lead to a decrease in PVN levels due to depletion of pre-pro CRF, with time. The micro-punch method to examinine individual nuclei would help determine which nucleus of the hypothalamus has increased levels. No effects of saline treated versus untreated rats were observed signifying that the treatment and time effects were specific to nicotine.

It would be expected that a significant rise in plasma corticosterone would be paralleled by a significant fall in the hypothalamic CRF and our results showed this. The present results indicated that after an initial rise at 15 minutes, CRF in the hypothalamus returned to baseline levels by 30 and 60 minutes after nicotine treatment. This suggested

an initial increase in processing of pre-pro CRF, which was followed by facilitation of

release and increased utilization. The return to baseline levels was expectedly due to the

corticosterone feedback inhibition. Notably, this initial increase in CRF after 15 minutes

was also paralleled by increased corticosterone levels, indicating that this time point may

be too early to detect feedback inhibition.

Acute and chronic stress have been shown to reduce CRF-like immunoreactiviy in

the ME while causing an increase in the anterior hypothalamus (Chappell, et al., 1986;

Feldman and Weidenfeld, 1996a). Notably, Swanson and Simmons, (1989), and Makino,

et al., (1994), showed that administration of increasing doses of corticosterone to

adrenalectomized animals caused a decrease in CRF mRNA in the hypothalamus

confirming the feedback inhibition. In animals and humans, increased anxiety or fear have

been reported to increase circulating levels of CRF and glucocorticoids (Nemeroff, et al.,

1991; Owens, et al., 1991) indicating a disregulation of the HPA axis. Studying effects of

nicotine directly on corticosterone synthesis in adrenal cortex would be useful in

determining the direct contribution of nicotine to circulating corticosterone and to clarify

why increased levels of corticosterone were observable when CRF levels had returned to

baseline after 30 and 60 minutes.

Paraventicular nucleus

Acute and chronic stress have been shown to cause no changes in CRF-like

immunoreactiviy in the paraventricular nucleus (PVN) (Chappell, et al., 1986; Feldman

and Weidenfeld, 1996a). In the present study, microdissections of the PVN showed that

there were no significant effects after 15 minutes treatment, for nicotine (0.4 mg/kg)

versus untreated versus saline injection. A slight increase in CRF was observed in 30

minutes but was not statistically significant. This would indicate that nicotine and the

saline injection induced the processing of pre-pro-CRF in PVN, which would unite with

existing pool of CRF get transported into the ME and be rapidly utilized to continue to

elicit ACTH release in pituitary and maintain elevated plasma corticosterone. It has been

reported by Sharp, et al., (1993), that c-Fos mRNA in hypothalamus was observable after

30 minutes of intraperitoneal nicotine which would confirm continued activation of the

HPA axis. This regulation of c-Fos by nicotine is modiated through brain stem

neurotransmitters (Valentine, et al., 1996). The question remains whether or not nicotine

would induce CRF mRNA. As time passes and approaches 60 minutes or more, would

pre-pro CRF be depleted enough to manifest a decrease in CRF levels for mRNA to be

induced? Would the induction of mRNA be counteracted by corticosterone feedback

inhibition? A higher dose of nicotine will be needed to assess effects on different

hypothalamic nuclei, using microdissections.

<u>Amygdala</u>

In amygdala macro-dissections, there were no significant treatment and time effects at

1.0 mg/kg nicotine. Even though there was an apparent increase in CRF levels in 15 and

30 min treatments, these were not significant. This would suggest that since the whole of

amygdala including the piriform cortex were dissected, CRF that had been released from

the CeA was still in the surrounding neurons, not yet metabolized, therefore detectable.

However, the apparent increase in CRF levels in 15 and 30 min treatments may suggest a typical effect on CRF releasing nuclei, because a significant increase was also observed in the hypothalamus. Administration of increasing doses of corticosterone to adrenalectomized animals has been reported to correspond to as much as five fold increase in CRF mRNA expression in the CeA of the amygdala, while CRF mRNA in the hypothalamus decreased (Makino, et al., 1994; Swanson and Simmons, 1989). Whether or not nicotine-induced corticosterone can cause such effects is not known.

Amygdaloid nuclei

In microdissections of amygdaloid nuclei, the time effect that was observed could not be taken as a clear cut indication of nicotine time effects, because there were no significant differences between saline treated or untreated rats at these time points. Treatment for 15 and 30 min with 0.4 mg/kg did not affect levels of CRF in the amygdala for nicotine versus saline versus untreated animals. Even though there was a slight increase in CRF for nicotine treated rats, at 15 minutes compared to saline treated and untreated, this was not significant. Therefore, 0.4 mg/kg nicotine slightly elevated CRF in the amygdala but the levels declined to the levels of untreated rats after 30 minutes. These findings poissibly follow the pattern of initial increase in content of CRF as seen with hypothalamus and amygdala data. A higher dose of nicotine will be needed to determine if there are any effects on CRF content in amygdaloid nuclei.

Brain stem

Macro-dissected brain stem showed no treatment or time effects on CRF content after 1.0 mg/kg nicotine treatments at all time points, 15, 30 and 60 minutes. This brain region was prone to having discrepancies in results due to two reasons; (I) inconsistencies in dissections which were more likely to occur with the brain stem and (II) this area was prone to damage during decapitations and removal from the cranium. Like all macro-dissections, in brain stem, effects of nicotine could have been masked by the fact that even after release from the nuclei, CRF was still in the neighboring CRF areas of the brain stem and would be detectable. In addition to the locus coeruleus/parabrachial nucleus (LCPB), other nuclei including nucleus solitarius, raphe nuclei and dorsal vagal complex express CRF immunoreactivity (Chappell, et al., 1986). As discussed earlier, CRF originating from the amygdala is believed to trigger activation of LC for catecholamine release and nicotine has been shown to cause noradrenergic activation and release of norepinephrine into the hypothalamus and amygdala for activation of CRF release (Fu, et al., 1997; Fu, et al., 1998; Matta, et al., 1993b; Mitchell, 1993). Since CRF expressing neurons originate from the amygdala, significant changes in amygdaloid CRF would need to occur before changes in the brain stem would be obvious.

Brain stem nuclei (Locus coeruleus and parabrachial nucleus)

The microdissected brain stem nuclei: locus coeruleus and parabrachial nucleus showed a nonsignificant decrease in CRF content after 15 minutes treatment. This initial decrease in CRF content followed by recovery to untreated levels is an opposite effect

compared to nicotine's effects on hypothalamus and amygdala. This is to be expected because the brain stem nuclei do not synthesize CRF and once utilized replenishment of CRF has to come from the amygdala. A possible role of nicotine to trigger CRF release which in turn triggers LC activation would be inferred by a decrease in content of CRF resulting from increased utilization in the brain stem. A higher dose of nicotine would need to be utilized to study effects on CRF in brain stem.

General conclusion

The present study examined effects of nicotine on CRF content in rat amygdala, hypothalamus and brain stem, using two doses of nicotine 0.4 and 1.0 mg/kg and two dissection methods at different treatment time points. The macro-dissection method revealed significant effects of nicotine (1.0 mg/kg) in the hypothalamus with 15 minutes treatment. No significant effects were seen in the amygdala and brain stem. Time dependent effects were observed between 15 and 30 minutes nicotine (1.0 mg/kg) treated rat hypothalami. The microdissection method using 0.4 mg/kg did not exhibit any treatment effects with all tested nuclei. Overall, no clear cut effects were observed with both dissections and doses of nicotine. Studies on CRF content in specific nuclei using varying doses of nicotine and various time points would help elucidate if indeed there are interactions.

Future directions

For further research, the method of choice would be the cryostat-microdissection method. With this method, the nuclei could be accessed individually and characteristic CRF levels detected for brain region nuclei. For example: (I) in the amygdala, the central nucleus (CeA) and basolateral nuclei (BLA) could be micro-punched individually, CRF levels should vary because the CeA contains more output CRF neurons (cell bodies) while basolateral has more receptors (terminals), (II) in the hypothalamus, PVN is the main source of CRF versus median eminence which contains the storage terminals, and other hypothalamic nuclei could be accessed, and (III) in brain stem, the locus coeruleus and parabrachial nucleus could be micro-punched separately and other nuclei including nucleus tractus solitarius and Barrington's nucleus can be accessed. Chappell, et al., (1986), was able micro-punch out several nuclei in the hypothalamus, amygdala and brain stem for CRF assessment. Determining CRF levels in the PVN and ME individually after 15, 30 and 60 minutes of treatment with nicotine (0.4 mg/kg and 1.0 mg/kg), would help clarify if indeed there are effects on CRF content, and the more rapid method of macro dissections could be utilized for these nuclei. Varying doses of nicotine could be utilized to determine dose response relationships on CRF content in nuclei of the hypothalamus, amygdala and brain stem.

Molecular biological techniques to examine CRF mRNA would help determine when the induction of CRF synthesis occurs after nicotine administration. Microdialysis techniques can be utilized to monitor CRF release after treatment with lower doses of nicotine; lower doses of the drug are used for direct delivery into the nuclei. Microdialysis

techniques would mostly be useful in the study of earlier time points from 0-20 minutes after nicotine treatment since nicotine accumulates in the brain within 5 minutes after intravenous administration. Effects of acute nicotine on CRF content in the brain could also be best determined between 5-20 minutes after administration, to coincide with peak levels. These earlier time points could be best understood if researched at specified time intervals for up to 30 or more minutes.

Immunocytochemical techniques and/or receptor binding techniques can be utilized to determine CRF receptors after acute or chronic nicotine treatment. The effects of chronic nicotine using minipumps and/or injections and the effects of nicotine withdrawal can be determined using these techniques. Nicotine administered acutely or chronically, and withdrawal from nicotine all exhibit different effects on animals (Helton, et al., 1993). In acute doses, nicotine naive rats could be compared to rats that have been pretreated with nicotine. With chronic nicotine treatments, the role that desensitization and tolerance play on CRF content still needs to be studied. Cognitive facilitation has been observed in rats up to four weeks after nicotine treatment (Levin, et al., 1992), therefore, effects of nicotine withdrawal would help in the elucidation of whether or not CRF is involved in the addictive effects of nicotine.

Future studies must involve the administration of neuronal nAChR antagonists namely: mecamylamine, dihydro-β-erythroidine or chlorisondamine (Dessirier, et al., 1998; Fu, et al., 1998; Malin, et al., 1994). This would help confirm whether or not effects are exerted on neuronal nAChR, that is, the specificity of nicotine. Whether or not nicotine stimulates CRF directly or indirectly through brain stem neurotransmitters is yet

to be documented. The interaction of CRF systems with cholinergic and noradrenergic systems and also with other systems could be investigated by injecting agonists or antagonist for these systems before nicotine injection.

Nicotine evokes different effects on stressed and unstressed animals and humans (Balfour, et al., 1975; Donnerer and Lembeck, 1990). The effects of nicotine-induced corticosterone will need to be assessed because stress reverses the inhibitory effects of high levels of corticosterone on CRF, causing a disregulation of the HPA axis (Makino, et al., 1995). Hexamethonium, a peripheral nAChR antagonist could be used to determine the role played in CRF modulation, by nicotine-induced adrenal corticosterone (Bugaski, et al., 1998; Malin, et al., 1997). Administration of metyrapone, a corticosterone synthesis inhibitor, before nicotine treatment would help determine the extent to which CRF is modulated by nicotine without the input from corticosterone.

The above mentioned techniques could be applied to stressed rats after acute or chronic nicotine treatment. This would demonstrate the effects of nicotine on stressed rats, and these are expected to vary from normal. The same techniques could be used on rats treated prenatally with nicotine to determine if the interaction of nicotine with CRF is manifest in the offspring. The plight of prenatal exposure to nicotine; effects on the offspring, was the impetus for this present study.

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Education:
West Virginia University, Morgantown, West Virginia (1995-1999).
  Master of Science: Pharmacology & Toxicology
  Thesis: Effects of nicotine on content of corticotropin releasing factor (CRF) in rat amygdala, hypothalamus and brain stem (Laboratory based research).

Indiana University of Pennsylvania, Indiana, PA. (1990-1992).
  Master of Science: Food and Nutrition Sciences
  Thesis: Lead Poisoning in Children: The awareness level of nutritionists on issues of lead poisoning and the role of nutrition in lead toxicity (42 item questionnaire).

University of Surrey, Guildford, Surrey, U.K. (1979-1983).
  Bachelor of Science (Honors):   Biochemistry (Medical)
  Final Year Project: Investigation of candidate enzymes for a simultaneous enzyme immunoassay of two thyroid hormones: triiodothyronine and thyroxine (Laboratory based research).

Professional Experience:
Graduate Research Assistant: Pharmacology/Toxicology, Box 9223, Morgantown, WV
                 26506 (1996-1998).
  • Neuropharmacology laboratory: whole animal studies.
  • Accurately handled paperwork/glassware/chemicals/animals/>400 samples.
  • Injected drugs intra-peritoneally; sacrificed rats; removed brains.
  • Utilized cryostat frozen brain sections; employed micro-punch technique.
  • Performed (micro)-centrifugation; lyophilization; radioimmunoassays.
  • Collaborated in the implementation of stress paradigm to rats.
  • Utilized computer for data entry/manipulation: Word Perfect 5.0-7.0; Microsoft Excel Spreadsheet, GB Stat, Reference Manager, Power Point.
  • Reviewed journal articles/delivered presentations to faculty and students.
  • Co-leader in medical students' conferences, on patient-based case studies.
  • Participated in WVU School of Medicine Annual Van Liere Research Convocation (March 1996).

GIS Class: Introduced to Geographic Information Systems (GIS), Fall 1998.

Research Assistant I: Tobacco Research, MBRCC, Box 9300, Morgantown, WV 26506.
       (4/1994-11/1994).
-   Assisted in two clinical trials for tobacco substitutes.
-   Proofread subject files for accuracy and completeness of collected data.

Graduate Teaching Assistant: Food & Nutrition, Ackerman Hall, IUP, Indiana, PA 15705.
       (1990-1991).
-   Supervised upperclassmen "Experimental Foods" laboratory.
-   Calibrated and ensured readiness of laboratory equipment.
-   Graded pre-laboratory assignments; coded scores into the computer.
-   Studied nutrients: sources, digestion, absorption and biological action, in health and disease.

Laboratory Intern: Biochemistry Department, Edgware General Hospital, Middlesex, U.K. (1981-1982).
-   Conducted assays for disease diagnosis.
-   Involved specimen handling/identity; centrifugation/separation; weighing balances; buffer/reagent preparation; pipetting; calorimetry; spectrophotometry; fluorimetry; chromatography (TLC, GLC, HPLC); electrophoresis; ELISA, RIA, use of auto-analyzers.

Laboratory Intern: Cardiff and South Glamorgan Area Hospitals, Wales, U.K.
       Diploma: Medical Laboratory Sciences/Technology (1977-1979).
-   Clinical laboratories: microbiology/immunology, hematology/serology, histopathology/cytology, clinical chemistry (8 weeks/Lab).
-   Prepared smears on glass slides; tissue embedding; microtome sectioning; staining procedures; microscopy; used Coulter Counter; culture media; bacterial cultures/cultivation/sterility/antibiotics.

Honors:
  Association for Women in Science, West Virginia Chapter (1997).
  BOT Minority Fellowship, West Virginia University (1996-1997).
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  Society for Neuroscience (1996-1999).
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Abstracts and Presentations:
Abstract: Corticotropin releasing factor (CRF) is increased in amygdala and hypothalamus of prenatally stressed rats. D.L. Birkle, M.S. Swatts, D.A. White, S. Masilela and E.M. Krouse. J. Neurochem 69:S216. (1997).

Poster: Does nicotine exert its central nervous system (CNS) effects by modulating corticotropin releasing factor (CRF) in selected brain regions?   Masilela, Sibonisiwe N., and D.L. Birkle. West Virginia University (WVU) School of Medicine "Research Day," (March 20, 1998).

Poster: Effects of nicotine on corticotropin releasing factor in the rat brain.   Sibonisiwe "Bonnie" Masilela. 1998 AWIS-WV Spring Symposium.

Abstract: The Knowledge and Role of WIC Nutritionists Regarding Lead Poisoning in Children." S. Masilela, J.B. Steiner, M.A. Cessna and R.M. Johnson. J. Am. Diet. Assoc. September 1993 Supplement, 93(9): p. A-25.

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Manuscript Reviews:
Reviewed *five manuscripts* on the subject of lead poisoning in children, for the serial, *Environmental Health Perspectives*. (1995-1998).

Volunteer Service:
Volunteer: Cheat Lake Elementary and Middle Schools, West Virginia (1994-99).
- Partaking in "Read aloud program."
- Chaperoned on School Field trips.
- Participated on "Science day" and "Sports Field Day."

Member: (MCAE) Monongalia County Association for Enrichment (1999).

Presentations : "Multi-culturalism and diversity" to Junior High and High School students (1998-1999).

Volunteer health educator: Cranwell International Center/YMCA, Virginia Tech, Blacksburg, VA. (1986-1987).

<u>Volunteer health educator</u>:   Cranwell International Center/YMCA, Virginia Tech,
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- Received (group) training from various health care givers in Montgomery County.
- Promoted awareness on access to health care, to newly arrived international students/families.
- Contributed to the publication international student pamphlet: "The health Resource Guide."

<u>WIC Clinic and Red Cross volunteer</u>:   Montgomery County Health Department,
Blacksburg, VA. (1983-1985).

- Hematocrit laboratory and anthropometric measures.
- Clerical work.