

ALTERATIONS OF THE CORTICOTROPIN-RELEASING HORMONE RECEPTOR
SYSTEM FOLLOWING EARLY LIFE STRESS AND CHRONIC ETHANOL SELF-
ADMINISTRATION IN RHESUS MONKEYS

BY

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A Thesis Submitted to the Graduate Faculty of

WAKE FOREST UNIVERSITY GRADUATE SCHOOL OF ARTS AND SCIENCES

in Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

Physiology and Pharmacology

August 2011

Winston Salem, North Carolina

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ACKNOWLEDGMENTS

I would like to thank several people for their unending supporting during my graduate career. First and foremost, I would like to thank God for giving me the strength, courage and perseverance to complete this degree and thesis. I thank my husband Trey, who has stood beside me through the years, offering guidance and support. He has been a strong rock to lean upon during this process and I would not have made it to the end without him. I thank my daughter Maggie who provides the inspiration and determination to continue everyday and her mere presence has encouraged me to push forward toward the completion of this degree. To them I dedicate this thesis.

I thank my mother, father, sisters and in-laws for offering continued time and support as I finish my degree and complete my thesis. Entering into the new frontier of parenthood, along with finalizing my degree, during the last several months would have been unbearably difficult without our family's' presence to lend a hand in our home.

I would like to thank my mentor David Friedman, PhD for his patience, guidance and continued instruction as I have written this thesis. I also thank the other members of the Friedman lab: April Davenport, Jim Daunais, PhD, Vicki Maxey, Diane Joyner, Liz Burnett and Gena Dolson who have offered continued advice and direction. They all have been an invaluable source of support and, at times, entertainment. I also thank my committee members Erik Johnson, PhD and Brian McCool, PhD for their time and assistance to complete this Masters thesis.

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LIST OF ABBREVIATIONS

Adrenocorticotropin (ACTH); Analysis of Variance (ANOVA); Area Under the Curve (AUC); Bed Nucleus of the Stria Terminalis (BNST); Blood Ethanol Concentration (BEC); Bovine Serum Albumin (BSA); Central nucleus of the Amygdala (CeA); Cyclic Adenylyl Monophosphate (cAMP); Corticotropin-releasing hormone (CRH); CRH Binding Protein (CRH-BP); CRH receptor type 1 (CRH-R1); CRH receptor type 2 (CRH-R2); Early Life Stress (ELS); Ethanol (EtOH); Ethylene Glycol Tetraacetic Acid (EGTA); γ -aminobutyric acid (GABA); G-protein Receptor Kinase (GRK); Femtomoles (fmol); Hippocampus (HIP); Hydrochloric Acid (HCl); Hypothalamic-Pituitary-Adrenal Axis (HPA); Lateral nucleus (Lat); Magnocellular accessory basal nucleus (ABmc); Magnocellular basal nucleus (Bmc); Maternal Separation (MS); Maltose Dextrin (MD); Medial nucleus of the Amygdala (MeA); Messenger Ribonucleic Acid (mRNA); Milligram (mg); Mother-Reared monkeys (MR); Norepinephrine (NE); Nursery-Reared monkeys (NR); Paraventricular Nucleus of the hypothalamus (PVN); Parvocellular accessory basal nucleus (ABpc); Periamygdaloid Cortex (PAC); Prefrontal Cortex (PFC); Protein Kinase A (PKA)

ABSTRACT

Early life stress (ELS) alters brain and neuroendocrine development leading to a higher risk of alcohol abuse. Corticotropin-releasing hormone (CRH), a primary mediator of the stress response in the brain, plays a regulatory role in ethanol (EtOH) consumption. The goal of this thesis was to identify how ELS and EtOH drinking affected hypothalamic-pituitary-adrenal (HPA) axis function and CRH receptor binding. We used an accepted ELS model of maternal separation with nursery-reared (NR) and mother-reared rhesus monkeys induced to self-administer EtOH or control solution for over 12 months. Subjects underwent endocrine challenges to probe the HPA axis before and after drinking. Brains were processed for *in vitro* receptor autoradiography using [¹²⁵I] Sauvagine and appropriate blockers to measure CRH receptor binding.

One year of EtOH drinking resulted in decreased cortisol and increased ACTH release. There were no initial rearing differences, but following EtOH self-administration there was decreased cortisol and ACTH release in NR monkeys. There was less dense CRH-R1 binding in all measured brain regions of EtOH drinking animals and increased CRH-R2 binding in the amygdala regardless of rearing condition. The most robust decreases in CRH-R1 binding ranging from 39%-50%, were seen in specific nuclei of the amygdala and in insular and entorhinal cortex. One year of EtOH self-administration was enough to alter HPA axis function and dramatically decrease extrahypothalamic CRH-R1 binding with rearing differences emerging only in the HPA axis following EtOH drinking.

INTRODUCTION

Early Life Stress and Alcohol Drinking

Over 17 million people in the US have DSM-IV characterized alcohol abuse or dependence problems (Grant et al., 2004). Alcohol use is the third leading preventable cause of death in the United States (Mokdad et al., 2004), and the economic costs of alcoholism, including the costs of treatment and medical consequences, lost earnings, alcohol-related accidents and criminal activities, are estimated at over \$184 billion per year (Harwood, 2000). It is clear that alcohol use and abuse not only have negative consequences on the quality of life, but also significant morbidity, and mortality as well.

Early adverse events are potential risk factors for developing alcoholism as an adult (Dube et al., 2002). There are over 3 million reports of childhood maltreatment every year, with 72% attributed to neglect (Sedlak et al., 2010). Traumatic stress initiates a cascade of psychological and physiological developmental pathways that can lead to behavioral dysfunction throughout life (De Bellis, 2001; Heim & Nemeroff, 2001; Teicher et al., 2003). Early stress exposure is implicated in conduct disorders, personality disorders and alcohol abuse during adolescence and adulthood, with an increased risk of maltreatment of one's own children later in life (De Bellis, 2001).

In a study of over 8500 participants surveyed for adverse childhood experiences, 30% experienced emotional neglect and 20% physical neglect. There was an increased incidence of alcohol use initiation before the age of 14 in participants who were emotionally or physically neglected (Dube et al., 2006). This study is supported by a meta-analysis of the literature that concluded childhood stress and early initiation of

alcohol use is highly correlated with heavy drinking in adulthood (Waldrop et al., 2007). The interaction of childhood stress and early alcohol drinking could alter neurobiological systems later in life (Heim & Nemeroff, 2001; Sanchez et al., 2001). Early life stress (ELS) and drinking both affect the hypothalamic-pituitary axis (HPA) and the extrahypothalamic corticotropin-releasing hormone (CRH) system, both of which are involved in coordinating the brain's response to stress (Higley 1991; 1992; Wand & Dobs, 1991; Fahlke et al., 2000; Richardson et al., 2008). This thesis will present data on the effect of ELS on these systems, the effect of alcohol consumption during adolescence on these systems and the interaction between ELS and later drinking. The data will elucidate how ELS alters the response to alcohol and may lead to abusive drinking.

Physiological Stress Response

Hans Selye first described his theory on chronic stress and coined the term “general adaptation syndrome” in 1936 (Seyle, 1998). He described a physiological response to stressors involving the central nervous system (hypothalamus), anterior pituitary release of adrenocorticotropin hormone (ACTH) into the bloodstream, and activation of ACTH receptors within the adrenal cortex to stimulate the release of glucocorticoids. Over time, chronic stress will lead to an adaptive state with increased release of glucocorticoids and other peptides followed by a state termed “exhaustion,” which leaves the system vulnerable to external stressors.

An early step in the stress response is the release of CRH, a 41 amino acid neuropeptide (Vale et al., 1981; Spiess et al., 1981), from the parvocellular cells of the paraventricular nucleus of the hypothalamus (PVN) into the portal system of the pituitary. Upon reaching the anterior pituitary, it binds to receptors that initiate the

Early Life Stress Models – Rodents and Monkeys

Rodent Maternal Separation

Many ELS studies performed in rodents use a maternal separation (MS) model in which either the rodent pup or the dam is removed from the home cage (Sanchez et al., 2001). Results from rodent ELS models vary greatly and are dependent on age of ELS, length of separation, number of separations and human handling of the rodent pups (Pihoker et al., 1993; Lehmann & Feldon, 2000; Sanchez et al., 2001). The literature suggests that repeated (Van Oers et al., 1998) and extended (Ladd et al., 2005) but not short-term maternal separations are crucial to initiating the long-term behavioral and neurobiological adaptations to stress (Sanchez et al., 2001).

Rodents subjected to 180 minutes of human handling maternal separation have greater alterations compared to 15 minutes with increased CRH messenger ribonucleic acid (mRNA) and decreased CRH-R1 binding; however, differential effects had diminished by adulthood (Ladd et al., 2005). Following a social isolation model of MS, there is increased basal CRH expression and CRH receptor type 1 (CRH-R1) expression in several brain regions (Pan et al., 2009; O'Malley et al., 2010). CRH receptors are described in more depth below.

In rodents that experienced MS in the first weeks of life, an acute stressor results in decreased CRH-R1 expression (O'Malley et al., 2010) and decreased CRH-R1 binding (Pihoker et al., 1993) in adulthood. Studies of mice lacking CRH-R1 receptors do not show typical HPA axis activation following maternal deprivation (Schmidt et al., 2010), providing further evidence that a functioning CRH system is essential for proper HPA activation following ELS.

Non-Human Primate Nursery Rearing Model

The influence of early experiences on primate behavior throughout life was first demonstrated by Harlow and colleagues beginning in the 1950s (Seay & Harlow, 1965; Griffin & Harlow, 1966; Suomi et al., 1971). Despite continuing behavioral studies, however, there has been little investigation of the neurobiological mechanism underlying the detrimental behavioral effects of ELS (Gutman & Nemeroff, 2003). The nursery rearing model entails separating rhesus monkeys from their mothers within 24 hours of birth and placing them in an incubator with a ‘surrogate’, a heated fleece object with springs that allow soothing, rocking movements. After fourteen days in the incubator, monkeys are moved to a cage within a quadrant rack with the ability to see, hear and smell surrounding monkeys. At thirty-seven days, monkeys are given two hour slots of time to play with other infants. This schedule continues until approximately seven months at which point nursery-reared and mother-reared monkeys are housed together in larger social groups (Shannon et al., 1998).

Nursery-reared (NR) monkeys have distinct behavioral and physiological differences from animals reared by their mothers in social groups (Higley et al., 1991; Shannon et al., 1998). Nursery-reared monkeys exhibit a trait-like chronic anxiety characterized by increased fearfulness (displayed by clinging to one another), decreased play with peers and increased self-directed behaviors (Higley et al., 1991; 1992). These monkeys also exhibit a blunted stress response with decreased plasma ACTH, decreased plasma cortisol and decreased serotonin metabolites in cerebrospinal fluid. They also tend to consume more EtOH (Higley et al., 1991; Friedman Lab unpublished data).

Using Non-human Primates in Research

The use of non-human primates in research modeling the human condition is beneficial because of the genetic, anatomical and physiological similarities between monkeys and humans. Monkey studies allow for more behaviorally complex, longitudinal investigations that can incorporate crucial developmental periods and long-term adaptations. The nursery rearing primate model of ELS allows a unique study of deprivation and neglect in childhood leading to distinct behavioral consequences in adulthood (Higley et al., 1991), that are also displayed in humans who have been neglected (Heim & Nemeroff, 2001).

Anatomically, a monkey model is advantageous because the monkey brain is more similar to the human brain than the rodent brain. One example of this similarity is the prefrontal cortex (PFC) where the architecture of cortical fields, the anatomical connections to subcortical areas and the unique array of granular, dysgranular and agranular fields are seen only in the primate brain (Carmicheal & Price, 1994). Prefrontal limbic cortical regions play a role in stress, emotion and drug self-administration, and the elaborated and distinct fields in the monkey and human brain comprise a more complex system with a more nuanced response to stress. There are also age-related changes in brain morphology and composition in non-human primates that parallel humans (Pierre et al., 2008).

Corticosteroid and CRH receptors are distributed differently in rats and primates, which suggests that they may respond differently to stress and alcohol use (Sanchez et al., 2000; Sanchez et al., 1999), an important factor when studying alterations in the stress system. In the primate, both types of CRH receptors are found in the anterior pituitary

and throughout the cerebral cortex where only CRH-R1 receptors are seen in rodents. There are no reports of CRH receptor binding in the central nucleus of the amygdala (CeA) in the rodent, while the monkey brain does have low levels of CRH-R2 labeling. There is profoundly more densely labeled CRH receptors in the hindbrain of primates than rodents (De Souza et al., 1985; Sanchez et al., 1999). The widespread and unique localization of CRH receptors in primates is another advantage of utilizing non-human primates in a model of ELS.

Alcohol Self-Administration Model

A non-human primate model of voluntary alcohol consumption provides similar EtOH consumption levels to humans and offers the opportunity to study alterations due to chronic self-administration as well as the interaction between ELS and drinking. Using a schedule-induced drinking model, subjects are trained to voluntarily self-administer large amounts of fluid without experimental confounds such as food restriction, sucrose solutions, vapor chambers or compulsory drinking (Falk, 1961). Grant and colleagues have developed a schedule-induced polydipsia method to induce various non-human primate species, with similar metabolism to humans, to drink EtOH in amounts that compare with 8-12 drinks per day (2.0-3.0 g/kg) in humans (Vivian et al., 2001; Friedman Lab unpublished data). Using this model, monkeys are induced to drink an EtOH or maltose-dextrin (MD) control solution over 4 months of schedule-induced polydipsia with increased doses of EtOH. The monkeys are then allowed *ad libitum* access to both EtOH and concurrently available water for one year. This model allows for the investigation of drinking behaviors during induction and open access. Drinking

behaviors during induction can be predictive of consumption across the span of the study (Grant et al., 2008).

Behavioral similarities between humans and monkeys are advantages in investigating the neurobiological interactions of chronic stress and alcohol as a model of human experiences. Recent studies using this model of early life stress and EtOH consumption to evaluate social and drinking behaviors suggest that increased stress due to social separation plays a role in consumption of both alcohol and other sweetened, palatable solutions (Higley et al., 1991; Fahlke et al., 2000). Though acute stress decreases EtOH consumption in some monkey models, chronic social stress (either social separation or ELS) consistently shows increased alcohol consumption (McKenzie-Quirk & Mizeck, 2008).

Utilizing both the model of ELS and chronic EtOH self-administration, enables an evaluation of neurobiological alterations due to the interactions of these factors. We used a 2x2 experimental design with monkeys from each rearing group self-administering either EtOH or maltose-dextrin, a control solution isocaloric with ethanol. Following approximately 14 months of open access to solutions, brain tissue was extracted and processed for *in vitro* autoradiography to investigate how CRH receptor binding was affected by the ELS alone, chronic EtOH alone and interactions of both conditions.

Corticotropin-Releasing Hormone

Neurobiology

Within the hypothalamus, CRH cell bodies are located within the parvocellular portion of the PVN (Swanson et al., 1983) and release the hormone into the portal system to bind with CRH-R1 receptors in the anterior pituitary. Acute stress results in increased

cellular activation in CRH-containing cells, shown by an increase in c-fos mRNA and CRH immunoreactivity (IR; Bonaz & Rivest, 1998; Carrasco & Van de Kar, 2003; Armario, 2010). Following chronic stress, CRH immunoreactivity and mRNA expression decreases in the PVN but increases in extrahypothalamic brain regions (Chappell et al., 1986; Bonaz & Rivest, 1998; Pinnock & Herbert, 2001). In rodents, the localization of cell bodies containing CRH in the cortex, amygdala (primarily the CeA), bed nucleus of the stria terminalis (BNST), hippocampus (HIP), and brainstem suggests that CRH exerts extrahypothalamic actions throughout the brain during periods of stress (Swanson et al., 1983; see Figure 1.a.).

Increased stress typically leads to increased CRH release throughout the CNS. There are increased levels of CRH in the CeA, brainstem, and the bed nucleus of the stria terminalis (BNST) following stress (Chappell et al., 1986; Cook, 2004); all of which are areas that influence the behavioral response to stress and reward through pathways shown in Figure 1. CRH containing neurons and CRH receptors are also located in many of these regions in the brains of non-human primates (Lewis et al., 1989; Sanchez et al., 1999; Bassett & Foote, 1992) and are altered following periods of stress and EtOH drinking, as described below. In this study, we primarily focused on four brain regions to investigate alterations to the CRH system, the amygdaloid complex, the PVN and the insula and entorhinal cortex.

Receptors

There are two subtypes of CRH receptors (1 & 2) differentially distributed throughout brain (De Souza et al., 1985; Chalmers et al., 1995). In the primate brain, there is significant expression of both receptors in the anterior pituitary, amygdaloid

complex, HIP, insular and entorhinal cortices. There are high levels of CRH-R2 in the PVN and high levels of CRH-R1 in the PFC, with lower levels of CRH-R1 and R2, respectively (Sanchez et al., 1999).

Along with differential distribution, the receptors have opposing actions. Activation of CRH-R1 is generally associated with excitation and anxiogenic behaviors, while activation of CRH-R2 is generally associated with inhibition and anxiolytic behaviors (Lee & Rivier, 1997; Bale & Vale, 2004). Both receptor subtypes are linked to G_s and increase cyclic adenosine monophosphate (cAMP) leading to increased phosphorylation of protein kinase A (PKA). Activation of these receptors has the ability to affect release of norepinephrine (NE), γ -amino butyric acid (GABA), glutamate, and serotonin.

In the dorsal raphe nucleus, for example, CRH-R1 is located on GABA neurons and CRH-R2 located on serotonin neurons (Lukkes et al., 2009), CRH will initiate the release of GABA during minimal acute stress, thereby inhibiting the release of serotonin to forebrain regions (Figure 1). Chronic and intense stress by contrast will activate CRH-R2 on the serotonin neurons and increase release (Lukkes et al., 2009). In the basolateral amygdala, CRH increases membrane excitability through postsynaptic CRH-R1 receptors on glutamatergic neurons and in the CeA, CRH reduces membrane excitability through pre-synaptic CRH-R1 on GABA neurons and postsynaptic CRH-R2 on glutamate neurons (Rainnie et al., 1992; Liu et al., 2004; Nie et al., 2004). Within the cortex, CRH receptors are located primarily on pyramidal neurons (Gallopín et al., 2006). CRH receptors activate various intracellular signalling pathways including protein kinase C–

phospholipase C, mitogen-activated protein kinase and extracellular signal-regulated kinase pathways (Hillhouse & Grammatopoulos, 2006).

There is also a binding protein that has the ability to regulate the concentration of brain and blood CRH. The CRH binding protein (CRH-BP) is predominately expressed in the cortex and subcortical limbic regions; and colocalized with CRH in cortical regions of the rodent and primate. CRH-BP binds with the CRH peptide and is thought to initiate a clearance mechanism to decrease 'free' CRH levels (Behan et al., 1995). In the amygdala, acute stress and CRH administration increased CRH-BP mRNA, an increase not affected by CRH antagonists. Corticosterone administration had no effect on CRH-BP mRNA (Herrington et al., 2006).

Factors Affecting Corticotropin-Releasing Hormone Release

CRH Neuron Activation

Several neurotransmitter systems have the ability to affect CRH release in the brain. A local GABA population can inhibit CRH release within the PVN (Bartanusz et al., 2004), possibly through a mechanism involving oxytocin (Bulbul et al., 2011). Norepinephrine-containing neurons project to the PVN and may stimulate the release of CRH (Dunn et al., 2004), as do serotonin projections from the raphe nuclei (Liposits et al., 1987).

Cortisol

Cortisol is a primary regulator of CRH release through feedback control (Imaki et al., 1995). Chronic, central glucocorticoid administration is shown to increase CRH gene expression in the CeA and BNST and simultaneously decrease of CRH gene expression in the PVN (Makino et al., 1994a; Makino et al., 1994b). The increase of CRH gene

expression in the extrahypothalamic CRH system is similar whether there is physiological stress or exogenous glucocorticoid administration (Chappell et al., 1986).

Alcohol

Rivier and colleagues (1984) showed that acute EtOH decreases hypothalamic CRH content and subsequent studies showed increased CRH in the CeA (Merlo Pich et al., 1995), but decreased CRH in the hypothalamus, PFC, and cingulate cortex in rodents after exposure to EtOH vapors (Ehlers et al., 1992; Slawecki et al., 1999; Richter et al., 2000). Cell culture findings provide evidence that EtOH increases CRH secretion, mRNA expression and gene transcription through cAMP/PKA dependent pathways (Li et al., 2005). In humans, acute alcohol stimulates the HPA axis, increasing ACTH and cortisol release; but chronic consumption blunts the HPA axis resulting in significantly less cortisol during a stress challenge (Roy et al., 1990; Wand & Dobs, 1991; Inder et al., 1995; King et al., 2006), an effect also seen in rodents (Lee et al., 2001; Richardson et al., 2008).

Amygdala

The amygdala is a major site of both CRH receptors in rodents and primates (De Souza et al., 1985, Van Pett et al., 2000, Sanchez et al., 1999; Wong et al., 1994), and stress increases release of CRH in the amygdala (Cook, 2004). Increased CRH release within the basolateral region of the amygdala significantly increases neuronal excitability and leads to increased anxiety behaviors (Bijlsma et al., 2011, Rainnie et al., 2004; Ugolini et al., 2008), while increased CRH release in the CeA results in increased neuronal inhibition (Bagosi et al., 2007, Gallagher et al., 2008) as described above. A decrease in anxiety-like behaviors due to increased environmental enrichment is

associated with decreased gene expression of CRH-R1 (Sztainberg et al., 2010), and possibly decreased receptor binding.

Amygdala Circuitry

The amygdaloid complex consists of several major nuclei that together project to most regions of the brain. There are amygdaloid projections to the hypothalamus and the brainstem from the CeA, medial (MeA), accessory basal (AB) and basal nuclei (Price, 2003). There are heavy labeled projections from the amygdala to all regions of the insula. The heaviest labeled projections terminate in the agranular region with decreasing densities across the dysgranular and granular fields. These projections arise primarily from the basal and accessory basal nuclei (Amaral & Price, 1984). There are also extensive projections from the lateral, basal and AB nuclei to the entorhinal cortex. The heaviest labeled projections originate in the lateral nucleus, parvocellular accessory basal nucleus (Abpc) and the paralaminar nucleus. These projections terminate primarily in the rostral entorhinal cortex (Pitkanen et al., 2002).

There are CRH-containing projections that originate in the CeA and terminate on NE neurons in the LC and raphe nuclei. (Dunn et al., 2004) Similarly, there are CRH projections from the CeA and BNST to the VTA (Rodaros et al., 2007). In non-human primates, CRH fibers are concentrated in the CeA and the lateral nucleus with CRH-containing cells located in the basal, lateral and cortical nuclei (Bassett & Foote, 1992).

Paraventricular Nucleus of the Hypothalamus

As mentioned above, there is considerable evidence demonstrating that stress alters the expression of CRH mRNA and CRH receptor binding in the hypothalamic PVN

(Kiss et al., 1996; Luo et al., 1994; Rivest et al., 1995). Hyperactivity of this CRH system could contribute to the development of anxiety and mood disorders.

PVN Neurocircuitry

The PVN contains several populations of neurons (including the CRH-containing neurons) that project to the median eminence (Laqueur, 1954; Antunes et al., 1977) where they terminate adjacent to the hypophyseal portal system (Paull et al., 1982; Kawata et al., 1982). There are also reciprocal projections to other nuclei of the hypothalamus (Sawchenko & Swanson, 1983) that may affect CRH release. Extrahypothalamic afferents from the forebrain include the CeA, MeA, BNST, lateral septum, hippocampus and prefrontal cortex (Reyes et al., 2005; Ottersen, 1980; Conrad & Pfaff, 1976). There are also an array of PVN inputs from the brain stem, including the parabrachial nucleus, locus coeruleus, dorsal raphe, ventral medulla and nucleus of the solitary tract, (McKellar & Loewy, 1981; Sawchenko & Swanson, 1983).

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Cortical Regions

In the rodent, CRH receptor expression and binding (primarily CRH-R1) are found throughout the cerebral cortex (De Souza et al., 1985; Chalmers et al., 1995; Van Pett et al., 2000). Sanchez and colleagues (1999) found extensive CRH-R1 labeling and less dense binding of CRH-R2 labeling in the rhesus insular and entorhinal cortices. In most cortical regions, CRH-R1 was diffuse across the layers, but typically denser in layers I-V than layer VI. In contrast, CRH-R2 had greater binding densities in layers IV

& V than the other layers. Overall, there were higher binding densities of CRH-R1 than CRH-R2 (Sanchez et al., 1999). CRH-R1 immunoreactivity is uniquely located in various cortical regions, as described below. CRH-containing cells are found primarily in layers II & III throughout the rodent neocortex (Swanson et al., 1983) and found differentially in cortical fields throughout the monkey as described below in the insular and entorhinal cortices (Lewis et al., 1989).

Insular Cortex Neurocircuitry

The insula contains reciprocal connections with various cortical regions, the basal ganglia, the thalamus and every subnucleus of the amygdala (Mesulam & Mufson, 1982; Friedman & Murray, 1986). The monkey insula consists of a rostroventral agranular field, a transitional dysgranular field and a posterior granular field. The agranular region has projections to the cingulate, entorhinal and periamygdaloid cortices with reciprocal connections from the entorhinal cortex. The dysgranular region projects to the frontal, parietal, the entorhinal and periamygdaloid cortices as well as several amygdaloid nuclei including medial, central, and lateral nuclei. The granular region has reciprocal connections with the frontal and parietal lobes and the retroinsular field, as well as the entorhinal cortex and the lateral nucleus of the amygdala (Augustine, 1996; Friedman et al., 1986).

In the insula, CRH- containing cells tend to be localized in layers I-III with some scattered labeling in layer IV. There were also a greater amount of total CRH processes as compared to cell bodies, especially in layers I & IV (Lewis et al., 1989). CRH-R1 IR is found throughout layers II-VI with the greatest density within layers V & VI (Kostich et al., 2004).

Entorhinal Cortex Neurocircuitry

The entorhinal cortex is easily distinguished due to the small clusters of stellate cells and patchy distribution of pyramidal cells in layer II, though this is best seen rostrally (Van Hoesen & Pandya, 1975). The entorhinal cortex is the primary projection to all regions of the hippocampal formation, but also projects to other temporal cortical regions (Kosel et al., 1982; Witter & Amaral, 1991) and has reciprocal connections with the insular cortex (Augustine, 1996). It also receives afferents from many subcortical structures, including the amygdala. Primary projections come from the lateral, accessory basal, paralaminar and the periamygdaloid cortex (Pitkanen et al., 2002; Insausti et al., 1987). Projections from the amygdala terminate primarily in the layers II and III of the rostral entorhinal cortex (Pitkanen et al., 2002).

CRH cell bodies are primarily located in layers II & III in this region of the cortex, whereas CRH processes are found primarily in layer IV (Lewis et al., 1989). CRH-R1 IR is found in layers II-VI throughout the temporal cortex with the most intense labelling on pyramidal neurons in layers III & V (Kostich et al., 2004).

A Role in Alcohol and Drug Abuse

The amygdaloid complex is involved in emotional learning and memory, attention and motivated behaviors. Overactivation can lead to increased anxiety, which can play a role in drug craving and drug-seeking behaviors. In addition, the amygdala is involved in other aspects of drug-seeking and taking behavior. The central and basolateral regions of the amygdala have both been shown to modulate the positive and negative aspects of drug self-administration. The CeA (with reciprocal connections with the basolateral nuclei and limbic cortices) is a part of an extended amygdala system including the BNST

and medial nucleus accumbens found to be interconnected with the mesocorticolimbic dopamine pathway (Koob, 1999). Drug and alcohol use alter the GABA, glutamate, CRH, and noradrenergic systems in the amygdala (McBride, 2002; Liu et al., 2004; Nie et al., 2004; Rainnie et al., 2004; Koob, 2009; Kryger & Wilce, 2010).

Lesions in the CeA decrease anxiety-like behaviors and voluntary EtOH consumption in rodents (Moller et al., 1997). In healthy rodents, EtOH withdrawal leads to increased anxiety-like behaviors and increased EtOH self-administration following withdrawal, reduced by the administration of a CRH antagonist into the amygdala (Funk et al., 2006). The literature supports dysfunction within the amygdala following alcohol use, possibly mediated by the CRH system.

Many drugs of abuse including opiates, ethanol, cannabinoids, nicotine and cocaine can activate CRH neurons of the PVN (Armario, 2010) as revealed by increased CRH gene expression following drug administration (Buckingham 1982; Rivier et al., 1984; Zhou et al, 2000). This could alter the ability of the PVN to activate the HPA axis.

Research investigating behavioral correlates of insula function is a relatively new but growing field. Various lesion studies show the importance of the insula in interpreting the interoceptive effects of drug taking and decision (see Naqvi & Bechara, 2010 for review). Insula damage reportedly decreased the urge to smoke cigarettes, leading to the speculation that the insula may be involved in maintaining an addiction. Neurocircuitry that supports this view includes insula connections to the amygdala and the prefrontal cortex which may relay information related to cue-induced urges (Naqvi & Bechara, 2010). CRH-R1 pharmacotherapies can target these brain regions and may aid in the suppression of drug taking urges.

The entorhinal cortex is a source of input to the HIP, which is known to play a role in drug abuse along with its primary function in memory formation. There is significant neurodegeneration in the entorhinal cortex following binge alcohol administration as well as the administration of cocaine or cannabinoids (Ellison et al., 1993; Obernier et al., 2002; Higuera-Matas et al., 2010). Neurodegeneration in layers II-IV throughout the entorhinal cortex can be caused by acute and chronic administration of EtOH (Ibanez et al., 1992; 1995). This degeneration indicates alcohol consumption could lead to possible disruption of entorhinal outputs and memory dysfunction (Obernier et al., 2002).

This Study

Utilizing our unique sets of nursery-reared monkeys and mother-reared controls from the same colony provides the opportunity to study stress and alcohol interactions in a primate model of ELS and EtOH self-administration that closely parallel the human condition. This thesis reports data collected from monkeys subjected to ELS followed by subsequent chronic EtOH self-administration later in life, with appropriate MR rearing and MD drinking controls. Along with the EtOH self-administration data, endocrine challenges investigate cortisol and ACTH levels following acute administration of saline, CRH and EtOH. *In vitro* receptor autoradiography provides insight into CRH-R1 and CRH-R2 binding alterations in the amygdala complex, PVN, insula and entorhinal cortex. We investigate dysfunction of the endocrine and CRH receptor systems due to ELS alone, chronic EtOH self-administration alone or EtOH drinking following ELS. The compilation of this data provides a clear picture of the impact of ELS and later alcohol drinking on neural stress systems.

MATERIALS AND METHODS

Subjects

These studies were performed in accordance with the Wake Forest University Animal Care and Use Committee and the Guidelines of the Committee on the Care and Use of Laboratory Animal Resources. Eighteen ethanol-naïve male Rhesus monkeys (*Macaca mulatta*) were used for this study. Half of the animals (n=9) were reared by their mothers (mother-reared, MR) and the other half (n=9) were removed from their mothers at birth and reared in a nursery (nursery-reared, NR) [Suomi Laboratory, National Institute of Child health and Development, Poolesville MD]. For the duration of the study, animals were housed individually within 76 x 60 x 70 cm³ stainless steel cages in a vivarium maintained at 21 ± 1° C, 30 - 50% humidity, and a 12:12 hour light: dark cycle with lights on at 7:00 am. Monkeys were fed a diet of Primate Food pellets (Research Diets Inc., Brunswick, N.J.) and fresh fruit; water was available ad-libitum.

Ethanol Drinking

Eight animals (four from each rearing group; mean age of 4.3 years) were trained to self-administer EtOH and the other ten (five from each rearing group; mean age of 4.8 years) were trained to self-administer an isocaloric maltose-dextrin (MD) solution. The apparatus and drinking model was adapted from the ethanol self-administration model developed by Grant and colleagues (Vivian et al., 2001). Briefly, monkeys were trained to operate the drinking panel in their home cage. Monkeys were then induced to consume water and then increasing amounts of EtOH or MD under scheduled pellet deliveries (i.e., schedule-induced polydipsia; Falk, 1961) in 16-hr sessions. The amount of ethanol

available increased in a stepwise fashion over 30-day epochs. Specifically, monkeys were induced to drink 0.5 g/kg solution/day for 30 consecutive days, 1.0 g/kg/day for 30 consecutive days, and finally 1.5 g/kg/day for 30 consecutive days. After four months of the induction procedure, the animals were allowed free access to either water or the 4% experimental solution (EtOH or MD) for 22 hours each day.

Blood Ethanol Concentrations

Blood samples were obtained for determination of blood ethanol concentrations (BEC). Twenty microliter (20- μ l) blood samples for blood ethanol concentration (BEC) were taken from the saphenous vein six times a month approximately 6-hours following the onset of the 22 hour free-access session and assayed for BEC. Samples were assayed using a gas chromatograph (Agilent 7890) equipped with a headspace autosampler and flame ionization detector.

Endocrine Profiles

In order to perform the pharmacological challenges and blood collection procedures under in awake animals under minimally stressful conditions, monkeys were trained to sit comfortably in a primate restraining chair and to present legs for blood draws. Femoral blood samples were obtained with a 22G x 1 inch Vacutainer needle and a 3 ml Vacutainer hematology tube (Becton Dickinson). All blood samples were stored on ice until centrifuged (approximately 5 minutes). Samples were spun at 3000 rpm for 15 minutes at 4°C in a Beckman Coulter refrigerated centrifuge (Model Allegra 21R). Plasma was pipetted into 2 ml microtubes in 100 μ l aliquots. Plasma samples for cortisol analysis were frozen at -20°C and samples for ACTH analysis were frozen at -80°C and stored until analyzed. Upon completion of all drug challenges the blood samples were

sent to the Yerkes Endocrine Core Laboratory (www.emory.edu/yerkes/div/rsrch/assay) for cortisol and ACTH analysis.

Saline Challenge Test

The saline challenge allowed a measure of hormone levels primarily due to the stress of being placed into the primate chair alone. Monkeys were fasted overnight. The following morning, monkeys were chaired and a baseline blood sample was taken followed by an I.M. injection of saline. Additional blood samples were obtained at 15, 60, 90 and 120 minutes and assayed for plasma ACTH and cortisol.

CRH Challenge Test

Pituitary response to CRH was assessed using the CRH challenge test (Gold et al., 1984; Sapolsky, 1989, Waltman et al., 1994). Briefly, the monkeys were fasted overnight; the following morning a 3 ml baseline blood sample was taken followed by administration of 1 µg/kg ovine CRH into the saphenous vein. Additional blood samples were obtained at 15, 30, 45 and 60 minutes following CRH challenge and assayed for plasma ACTH and cortisol.

EtOH Challenge Test

Ethanol produces changes in circulating cortisol (Schuckit et al., 1987; Gianoulakis et al., 1996). We first tested a dose of 1.0 g/kg ethanol; five days later we tested a higher dose of 1.5 g/kg EtOH. Animals were fasted overnight. The following morning they were seated in primate restraint chairs and then a baseline blood sample was taken, 30 minutes later monkeys received the challenge dose of EtOH (30% w/v in tap water) intragastrically. Additional blood samples were then drawn at 15, 60, 90 and 120 min following administration of EtOH for plasma ACTH and cortisol.

Tissue collection and preparation

Necropsies for tissue collection were scheduled just prior to the onset of a daily drinking session. Animals were anesthetized with ketamine (15 mg/kg, IM) and then brought to a deep surgical plane of anesthesia with intravenous sodium pentobarbital (30-50 mg/kg). After a craniotomy, the animals were perfused transcardially with ice cold, oxygenated PBS for 90 seconds. The brains were rapidly removed, blocked and frozen in isopentane at -40°C and stored at -80°C until processed for *in vitro* receptor autoradiography. Twenty micron coronal sections were collected in a cryostat maintained at -20°C, and thaw mounted on plus-charged slides (Brain Research Laboratories, Newton MA). The slides were vacuum desiccated overnight at 4°C, at which time they were transferred to -80°C and stored until use.

Corticotropin Releasing Hormone Receptor Autoradiography

Procedures for *in vitro* receptor autoradiography for CRH-R1 and CRH-R2 were adapted from Sanchez et al. (1999) and Reubi et al. (2003). Briefly, duplicate sections at two levels were preincubated twice for 15 minutes each in room temperature 0.05M Tris-Hydrochloric acid (HCl; pH 7.4) buffer. Assessment of total CRH binding was performed in the same buffer containing 0.2nM [¹²⁵I] Tyr-Sauvagine, 5mM magnesium chloride, 2mM ethylene glycol tetraacetic acid (EGTA), 0.1% bovine serum albumin (BSA), 0.1mM bacitracin, and 0.3μM aprotinin. Evaluation of CRH-R2 was performed in the same buffer with the addition of 20nM Stressin₁ (for blockade of CRH-R1) or with 20nM Astressin₂-B (for blockade of CRH-R2) for 2.5 hours at room temperature. Adjacent sections at each level were incubated in the same buffer containing 1μM unlabelled sauvagine for non-specific binding. The sections were washed 5 times for 2

minutes each in ice-cold assay buffer (0.05M Tris-HCl; 0.3% BSA, pH 7.4), dipped twice in 4° ddH₂O and dried under a cool stream of air. Sections were apposed to phosphorimaging plates along with [¹⁴C] microscale standards (GE Bioscience) for 72 hours and developed using a Fuji BAS5000 phosphorimaging system.

Statistical Analysis

Mean daily and total ethanol consumption (g/kg) measures were calculated for the ethanol-drinking monkeys. Daily means were analyzed using two-way repeated measures ANOVA. BECs (mg%) were determined and correlated with ethanol consumed. Peak response and AUC values were calculated for cortisol (ug/dl) and ACTH (pg/ml) levels for each pharmacological challenge (saline, CRH, EtOH 1.0 g/kg and EtOH 1.5 g/kg). Analysis of variance was calculated with rearing groups and EtOH conditions as main factors and a multiple pairwise comparison (Holms-Sidak method) when appropriate. Quantitative densitometric analysis of autoradiograms was performed using AIS/C image analysis system (Imaging Research, St. Catherines, Ontario). Digitized images of the isotope-labeled section and an adjacent Nissl stained section were overlapped in Photoshop to accurately identify and measure specific nuclei and cortical fields within each region. Optical density was measured for two regions of interest within each field that lie entirely within the boundary of the target layer. Optical density was converted to fmol/mg of wet weight tissue by reference to the GE Bioscience [¹⁴C] microscale standards. Rearing and treatment effects were evaluated using ANOVA. There were no differences detected between the groups as a result of rearing. The data were then analyzed using treatment and brain regions as main factors with ANOVA followed by multiple pairwise comparisons (Holms-Sidak method) where appropriate.

Percent difference of the density means for EtOH and control groups were calculated for each measured region.

RESULTS

Ethanol Drinking and Blood Ethanol Concentrations

Following one year of 22 hour free access to EtOH, nursery-reared monkeys had consumed significantly more EtOH than mother-reared monkeys (two-way ANOVA, $p < 0.01$; Figure 2 and Table I). Blood ethanol concentrations that were sampled approximately every six days during the study correlated with EtOH dose at the time of blood draw ($r^2 = 0.67$, $p < 0.001$; Figure 3).

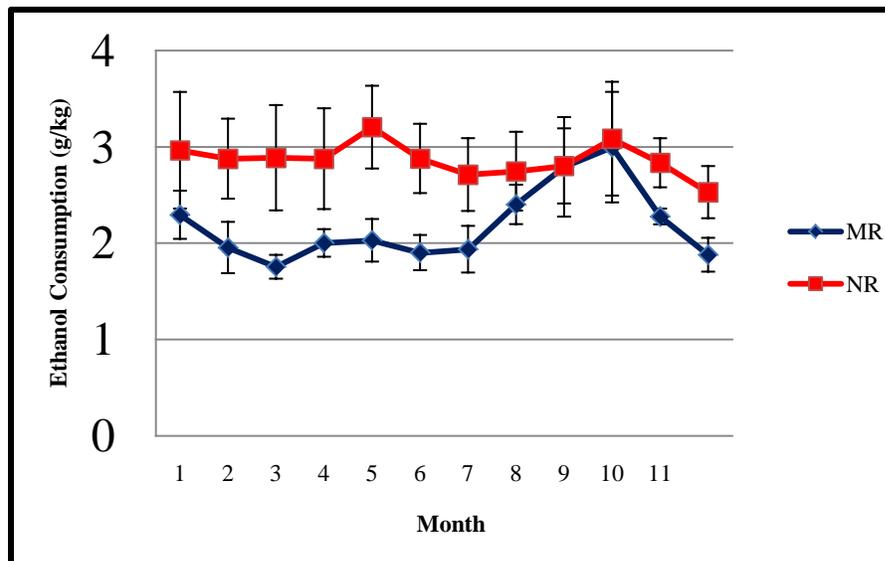


Figure 2. Graphical representation of mean daily EtOH consumption (g/kg) in monthly increments across 12 months of open access. Two-way ANOVA reveals that nursery-reared monkeys drank significantly more EtOH than mother-reared monkeys ($p < 0.01$).

Table I. Ethanol consumption across the entire length of the study for individual monkey. These totals include the 12 month open access period and the continued open access through endocrine profiles to necropsy, a total of approximately 420 days.

	<i>Monkey</i>	<i>Average Daily Intake (SEM)</i> (g/kg)	<i>Total Intake</i> (g/kg)
MR	1466	2.25 ± 0.03	952
	1469	1.90 ± 0.03	799
	1470	2.23 ± 0.06	951
	1471	2.12 ± 0.03	908
NR	1467	1.98 ± 0.03	824
	1468	3.37 ± 0.05	1412
	1472	3.46 ± 0.05	1493
	1473	2.38 ± 0.03	1034

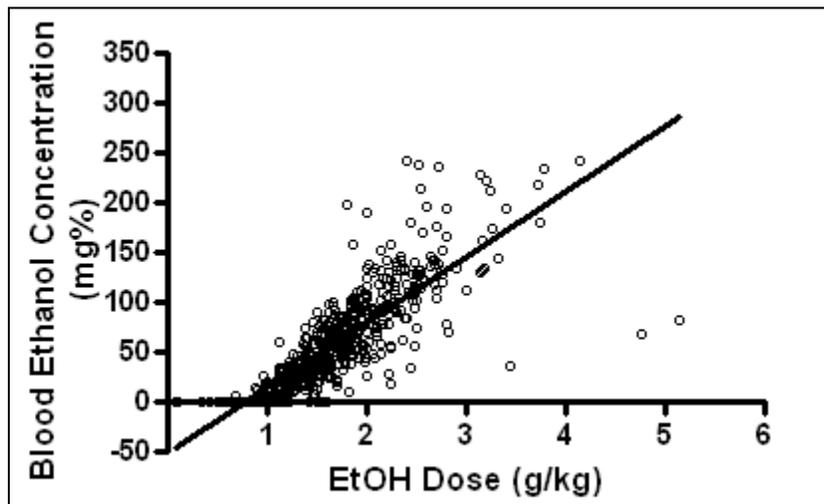


Figure 3. Blood EtOH concentration (mg%) sampled 6 hours post session start during 22 hour open access to EtOH and corresponding EtOH consumption. There is a significant correlation between blood EtOH concentration and EtOH consumption ($r^2=0.67$, $p<0.001$).

Endocrine Profiles

Baseline Cortisol and Adrenocorticotropin Levels

There were no significant differences in the resting (awake blood draw in the home cage) values for cortisol or ACTH between rearing conditions at pretreatment profiles (Table II), although there was a tendency for higher cortisol among the nursery-reared monkeys (ANOVA, $p=0.1$). Following long-term EtOH self-administration, monkeys exhibited lower resting values for cortisol than their Pre-EtOH levels (ANOVA, $p<0.01$; Table III). Pairwise comparisons revealed significantly lower cortisol levels in both rearing conditions ($p<0.05$). There were no baseline differences in ACTH levels.

Table II. Resting cortisol (ug/dl) and ACTH (pg/ml) levels measured from within cage, minimal stress blood draws. Samples were taken from Cohorts 1 and 2 in pretreatment conditions before EtOH or MD consumption. Nursery-reared monkeys tended to have higher cortisol levels (ANOVA, $\#p=0.1$) as compared to mother-reared monkeys.

	Cortisol		ACTH	
	<i>MR</i>	<i>NR</i>	<i>MR</i>	<i>NR</i>
Cohort 1	10.40 \pm 3.0	12.59 \pm 2.2 [#]	54.70 \pm 5.7	51.26 \pm 4.9
Cohort 2	5.79 \pm 1.1	10.67 \pm 1.7	57.71 \pm 7.2	65.47 \pm 3.1

Table III. Resting cortisol (ug/dl) and ACTH (pg/ml) levels measured within cage, minimal stress blood draws. Samples were taken from Cohort 1 before and after one year EtOH self-administration. Regardless of rearing group, monkeys exhibited lower resting values of cortisol during Post-EtOH conditions as opposed to Pre-EtOH conditions (ANOVA, $*p<0.01$).

	Cortisol		ACTH	
	<i>MR</i>	<i>NR</i>	<i>MR</i>	<i>NR</i>
Pre-EtOH	10.40 \pm 3.0	12.59 \pm 2.2	54.70 \pm 5.7	51.26 \pm 4.9
Post-EtOH	3.06 \pm 1.1	5.74 \pm 1.7 [*]	59.84 \pm 7.4	58.71 \pm 10.5

Effect of Ethanol on Endocrine Challenge Responses

Analysis of endocrine profile challenges revealed significant changes following one year of EtOH self-administration (Tables IV & V). There were alterations in several of the profile challenges.

ACTH Release

Saline Challenge

Rearing effects: There was a significant main effect of rearing on decreased ACTH release in nursery-reared monkeys (peak, $p<0.01$; AUC, $p<0.01$) following drinking. Post-hoc analysis revealed significantly lower ACTH levels in Post-EtOH nursery-reared monkeys (peak, $p<0.001$; AUC, $p<0.001$).

EtOH Effects: There was a significant main effect of EtOH on increased ACTH release (peak, $p<0.01$), with higher levels of ACTH in the mother-reared group (peak, $p<0.001$; AUC, $p<0.01$).

CRH Challenge

Rearing Effects: There was a significant main effect of rearing on decreased ACTH release in nursery-reared monkeys (peak, $p<0.05$; AUC, $p<0.01$).

EtOH Effects: There was a significant main effect of EtOH on increased ACTH release (peak, $p<0.01$; AUC, $p<0.01$) with higher peak values of ACTH ($p<0.05$) in the Post-EtOH condition for both rearing groups while the nursery-reared group had significantly higher AUC values of ACTH ($p<0.01$).

EtOH 1.0 g/kg Challenge

Rearing Effects: There was a significant main effect of rearing on decreased ACTH release in nursery-reared monkeys (AUC, $p<0.05$).

EtOH Effects: There was a significant main effect of EtOH on increased ACTH release (peak, $p < 0.001$; AUC, $p < 0.01$) with significantly higher levels of ACTH in the Post-EtOH condition for both rearing groups (peak, $p < 0.01$) and higher levels of ACTH in the mother-reared group (AUC, $p < 0.05$).

EtOH 1.5 g/kg Challenge

Rearing Effects: There was a significant main effect of rearing on decreased ACTH release in nursery-reared monkeys (AUC, $p < 0.05$).

EtOH Effects: There was a significant main effect of EtOH on increased ACTH release (peak, $p < 0.01$; AUC, $p < 0.001$) with significantly higher levels of ACTH in the Post-EtOH condition for both rearing groups (AUC, $p < 0.01$) and higher levels of ACTH in the mother-reared group (peak, $p < 0.05$) Pre- and Post-EtOH.

Following EtOH, both rearing groups had increased ACTH across all challenges, though between rearing groups, the nursery-reared animals had decreased ACTH compared to mother-reared animals. There was no effect of EtOH dose.

Cortisol Release

Saline Challenge

Rearing Effects: There was a significant main effect of rearing on decreased cortisol release in nursery-reared monkeys (peak, $p < 0.01$; AUC, $p < 0.05$) and lower cortisol levels in Post-EtOH nursery-reared monkeys (peak, $p < 0.01$; AUC, $p < 0.05$).

EtOH Effects: There was no significant effect of EtOH on cortisol levels in the saline challenge.

CRH Challenge

Rearing Effects: There was no significant effect of rearing on cortisol levels in the CRH challenge.

EtOH Effects: There was a significant main effect of EtOH on decreased cortisol release (AUC, $p < 0.05$).

EtOH 1.0 g/kg Challenge

Rearing Effects: There was no significant effect of rearing on cortisol levels in the EtOH 1.0 g/kg challenge.

EtOH Effects: There was no significant effect of EtOH on cortisol levels in the EtOH 1.0 g/kg challenge.

EtOH 1.5 g/kg Challenge

Rearing Effects: There was a significant main effect of rearing on decreased cortisol release in nursery-reared monkeys (AUC, $p < 0.05$) with significantly lower cortisol levels in Post-EtOH nursery-reared monkeys (AUC, $p < 0.05$).

EtOH Effects: There was no significant effect of EtOH on cortisol levels in the EtOH 1.5 g/kg challenge.

One year of EtOH exposure led to lower cortisol levels at baseline and following the CRH challenge despite generally increased adrenocorticotropin (ACTH) levels in the saline, CRH, and EtOH challenges. Though the rearing groups were similar at pre-treatment levels, differences emerged following one year of EtOH with nursery-reared monkeys exhibiting decreased cortisol release in the saline and EtOH challenges and decreased ACTH release in the saline, CRH and EtOH challenges.

Table IV. ACTH levels (mean \pm SEM) as measured by AUC and peak (pg/ml) values from blood sampled during endocrine profiles. Samples from Cohort 1 during endocrine profiles at Pre- and Post-EtOH self-administration. An ANOVA revealed a significant main effect of EtOH (represented by *) with indicated pairwise comparisons (* p <0.05, ** p <0.01, *** p <0.001) within each challenge. An ANOVA revealed a significant main effect of rearing (represented by #) with indicated pairwise comparisons (# p <0.05, # p <0.01, # p <0.001) within each challenge.

		Area Under the Curve Values		Peak Values	
		<i>MR</i>	<i>NR</i>	<i>MR</i>	<i>NR</i>
Saline	Pre-EtOH	6038 \pm 143	5787 \pm 371	63.4 \pm 4.0	58.9 \pm 5.0
	Post-EtOH	8927 \pm 956 **	4749 \pm 682 ###	109.8 \pm 6.8***	61.3 \pm 10.1###
CRH	Pre-EtOH	4854 \pm 222	3481 \pm 250	88.6 \pm 5.0	67.0 \pm 4.8
	Post-EtOH	5725 \pm 447	5188 \pm 249*##	117.9 \pm 12.4*	99.7 \pm 5.2**
EtOH 1.0 g/kg	Pre-EtOH	6642 \pm 367	5123 \pm 527	69.7 \pm 4.2	62.8 \pm 1.8
	Post-EtOH	9102 \pm 1098*	7277 \pm 767#	101.0 \pm 5.0**	91.3 \pm 8.5**
EtOH 1.5 g/kg	Pre-EtOH	5842 \pm 382	4187 \pm 770	66.5 \pm 6.2	52.8 \pm 5.0
	Post-EtOH	9391 \pm 796**	7384 \pm 710***#	99.7 \pm 12.4*	80.2 \pm 10.9

Table V. Cortisol levels (mean \pm SEM) as measured by AUC and peak (ug/dl) values during endocrine challenges. Samples were taken from Cohort 1 before and after one year of EtOH self-administration. There was a significant main effect of EtOH (represented by *) and significant pairwise comparisons are indicated (* p <0.05). There was also a significant main effect of rearing (represented by #) with pairwise comparisons as indicated (# p <0.05, # p <0.01).

		Area Under the Curve Values		Peak Values	
		MR	NR	MR	NR
Saline	Pre-EtOH	2614 \pm 122	2378 \pm 267	27.6 \pm 2.1	24.1 \pm 2.3
	Post-EtOH	2680 \pm 73	1805 \pm 291 [#]	29.3 \pm 0.1	20.6 \pm 2.6 ^{##}
CRH	Pre-EtOH	1730 \pm 118	1587 \pm 118	32.3 \pm 2.7	29.9 \pm 2.2
	Post-EtOH	1467 \pm 40 [*]	1349 \pm 90 [*]	29.4 \pm 0.5	25.6 \pm 1.5
EtOH 1.0 g/kg	Pre-EtOH	2530 \pm 173	2086 \pm 284	27.4 \pm 1.4	21.8 \pm 2.9
	Post-EtOH	2792 \pm 161	2515 \pm 380	25.4 \pm 1.2	23.8 \pm 3.4
EtOH 1.5 g/kg	Pre-EtOH	2461 \pm 196	1810 \pm 220	24.8 \pm 2.1	21.3 \pm 3.1
	Post-EtOH	3150 \pm 196	2144 \pm 506 [#]	29.1 \pm 1.4	21.1 \pm 4.0

Cohort Differences to Endocrine Challenges

To examine differences within each rearing condition between the cohort of monkeys that drank ethanol (Cohort 1) and the control cohort that drank a maltose-dextrin solution isocaloric with ethanol (Cohort 2), baseline profiles (prior to treatment) were analyzed to compare the two groups. An ANOVA revealed a significant main effect of Cohort in several of the endocrine profile challenges (Tables VI & VII).

ACTH Release

Saline Challenge

There was a significant main effect of cohort with Cohort 2 having significantly higher ACTH release (peak, $p < 0.05$; AUC, $p < 0.05$).

CRH Challenge

There was a significant main effect of cohort, with Cohort 2 having significantly higher ACTH release (AUC, $p < 0.05$). Posthoc analysis showed significantly higher levels of ACTH in nursery-reared monkeys (AUC, $p < 0.05$).

EtOH 1.0 g/kg Challenge

There was a significant main effect of cohort revealed Cohort 2 having significantly higher ACTH release (AUC, $p < 0.05$).

EtOH 1.5 g/kg Challenge

There was a significant main effect of cohort with Cohort 2 having significantly more ACTH release (peak, $p < 0.05$; AUC, $p < 0.01$) with significantly higher levels of ACTH in nursery-reared monkeys (AUC, $p < 0.05$).

Cortisol Release

Saline Challenge

There was a significant main effect of cohort with Cohort 2 having significantly higher blood levels of cortisol (peak, $p < 0.001$; AUC, $p < 0.01$); specifically with significantly higher levels of cortisol in both nursery-reared (peak, $p < 0.01$; AUC, $p < 0.01$) and mother-reared (peak, $p < 0.05$) animals.

CRH Challenge

There was no significant effect of cohort on cortisol levels in the CRH challenge.

EtOH 1.0g/kg Challenge

There was a significant main effect of cohort with Cohort 2 having significantly increased cortisol release (peak, $p<0.05$; AUC, $p<0.05$); specifically with significantly higher levels in nursery-reared monkeys (peak, $p<0.05$; AUC, $p<0.05$).

EtOH 1.5 g/kg Challenge

There was a main effect of cohort with Cohort 2 having significantly increased cortisol release (peak, $p<0.05$; AUC, $p<0.01$); specifically with significantly higher levels of cortisol in nursery-reared monkeys (peak, $p<0.05$; AUC, $p<0.01$).

Endocrine challenges of cohorts at pre-treatment conditions revealed a significant effect of cohort across almost every challenge. Cohort 2 had an increased responsiveness to a stressor during the profiles, depicted by higher ACTH and cortisol levels.

Table VI. ACTH levels (mean \pm SEM) as measured by AUC and peak (pg/ml) values during endocrine challenges. Samples were taken from Cohorts 1 and 2 before EtOH and MD self-administration. An ANOVA reveal a significant main effect of cohort with Cohort 2 showing higher cortisol levels, pairwise comparisons are indicated ($^{\wedge}p<0.05$)

		Area Under the Curve Values		Peak Values	
		MR	NR	MR	NR
Saline	Cohort 1	6038 \pm 143	5787 \pm 371	63.4 \pm 4.0	58.9 \pm 5.0
	Cohort 2	10250 \pm 2314	9470 \pm 1832 [^]	115.6 \pm 24.2	99.5 \pm 20.9 [^]
CRH	Cohort 1	4854 \pm 222	3481 \pm 25	88.6 \pm 5.0	67.0 \pm 4.8
	Cohort 2	5510 \pm 694	5234 \pm 656 [^]	75.2 \pm 18.2	102.9 \pm 12.8
EtOH 1.0 g/kg	Cohort 1	6642 \pm 367	5123 \pm 527	69.7 \pm 4.2	62.8 \pm 1.8
	Cohort 2	10208 \pm 2284	7837 \pm 637 [^]	140.1 \pm 52.9	96.7 \pm 17.7
EtOH 1.5 g/kg	Cohort 1	5842 \pm 382	4187 \pm 770	66.5 \pm 6.2	52.8 \pm 5.0
	Cohort 2	8503 \pm 1403	7500 \pm 620 [^]	94.7 \pm 17.4	76.4 \pm 5.8 [^]

Table VII. Cortisol levels (mean \pm SEM) as measured by AUC and peak (ug/dl) values during endocrine challenges. Samples were taken from Cohorts 1 and 2 before EtOH and MD self-administration. An ANOVA reveal a significant main effect of Cohort with Cohort 2 showing higher cortisol levels, pairwise comparisons are indicated ($^{\wedge}$ p<0.05, $^{\wedge}$ p<0.01).

		Area Under the Curve Values		Peak Values	
		MR	NR	MR	NR
Saline	Cohort 1	2614 \pm 122	2378 \pm 267	27.6 \pm 2.1	24.1 \pm 2.3
	Cohort 2	3420 \pm 285	3760 \pm 461 $^{\wedge}$	38.4 \pm 7.3 $^{\wedge}$	42.4 \pm 4.6 $^{\wedge}$
CRH	Cohort 1	1730 \pm 118	1587 \pm 118	32.3 \pm 2.7	29.9 \pm 2.2
	Cohort 2	1963 \pm 356	1763 \pm 119	36.0 \pm 6.37	33.8 \pm 1.5
EtOH 1.0 g/kg	Cohort 1	2530 \pm 173	2086 \pm 284	27.4 \pm 1.4	21.8 \pm 2.9
	Cohort 2	3658 \pm 560	3609 \pm 522 $^{\wedge}$	35.9 \pm 4.9	37.0 \pm 6.1 $^{\wedge}$
EtOH 1.5 g/kg	Cohort 1	2461 \pm 196	1810 \pm 220	24.8 \pm 2.1	21.3 \pm 3.1
	Cohort 2	3078 \pm 390	3236 \pm 413 $^{\wedge}$	29.1 \pm 4.47	33.5 \pm 3.0 $^{\wedge}$

Corticotropin-Releasing Hormone Autoradiography

The distribution of specific binding of [125 I] Tyr-Sauvagine for both CRH-R1 and CRH-R2 in both control animals and drinkers was comparable to published literature with dense binding throughout the amygdaloid complex and cortical regions ventral to the insula (Figures 4 & 5; Sanchez et al., 1999). CRH receptor binding densities were at background in the CeA and the PVN in our animals.

There were no significant differences between rearing groups in any brain region, as revealed by a two-way ANOVA. Based on this result, the rearing groups were pooled to compare differences in CRH receptor binding between the EtOH and control drinkers.

Figure 4 depicts a representative autoradiogram of total CRH receptors at the level of the hypothalamus and amygdala for the EtOH and control groups. Binding was measured in detectable areas of the amygdala with distribution of ABpc > PAC > Lat > ABmc > Bmc. There was a significant main effect of EtOH in the amygdaloid complex with 30.0% ($p < 0.01$) less CRH-R1 binding overall and 15.1% ($p < 0.05$) more CRH-R2 binding overall (Figures 5 & 6 and Table VIII). Pairwise comparisons reveal significantly less CRH-R1 binding in the ABpc (42.0%, $p < 0.01$) and the lateral nuclei (42.0%, $p < 0.05$).

There was a significant main effect of EtOH in the insular and entorhinal cortex, with 48.4 % ($p < 0.01$) less dense CRH-R1 binding and no significant changes to CRH-R2 binding (Figures 5 & 7 and Table IX). Pairwise comparisons revealed significantly decreased CRH-R1 binding the entorhinal cortex (50.1%, $p < 0.01$) primarily in layers I-III, dysgranular insular cortex (50.1%, $p < 0.01$) primarily in layers I-V, and the granular insular cortex (41.2%, $p < 0.05$) primarily in layers I-V.

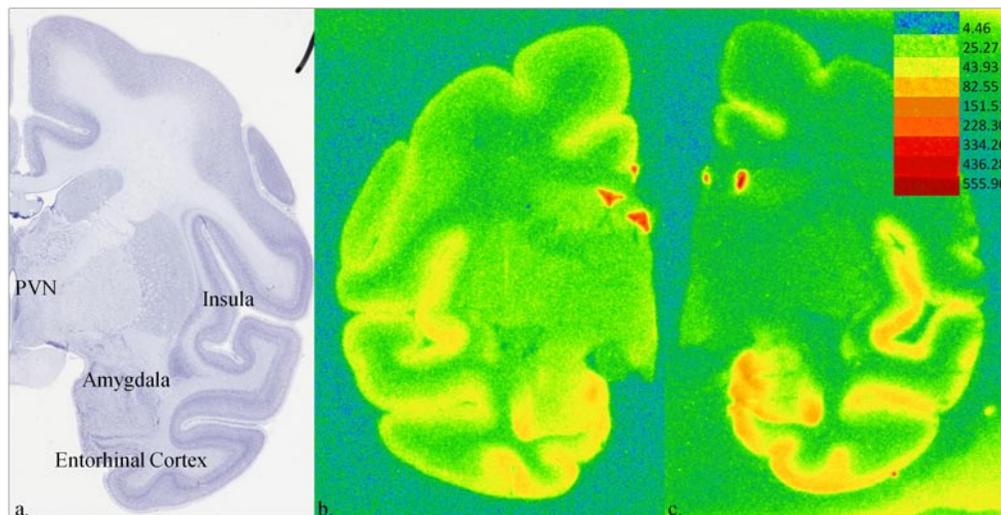


Figure 4. Representative nissl section (a.) (adapted from www.brainmaps.org) with measured brain regions labelled and total CRH receptor binding density autoradiogram from an EtOH drinker (b.) and a control drinker (c.).

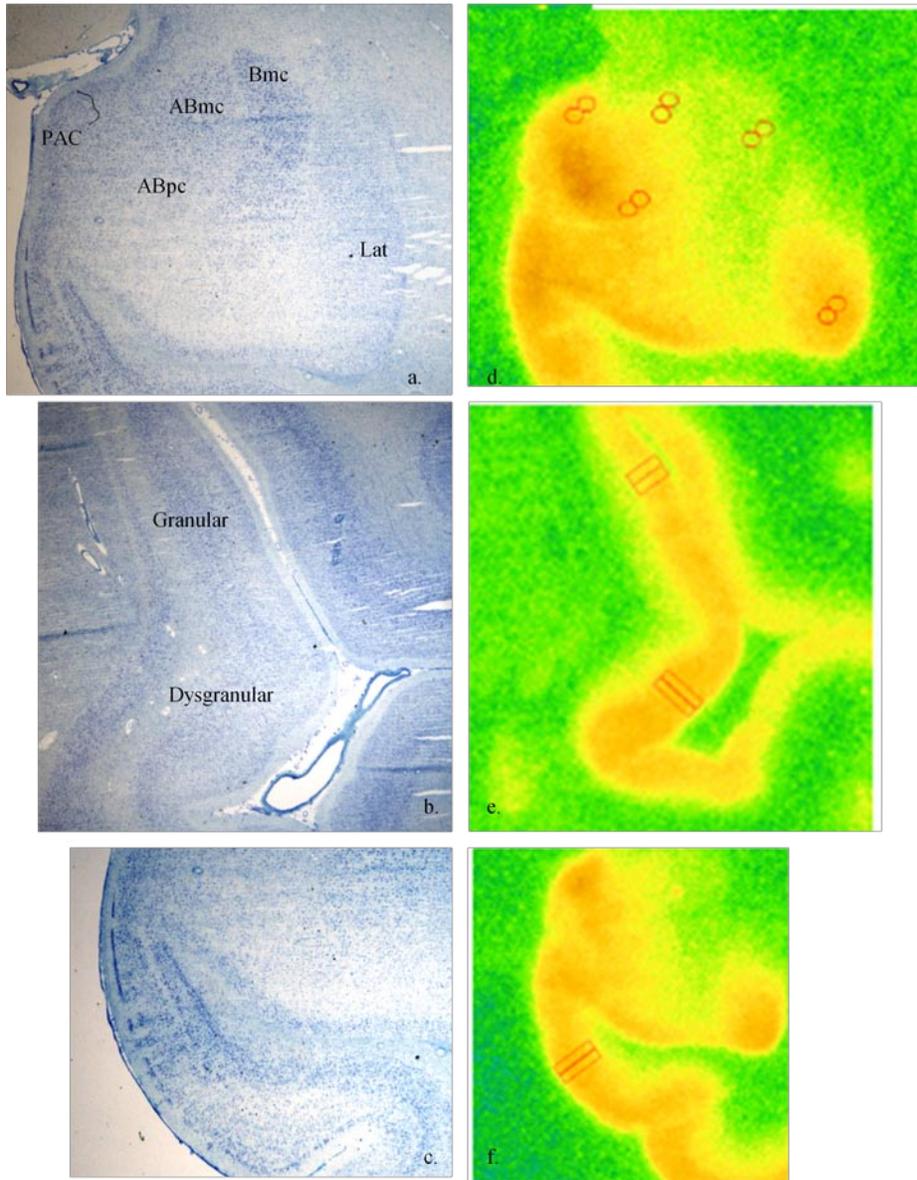


Figure 5. Representative nissl and autoradiogram sections from amygdala (a. & d.) with nuclei labelled, insula (b. & e.) with regions labelled and entorhinal cortex (c. & f.).

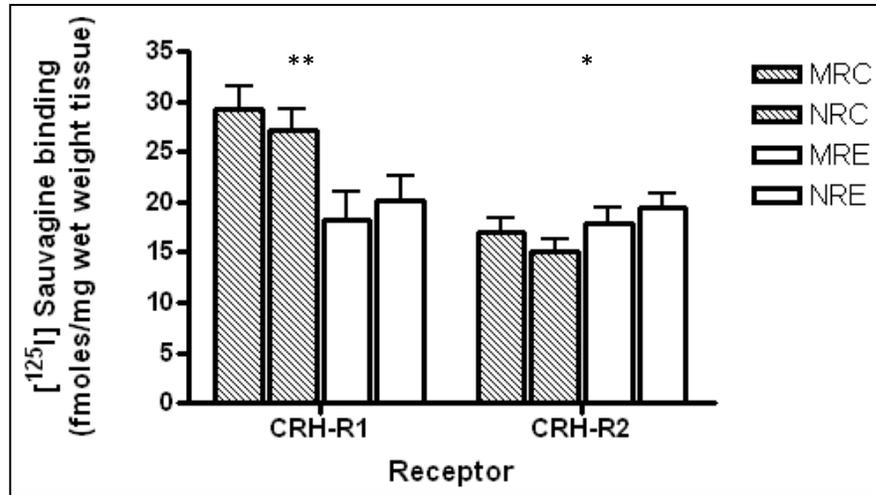


Figure 6. Graphical representation of CRH receptor densities represented as mean (SEM) of measured ROIs from sampled areas within the amygdala given in [¹²⁵I] Tyr-Sauvagine binding (fmols/mg of wet-weight tissue). There were no significant rearing differences. There was a significant effect of EtOH with decreased overall CRH-R1 binding (ANOVA, **p<0.01) and increased CRH-R2 binding (*p<0.05) in EtOH drinkers as compared to control drinkers when rearing groups were pooled.

Table VIII. CRH receptor densities represented as mean (SEM) of measured ROIs from sampled areas given in [¹²⁵I] Tyr-Sauvagine binding (fmols/mg of wet-weight tissue). There was a significant effect of EtOH with decreased overall CRH-R1 binding (ANOVA, **p<0.01) and increased CRH-R2 binding (*p<0.05). Specifically, the parvocellular cells of the accessory basal nucleus (**p<0.01) and the lateral nucleus (*p<0.05) showed significantly decreased CRH-R1 binding.

	CRH-R1			CRH-R2		
	<i>Control</i>	<i>EtOH</i>	<i>Percent Difference</i>	<i>Control</i>	<i>EtOH</i>	<i>Percent Difference</i>
ABmc	13.8 ±2.2	12.2 ±1.1	-11.5	11.7 ±0.9	14.7 ±2.5	20.2
ABpc	46.2 ±4.3	26.8 ±5.1	-42.0 **	22.6 ±1.8	23.3 ±2.2	3.1
Bmc	4.5 ±1.2	3.8 ±0.9	-15.9	7.3 ±1.1	9.2 ±1.9	20.9
Lat	36.9 ±2.2	21.4 ±3.0	-42.0 *	17.7 ±1.0	21.3 ±4.6	16.7
PAC	39.0 ±5.5	32.8 ±5.5	-15.8	20.6 ±1.8	25.6 ±2.3	19.5
Overall	28.1 ±1.5	19.4 ±1.70	-30.9 **	16.0 ±0.9	18.8 ±1.0	15.1 *

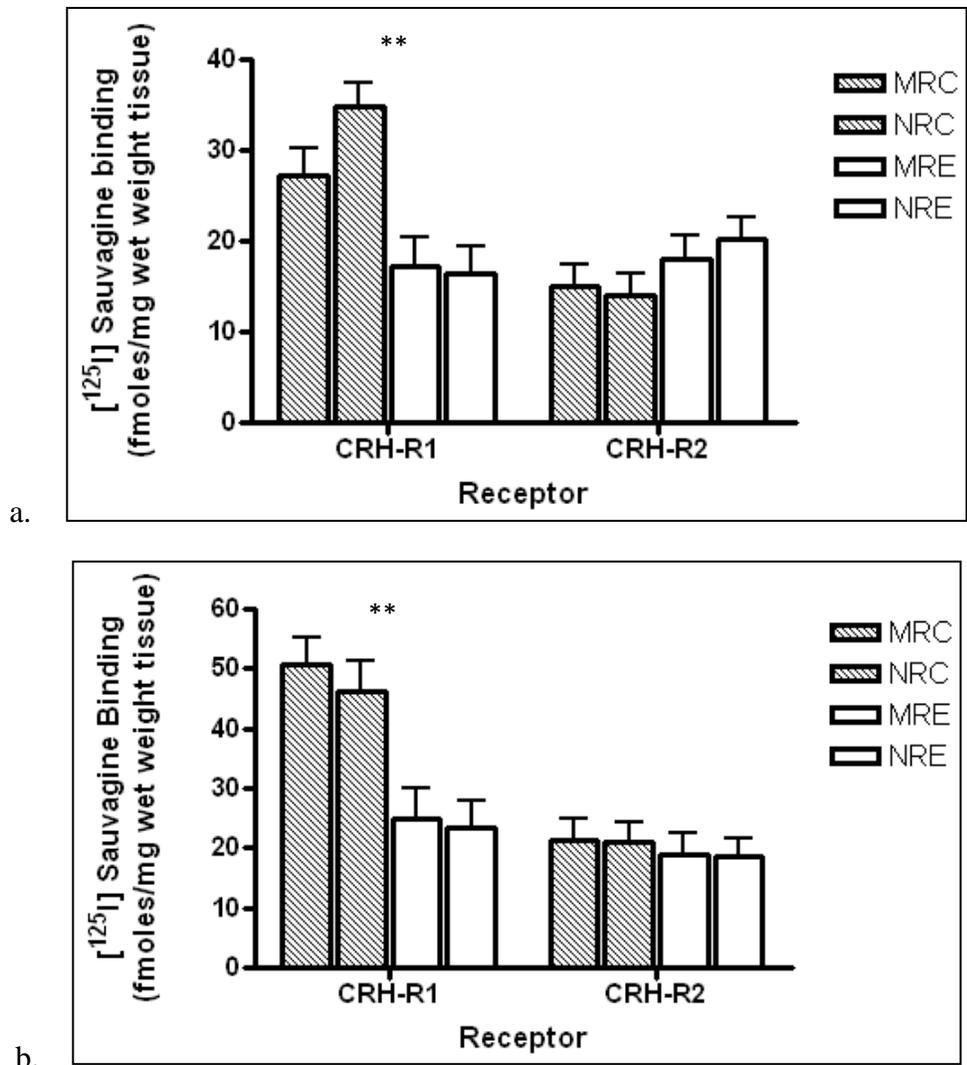


Figure 7. Graphical representation of CRH receptor densities in the Insula (a) and the Entorhinal (b) cortices represented as mean (SEM) of measured ROIs from sampled areas given in [¹²⁵I] Tyr-Sauvagine binding (fmols/mg of wet-weight tissue). There were no significant rearing differences. There was a significant effect of EtOH with overall decreased CRH-R1 binding (ANOVA, **p<0.01) in EtOH drinkers as compared to control drinkers when rearing groups were pooled.

Table IX. CRH receptor densities represented as mean (SEM) of measured ROIs from sampled cortical areas given in [¹²⁵I] Tyr-Sauvagine binding (fmols/mg of wet-weight tissue). There was a significant effect of EtOH with overall decreased CRH-R1 binding (ANOVA, **p<0.01). Individually, CRH-R1 binding was decreased in the entorhinal (**p<0.01), dysgranular insula (**p<0.01) and the granular insula (*p<0.05) cortices.

	CRH-R1			CRH-R2		
	<i>Control</i>	<i>EtOH</i>	<i>Percent Difference</i>	<i>Control</i>	<i>EtOH</i>	<i>Percent Difference</i>
Entorhinal	48.9 ±4.0	24.2 ±3.4	-50.1 **	21.8 ±1.4	18.8 ±2.8	-13.9
Dysgranular Insula	38.4 ±4.1	19.2 ±1.7	-50.1 **	17.8 ±1.1	19.3 ±3.1	8.1
Granular Insula	24.7 ±3.2	14.5 ±1.3	-41.2 *	12.9 ±1.1	17.7 ±3.2	27.0
Overall	37.3 ±1.8	19.3 ±1.9	-48.4 **	16.7 ±1.6	19.0 ±1.3	12.0

DISCUSSION

One year of ethanol (EtOH) self-administration was sufficient to significantly decrease corticotropin-releasing hormone type 1 (CRH-R1) receptors in the amygdaloid complex, insula and entorhinal cortex of rhesus monkeys. Binding densities for CRH-R1 decreased by 30-50% in the measured brain regions (Figures 5, 6, & 7) and densities for CRH-R2 increased by 15% in the amygdala.

This study also revealed that one year of EtOH exposure led to decreased cortisol levels at home cage baseline and following the CRH challenge. We also observed dissociation between ACTH and cortisol release. Specifically, there was increased adrenocorticotropin (ACTH) release following the saline, CRH, and EtOH challenges, with either no change (saline, EtOH) or decreased (CRH) cortisol release. The rearing groups responded similarly before drinking, but the nursery-reared (NR) monkeys showed lower ACTH response to all challenges and lower cortisol response during the saline and EtOH challenges after drinking than mother-reared (MR) monkeys. Hormones were measured by area under the curve (AUC) and peak levels, both of which assess hypothalamic-pituitary-adrenal axis function and primarily complement one another throughout our findings.

This study is the first of its kind to investigate neurobiological alterations following early life stress (ELS) and chronic EtOH drinking. Utilizing the schedule-induced drinking model in monkeys allowed us to study voluntary drinking behaviors across an extended time period and investigate how these behaviors are affected by early maternal separation, a model that parallels similar experiences in humans. This design

provided the opportunity to probe the HPA axis before and after chronic EtOH consumption and is also the first report of CRH receptor binding alterations following chronic EtOH self-administration.

Chronic Ethanol Alters Corticotropin-Releasing Hormone Receptor Densities

Though the literature does not describe alterations of CRH receptor binding in extrahypothalamic brain regions following alcohol exposure, our results do agree with rodent studies that report decreased CRHR-1 transcripts following chronic self-administration in non-dependent animals (Lee et al., 2001; Hansson et al., 2007). In another study, CRH-R1 transcripts increased following drinking in dependent rodents (Sommer et al., 2008). We found a robust decrease in CRH-R1 binding in all measured brain regions and a small increase in CRH-R2 binding in the amygdala.

The lack of detectable CRH receptor binding in the paraventricular nucleus of the hypothalamus (PVN) and central amygdala (CeA) is unexpected and differs somewhat from reports in monkeys (Sanchez et al., 1999) and rodents (De Souza et al., 1985), though some rodent studies report findings similar to ours (Sommer et al., 2008; Makino et al., 2005).

Low levels of both receptor types were reported in the PVN and CeA in normal rhesus monkeys (Sanchez et al., 1999), but in our study, binding in these two structures was at background. Despite being unable to detect CRH receptors in the CeA, other nuclei of the amygdala exhibited the same binding pattern found by the Sanchez group with relative densities in the accessory basal > periamygdaloid cortex > lateral > basal nucleus matching in both studies. In the Sanchez report, CeA CRH-R1 binding levels

were at 25% of ABpc levels and in our study, 25% of ABpc CRH-R1 measured below background binding which could account for differential measures in the two studies.

The differential results may be due to the use of different blockers in the [¹²⁵I] Tyr-Sauvagine incubation solutions or differing incubation times. Sanchez and colleagues used CP-154,526, a selective CRH-R1 antagonist to measure CRH-R2 binding and calculated CRH-R1 binding. In the present study, we used Stressin₁, a selective CRH-R1 antagonist to measure CRH-R2 and Astressin_{2B}, a selective CRH-R2 antagonist to measure CRH-R1 (Reubi et al., 2003). Another difference between these studies is the method of film exposure, our study used phosphorimaging plates with 3 day exposure to minimize possible saturation while the Sanchez study used BioMax MR film with a 4 day exposure.

Further analysis of CRH receptor densities and EtOH consumption levels reveal no correlation between the variables. Monkeys that drank >3.0 g/kg daily did not have significantly different binding densities than drinkers who consumed an average of 2.14 g/kg daily. It, therefore, seems that significantly reduced CRH-R1 binding can be induced after a certain threshold level of drinking is attained rather than related to dose.

Downregulation of Corticotropin-Releasing Hormone Receptors

It is clear from the CRH receptor binding results as well as the CRH endocrine challenge that CRH receptors are profoundly different following one year of EtOH self-administration. The CRH endocrine challenge examines receptor function at the level of the anterior pituitary by the ability of receptor activation to produce the release of ACTH. After one year of EtOH exposure, ACTH levels are increased across every challenge. Chronic stress has been reported to cause downregulation of CRH-R1 binding and gene

expression in the pituitary (Dave et al., 1986; Zhou et al., 2000), although, an increase in corticotroph responsiveness has also been reported (Aguilera et al., 2001). Therefore, robust release of ACTH can be achieved even with a decrease in available receptors. Downregulation of the CRH-R1 receptors in the pituitary is associated with the activation of protein kinase A (PKA; Moriyama et al., 2005). Increased ACTH release during times of stress is interpreted throughout the literature as an increased vulnerability to stressors in humans and animals that have experienced chronic adversity (Aguilera et al., 2001; Sanchez, 2001; 2006).

A decrease in CRH-R1 binding within the amygdala and cortical regions supports the notion that this receptor system is affected by chronic EtOH exposure, possibly by increased CRH release. Agonist binding to CRH receptors results in the initiation of various intracellular pathways (Hillhouse & Grammatopoulos, 2006; Refojo & Holsboer, 2009), including the recruitment of several G protein-coupled receptor kinases (GRK) to the membrane. Activated kinases permit receptor interaction with beta-arrestins, leading to uncoupling from G proteins and desensitization of the receptor (Hillhouse & Grammatopoulos, 2006; Holmes et al., 2006). Excessive receptor activation may lead to the internalization and degradation of the receptor (Refojo & Holsboer, 2009). This may explain the decrease in CRH-R1 binding, but does not offer explanation for the small but significant increase in CRH-R2 binding in the amygdala.

Ethanol has the ability to increase CRH in the amygdala and other brain regions (Merlo Pich et al., 1995). EtOH increases CRH release, mRNA expression and gene transcription through cAMP/PKA dependent pathways in cell culture (Li et al., 2005) possibly through the activation of the EtOH-sensitive membrane-bound type 7 adenylyl

cyclase that enhance G protein-coupled receptor activation (Pronko et al., 2010). In rodents and humans, administration of EtOH activates the HPA axis depicted by increased plasma ACTH and cortisol (Rivier et al., 1984; Wand & Dobs, 1991; Richardson et al., 2008). One year of EtOH self-administration, at moderate levels, alters the HPA axis and available CRH receptors in the amygdala and cortical regions regardless of rearing conditions.

One mechanism that could not be addressed in this study is the action of the CRH binding protein (CRH-BP) within the measured areas. The CRH-BP is a freely circulating protein that acts to clear out available CRH (Behan et al., 1995). This protein is found in the amygdala of monkeys and rodents, though one study shows CRH-BP mRNA levels in the amygdala were not affected by chronic EtOH consumption (Lack et al., 2005). If CRH-R1 receptor downregulation is due to increased CRH levels, it would appear that levels of the binding protein were insufficient to buffer the higher levels of free CRH.

Alterations to endogenous urocortin levels could also play a role in the regulation of CRH receptors. Urocortin binds to the CRH-BP as well as both CRH receptors, but urocortin binds with higher affinity to CRH-R2 than the CRH-R1 (Hillhouse & Grammatopoulos, 2006). Though we had a small but significant overall increase of CRH-R2 binding in the amygdala, there are few if any urocortin projections to the measured regions of the amygdala, insula and entorhinal cortices in the monkey (Vasconcelos et al., 2003). Alterations to the urocortin system do not presently explain the current changes to the CRH receptor system.

Chronic Ethanol Alters Hormone Release in the HPA Axis

Chronic EtOH led to a decrease in blood plasma levels of cortisol at baseline and decreases are also seen following acute stressors and in rodents (Van Waes, et al., 2006) and in human alcoholics (Wand & Dobs, 1991; King et al., 2006). In contrast, one year of EtOH exposure led to increased levels of ACTH release during endocrine challenges. EtOH administration is known to increase the release of CRH (Vale et al., 1984) and increased activation of CRH-R1 receptors will lead to increased release of ACTH despite the dissociation with cortisol, shown in our results.

The dissociation in the levels of ACTH and cortisol in the blood has been observed in other cohorts and species in our lab (unpublished results) and has been reported by others as well (see Bornstein et al., 2008 for review). This dissociation may be the result of the actions of EtOH or other factors modulating cortisol release including fluctuating levels of neuropeptides, cytokines, endothelial derived factors and adipocyte-derived factors or a dysfunction of the ACTH receptors within the adrenal gland (Bornstein et al., 2008). Many of the results in the endocrine profiles reveal complementary AUC and peak alterations, though some instances reveal increases in AUC without significant changes in peak levels. AUC measures hormone release over time and may detect alterations of feedback control. The dissociation of AUC and peak may be representing alteration due to the extraneous factors regulating hormone release or a disruption in the feedback systems.

Pre-treatment Hormone Levels Were Different Between Cohorts

In this study, EtOH consuming animals and the MD consuming control group were in different cohorts. An analysis comparing pre-treatment HPA axis measures at

baseline and following endocrine challenges reveal some endocrine differences between these two cohorts of monkeys that may have affected our findings. During the pre-treatment profiling, Cohort 2 (MD) had significantly higher levels of ACTH across all challenges and higher levels of cortisol in the saline and EtOH challenges. The cohorts had similar cortisol levels following the CRH challenge.

Increased cortisol has the ability to decrease CRH protein and receptor protein levels in the PVN and increase in extra-hypothalamic regions (Makino et al., 1994; 1995). The effect of increased plasma cortisol alone, however, cannot explain the alterations to the CRH receptor system because increased activation of glucocorticoid receptors leads to a downregulation of CRH receptors (Yao et al., 2008). Our results reveal a downregulation of CRH receptors in Cohort 1 (EtOH) which display lower levels of ACTH and cortisol in the pretreatment condition. Also, while our results depict an overall change in CRH receptors in the amygdala, pairwise comparisons reveal the changes are limited to the accessory basal nucleus and the lateral nucleus. In addition, the directionality of receptor binding is specific to receptor subtype with decreases in CRH-R1 and increases in CRH-R2 binding densities in the amygdala.

Both cohorts of monkeys were transferred together from the National Institutes of Health to Wake Forest University. Cohort 1 was taken immediately to the testing facility where they were acclimated, trained and entered the study, while Cohort 2 was transferred to a different campus where they lived in a social colony for approximately 1.5 years before entering into the study. This observed cohort effect could be in part due to these differing experiences. Cohort 1 was slightly younger when they entered into the study (6 months), though it is not likely that this small age difference would cause the

observed differences in plasma cortisol and ACTH, because studies with human children show that they reach adult plasma cortisol levels by age 3 (Sippell et al., 1980). This provides additional evidence that the decrease in CRH-R1 binding is due to the EtOH exposure.

Rearing Differences Emerged Following Ethanol Exposure

There were no detected differences between NR and MR monkeys in the pretreatment endocrine profiles or the CRH receptor binding measures. The endocrine results are in contrast to a study that examined rhesus monkeys reared under similar conditions that reported to have lower baseline cortisol and ACTH levels, but tested at a much younger age of 10 months and under ketamine anesthesia (Clarke et al., 1998). Falke and colleagues (2000) reported no baseline cortisol differences following adverse rearing conditions in 8 month old monkeys tested without anesthesia. Aged matched male rhesus monkeys that experienced the same rearing conditions as the monkeys in our study did not differ from MR monkeys with baseline and EtOH challenged ACTH and cortisol levels (Barr et al., 2004).

One year of EtOH self-administration revealed rearing differences between the groups in the endocrine challenges. After drinking, NR monkeys had significantly lower levels of ACTH following all challenges and lower levels of cortisol following the saline and EtOH challenges. This is consistent with human epidemiological and other non-human primate findings that report correlations between ELS and a later blunted stress response to ethanol in humans (Schuckit et al., 1987) and rodents (Van Waes et al., 2006).

Behavioral Outcomes of a Dysfunctional CRH System

Assuming that decreased CRH-R1 binding densities are the result of EtOH-induced increased CRH, it is imperative to understand behavioral outcomes of this overactivated system. Within the amygdala, CRH release leads to increased anxiety-related behaviors (Sajdyk & Gehlert, 2000; Rainnie et al., 2004; Spiga et al., 2006) and activation of the fear memory consolidation process (Hubbard et al., 2007; Roozendaal et al., 2008). Activation of CRH receptors located on glutamatergic neurons within the amygdala leads to increased excitation of the cells as shown through a reduction in inhibitory post synaptic potentials (Rainnie et al., 2004), an increase in the amplitude of field potentials (Ugolini et al., 2008) and an increase in compound post synaptic potentials (Chung & Moore, 2009).

Koob and colleagues have highlighted the importance of CRH in addiction and outlined the ability of CRH to drive dependence and compulsivity in addiction (Koob & Zorrilla, 2010). During EtOH withdrawal, there is an increase of CRH in the amygdala (Merlo-Pich et al., 1995), leading to an increase of anxiety-like behaviors that can be reversed by CRH antagonists (Baldwin et al., 1991; Wills et al., 2009). Application of CRH antagonists also decreases self-administration in post-dependent rodents (Valdez et al., 2002; Heilig & Koob, 2007).

With the ability of CRH antagonists to decrease anxiety-like behaviors that may lead to relapse in an addicted individual (Heinrichs & Koob, 2004; Koob & Kreek, 2007), it is hypothesized that this system may play a role in the withdrawal/negative affect of drug use with addicts using a particular drug (*i.e.*, alcohol) to rid themselves of these

negative feelings (Sarnyai et al., 2001; Koob, 2008). Utilizing receptor antagonists may be a possible therapy to assist addicted individuals from drug relapse. Various studies show that anxiety-related behaviors are reversed following administration of a CRH-R1 antagonist (Spiga et al., 2006; Valdez et al., 2002). Many treatments have reached the clinical trial phase and provide promising therapeutic values for patients with psychiatric disorders (Holsboer & Ising, 2008; Lowry & Thiele, 2010). Our study represents a step forward in understanding how chronic stress and alcohol can affect receptor systems within the brain and continue non-human primate research will continue to provide insight into the ability of stress to dramatically affect neurological systems.

There is a growing consensus that chronic and early stress can lead to negative effects on CRH systems in the brain (Sanchez et al., 2001; Heim & Nemeroff, 2002; Heim et al., 2008). These effects may lead to an increased vulnerability to stress in afflicted individuals. Rodents that experienced early life stress display a heightened vulnerability to stress in behavioral tasks (Aisa et al., 2008) indicated by decreases in glucocorticoid receptors and concomitant increases in anxiety-like behaviors. Within the CRH system, this may be primarily due to the downstream targets of CRH including modulation of the norepinephrine and serotonin systems in the brainstem (Bale, 2006; Lukkes et al., 2009). Rodents deficient in limbic CRH-R1 have less anxiety-like behaviors than wild type littermates; however, following a restraint stress challenge, these rodents have increased stress sensitivity with elevated release of ACTH and cortisol (Muller et al., 2003). Other possible mechanisms include fluctuating levels of cortisol and catecholamines leading to an imbalance of the stress system (Pervanidou, 2008) or dysfunctional cortico-limbic connections as shown through electroencephalographic

abnormalities. (Teicher et al., 2003) and decreased enzymatic activity in the amygdala, hippocampus and parietal cortex (Silveira et al., 2011).

Utilizing a non-human primate model of ELS in combination with voluntary and excessive alcohol drinking, we have evidence that supports the HPA axis and extrahypothalamic stress systems are profoundly altered following these experiences. The benefit of using a non-human primate model allowed us to investigate receptor changes in complex brain regions. In this case, one year of EtOH self-administration is sufficient to robustly alter the physiological response to acute stressors, which may be the result of altered CRH receptor binding densities shown in our study. The unique nursery-rearing paradigm enabled us to conclude that ELS affects the function of the HPA axis only following the chronic consumption of alcohol. The alteration of the CRH system may have anxiety-related and addiction-related behavioral consequences in adulthood.

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CURRICULUM VITAE

Education

Wake Forest University School of Medicine, Graduate Student, Aug 2008 - Present
Department of Physiology and Pharmacology

Appalachian State University, B.S. with Honors in Psychology, Aug 2004 - May 2008
Concentration: Natural Science
Minors: Biology & Chemistry
Honors Thesis: *Delayed Alternation Learning and Memory are Affected by Sound-Induced Seizures in Adult but not Juvenile or Adolescent Rats*

Research Experience

Thesis Project Jan 2009- present
Title: Corticotropin releasing hormone receptor binding in rhesus monkeys that experienced early life stress and chronic alcohol self-administration
Advisor: David Friedman, PhD. Wake Forest University School of Medicine
Techniques: EtOH self-administration in non-human primates including behavioral training, experimental set up, medicine administration, femoral blood draw, saphenous blood draw, autoradiography

Lab Rotation Aug 2008-Dec 2009
Advisor: Brian McCool, PhD. Wake Forest University School of Medicine
Techniques: Extracellular field recording, patch clamp electrophysiology

Behavioral Neuroscience Lab Fall 2005 – May 2008
Advisor: Mark C. Zrull, Appalachian State University Department of Psychology
Techniques: audiogenic seizure priming and induction, behavioral tests including- object and location preference, open field exploration, and delayed alternation task; intracardial perfusions, brain tissue extraction, free floating tissue preparation, immunohistochemistry and microscopy.

Professional Affiliations

Society for Neuroscience 2007 - present
Western North Carolina Society for Neuroscience 2009 - present
Research Society on Alcoholism 2009 - present

Grants and Appointments

NIAAA Alcohol Training Grant 2009-2011
2T32-AA007565-16; Program Director: Brian A. McCool

Outreach and Extracurricular Activities

Wake Forest University School of Medicine Brain Awareness Council

School Visit Volunteer Aug 2008 – present
Movie Night Coordinator Aug 2009 – present
Lending Library Committee June 2010

Partnership for Drug Free America

Certification in PACT360 program Oct 2010

Teaching Experience

Tutoring

Applied Human Physiology PHT 5401 June 2009 – July 2009
WSSU, Department of Physical Therapy

Clinical Pharmacology PHT 6203 Aug 2009 – Nov 2009
WSSU, Department of Physical Therapy

Lecturer

Applied Human Physiology PHT 5401 July 2010; July 2011
WSSU, Department of Physical Therapy

Clinical Pharmacology PHT 6203 Oct 2010
WSSU, Department of Physical Therapy

Neuroscience of Drug Abuse March 2011
NCCU, Department of Biology

Publications and Presentations

Trimnal, M.W., Davenport, A.T., Grant, K.A., and Friedman, D.P. Chronic ethanol self-administration does not alter GABA_A receptor subunit density in the monkey amygdala. *Soc Neurosci Abstr.* 2009.

Trimnal, M.W. and Friedman, D.P. A Review of Alcohol and Corticotropin Releasing Hormone Interactions. *Soc Neurosci Abstr.* 2009.

Trimnal, M.W., Davenport, A.T., Burnett, E.J., Maxey, V.M., and Friedman, D.P. Chronic ethanol self administration but not early life stress alters corticotropin releasing hormone receptors in monkey amygdala. *Research Soc. Alcohol. Abstr.* 2010.

Trimnal, M.W., Davenport, A.T., Bennett, A.J., Suomi, S.J., and Friedman, D.P. Effect of chronic ethanol and early life stress on serotonin transporter densities in the prefrontal cortex of rhesus macaques. *Soc Neurosci Abstr.* 2010.

Undergraduate Career

Research Honors and Funding

Delegate to Research Day at the Capital, NC	April 2007
Office of Student Research	
Research Grant	2007
Travel Grant (3),	2006-2008
Wise Travel Grant (3),	2006-2007

Publications and Presentations

Glenn, E.A., **Waddell, M.L.**, Will, E. (Sherwood, K., Zrull, M.C.) Juvenile audiogenic seizures affect location exploration and preference. *National Conference on Undergraduate Research (NCUR) Poster Presentation*. 2006.

Hodgin, E.L., Ostertag, M., Glenn, E., Jones, W., **Waddell, M.**, Price, V. and Zrull, M.C. Juvenile audiogenic seizures affect location preference and working memory. *Soc Neurosci Abstr*. 2006.

Lemmerman, S., **Waddell, M.L.**, Lawrence, J., Adams, M.R. (Zrull, M.C.) The severity of sound induced seizures was not altered by acoustic "Rehabilitation." *National Conference on Undergraduate Research (NCUR) Poster Presentation*. 2008.

Price, V.A., Glenn, E.A., **Waddell, M.L.**, Adams, M.R. (Zrull, M.C.) Audiogenic seizures affect object preference of adult but not juvenile rats. *National Conference on Undergraduate Research (NCUR) Poster Presentation*. 2007.

Waddell, M.L., Glenn, E.A., Jones, W.S. (Zrull, M.C., Hodgin, E.L.) Open field activity is affected by juvenile sound-induced seizures. *National Conference on Undergraduate Research (NCUR) Poster Presentation*. 2006.

Waddell, M.L., Jones, W.S., Snyder, J.L., Price, V.A. (Zrull, M.C.) Learning and performing a task that requires working memory is affected by sound-induced seizures in adult rats. *National Conference on Undergraduate Research (NCUR) Poster Presentation*. 2007.

Waddell, M.L., Jones, W.S., Glenn, E.A., Snyder, J.L., Hazel, O. and Zrull, M.C. Sound-induced seizures in adult but not juvenile or adolescent rats affect delayed alternation learning and memory. *Soc Neurosci Abstr*. 2007.

Waddell, M.L., Lemmerman, S., Lawrence, J., Cosgrove, C.K., Adams, M.R., & **Zrull, M.C.** Neither acoustic activation nor desensitization reduces audiogenic responses in a model of acquired reflex epilepsy. *Soc Neurosci Abstr*. 2008.

Will, E.A., Jones, W.S., **Waddell, M.L.**, Barnett, J.B. (Hodgin, E.L., Zrull, M.C.) Age and seizure frequency affect severity of sound-induced seizures in juvenile rats. *National Conference on Undergraduate Research (NCUR)* Poster Presentation. 2006.