ABSTRACT

Regulation of Anxiety and Alcohol Drinking Behavior by Neuropeptide Y and Corticotropin Releasing Hormone in the Central Nucleus of the Amygdala

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The rising consumption of alcohol in Western societies and subsequent alcohol-related life problems that develop has been labeled a major health and social issue. Finding an effective treatment for alcohol dependence has been faced with difficulty, as this is a complex phenomenon that has its roots in a variety of factors. Anxiety and stress have a strong correlation with alcohol intake behavior, and cyclic bouts of alcohol drinking and withdrawal lead to enhanced anxiety reactivity. Recently, two neuromodulators have been implicated in the regulation of anxiety and ensuing alcohol intake: neuropeptide Y (NPY) and corticotropin releasing hormone (CRH) both of which exert their effects on the central nucleus of the amygdala (CeA), a structure implicated in anxiety disorders. This review examines how these two neuromodulators interact, the cellular and molecular mechanisms by which they exert their effects, how these mechanisms affect neuronal excitability within the CeA, and how ethanol relates to both NPY and CRH which will hopefully answer questions with regards to the neurophysiology of alcohol intake behavior.

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REGULATION OF ANXIETY AND ALCOHOL DRINKING BEHAVIOR BY NEUROPEPTIDE Y AND CORTICOTROPIN RELEASING HORMONE IN THE CENTRAL NUCLEUS OF THE AMYGDALA

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LIST OF ABBREVIATIONS

aCSF	artificial cerebrospinal fluid
ADX	adrenolectomized
APP	avian pancreatic polypeptide
AVP	arginine vasopressin
BLA	basolateral nucleus of the amygdala
BNST	bed nucleus of the stria terminalis
BPP	bovine pancreatic polypeptide
CaMK	Ca^{2+}/calmodulin kinase
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
CeA	central nucleus of the amygdala
CeC	capsular subdivision of CeA
CeI	intermediate subdivision of CeA
CeL	lateral subdivision of CeA
CET	chronic ethanol treated
CORT	corticosterone
CREB	cAMP response element binding
CRF	corticotropin releasing factor
CRH	corticotropin releasing hormone
CRH BP	CRH binding protein
CNS	central nervous system

DA	dopamine
DAG	diacylglycerol
DNA	deoxyribonucleic acid
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders
EEG	electroencephalogram
EPSC	excitatory post-synaptic current
GABA	gamma aminobutyric acid
GAD	generalized anxiety disorder
GTP	guanosine triphosphate
HAD rats	high alcohol dependence rats
HPA axis	hypothalamic-anterior pituitary-adrenal axis
ht	haplotype tagging
ICV	Intracerebroventricular
IP_3	inositol 1,4,5-trisphosphate
IPSC	inhibitory post-synaptic current
IPSP	inhibitory post-synaptic potential
ir	immunoreactivity
K_{Ca}	calcium-dependent potassium channel
KO	knockout
LAD rats	low alcohol dependence rats
LDB	light/dark box
MAPK	mitogen activated protein kinase

MeA	medial nucleus of the amygdala
mRNA	messenger ribonucleic acid
MTIP	3-(4-Chloro-2-morpholin-4-yl-thiazol-5-yl)-8-(1-ethylpropyl)-2,6-dimethyl-imidazo[1,2-b]pyridazine
NAc	nucleus accumbens
NMDA	N-methyl D-aspartate
NP rats	alcohol non-preferring rats
NPY	neuropeptide Y
NPY-OX	NPY gene overexpression
OBX	olfactory bulbectomized
P rats	alcohol preferring rats
pCREB	phosporylated cAMP response element binding
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	cAMP-dependent protein kinase/protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PPR	paired-pulse ratio
PTSD	post traumatic stress disorder
PVN	parvocellular nucleus
PYY	peptide YY

SNP	single nucleotide polymorphism
UNC	urocortin
$Y_1 – R$	Y_1 receptor
Y_2-R	Y_2 receptor

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CHAPTER ONE

Background Information

Alcohol is one of the most commonly used psychoactive drugs in the United States, second only to caffeine. Although it possesses a simple chemical structure, it has a high potential for abuse and dependence, the latter being labeled a major health and social issue (Edenberg & Foroud, 2006). In Western societies, roughly 90% of people consume alcohol at some point in their lives and 30% or more of these develop alcohol-related life problems (Sher, 2005). Alcohol dependence (alcoholism) is observed at some time in their lives in 10% of men and in 3-5% of women (Sher, 2005). Due to the fact that alcohol dependence is a complex phenomenon that has its roots in psychological, neurobiological, genetic, and sociocultural factors (Enoch, 2006; Meyer & Quezner, 2004), finding an effective treatment and criteria to define has been difficult. For instance, alcoholism has a heritability factor of 50-60% (Goldman, Oroszi, & Ducci, 2005) which interacts with environmental factors, such as alcohol availability, peer pressure, and parental attitudes (Koob, Everitt, & Robbins, 2008). Learning paradigms, such as the subsequent negative effects of drinking including hangover, legal costs, and personal problems, also affect alcoholism as these consequences are viewed as being too separated in time from the time of alcohol consumption (Koob et al., 2008). Other factors, such as childhood stressors and physical sexual abuse have also been implicated in increased risk for alcoholism (Enoch, 2006). Nevertheless, the Diagnostic and Statistical Manual of mental Disorders (DSM-IV) (American Psychological Association, 1994) has labeled a series of diagnostic criteria that identifies behavioral, cognitive, and physical

characteristics and has also differentiated between alcohol abuse and alcohol dependence (Table 1.1).

Table 1.1
DSM-IV criteria for alcohol dependence

A maladaptive pattern of alcohol use, leading to clinically significant impairment or distress as occurring at any time in the same 12-month period:
1. Need for markedly increased amounts of alcohol to achieve intoxication or desired effect; tolerance or markedly diminished effect with continued use of the same amount of alcohol
2. The characteristic withdrawal syndrome for substance; or alcohol is taken to relieve or avoid withdrawal symptoms
3. Persistent desire or one or more unsuccessful attempts to cut down or control alcohol use
4. Alcohol used in larger amounts or over a longer period than person intended
5. Important social, occupational, or recreational activities given up or reduced because of alcohol use
6 A great deal of time spent in activities necessary to obtain alcohol, to use alcohol, or to recover from its effects
7. Continued alcohol use despite knowledge of having a persistent problem that are likely to be caused or exacerbated by alcohol use

Alcoholism is characterized by tolerance, which is defined as the need for increased amounts of alcohol to achieve desired effect and/or diminished continued use of the same amount of alcohol, uncontrolled heavy drinking and a chronic relapse, and withdrawal symptoms when alcohol intake is decreased (Dackis & O'Brien, 2005). Compulsion to seek and take the drug, loss of control in limiting intake, and the emergence of a negative emotional state (Koob & Le Moal, 1997) have also been criteria that define alcoholism. Saitz (2005) has placed alcohol-use disorders into four diagnostic categories, each increasing in severity: risky use, problem drinking, alcohol abuse, and alcohol dependence. Loss of control over alcohol intake, tolerance, and physical dependence characterize alcohol dependence (Saitz, 2005), while alcohol abuse is characterized by individuals that have repeated legal, interpersonal, social, or

occupational impairments related to alcohol consumption but are not themselves dependent on alcohol (Meyer & Quezner, 2004; Saitz, 2005).

The transition from alcohol use to alcohol dependence has aspects relating to impulse control disorders and compulsive disorders as well as a transition from positive reinforcement to negative reinforcement (Koob, 2003), as the initial transition takes place. Impulse control disorders are characterized by an increasing sense of tension before committing an impulsive act; pleasure, gratification or relief is felt at the time of committing the act, and following the act, there may or may not be regret or guilt (American Psychological Association, 1994). On the other hand, compulsive disorders are distinguished by anxiety and stress prior to committing a compulsive repetitive behavior and relief from stress by performing the compulsive behavior. There is also a shift from positive reinforcement being the main driving factor in alcohol consumption to negative reinforcement being the main driving factor. The positive reinforcing characteristics of alcohol are linked to the hedonistic aspects of intoxication (Wand, 2005) including euphoria, relaxation, and social disinhibition. Current research suggests that these positive reinforcing properties act through signal transduction systems affecting the mesocorticolimbic dopamine (DA) pathways, with the nucleus accumbens, a structure at the base of the corpus striatum, appearing to be a major factor in this pathway (Tupala & Tiihonen, 2004). Repeated alcohol exposure causes tolerance to these positive reinforcing effects, leading to a diminished euphoric experience. As increased tolerance to the positive reinforcement properties occurs, alcohol intake becomes driven by the negative reinforcement properties when it is used to alleviate symptoms of alcohol withdrawal. Alcohol withdrawal is characterized by impaired physiological function and

enhanced negative affect (Valdez & Koob, 2004). Physical withdrawal symptoms include disturbed sleep patterns, convulsions, tremor, perspiration, nausea, and vomiting (Hershon, 1973, 1977). Negative affect in withdrawing alcoholics includes depressed mood and anxiety, both of which have been associated with relapse (Cloninger, 1987).

It has been suggested that the negative reinforcement in relieving the negative affect associated with withdrawal symptoms is a major contributing factor in relapse in alcoholics. More specifically, increased anxiety and stress levels seem to be strongly correlated with increased alcohol intake (Michael, Zetsche, & Margraf, 2007; Thomas, Randall, Book, & Randall, 2008). Cranford, Nolen-Hoeksema, & Zucker (2011) found that alcohol dependence and major depressive episodes are strongly correlated, with individuals who experience both major depressive episodes and alcohol dependence reporting more drinking days, number of days intoxicated, and number of days where individuals drank more than two times in the past year. This effect was more pronounced in females than in males. It has also been found that various types of anxiety disorders, such as panic disorder, agoraphobia, social phobia, simple phobia, generalized anxiety disorder (GAD), and post-traumatic stress disorder (PTSD) are strongly correlated with increased alcohol intake (Michael, Zetsche, & Margraf, 2007). An early symptom of alcohol withdrawal is anxiety. It is thought that early withdrawal anxiety is an important factor in the continued use of alcohol in alcohol dependent individuals (Koob, 2003). Self-medication may underlie a strong correlation between alcohol intake, decreased anxiety-stress process. Alcohol has been found to act as an anxiolytic, effectively reducing cognitive responses to stressful and threatening signals (Sripada, Angstadt, McNamara, King, & Phan, 2011) and attenuating limbic responses to fearful stimuli

(Gilman, Ramchandani, Davis, Bjork & Hommer, 2008). Since there is such a strong correlation between anxiety and alcohol intake, it seems plausible that many individuals drink alcohol for its anxiolytic effects. This could explain why there is such an increase in alcohol intake during withdrawal.

Self-medication of anxiety symptoms with alcohol may lead to the development of alcohol use and dependence. For instance, Buckner, Ecker & Proctor (2011) have found that social anxiety disorder in adolescence was a unique and significant risk factor for the development of alcohol dependence in adulthood. Anxiety disorders are also highly likely to predate the onset of alcoholism (Koob, 2003) and there is evidence that innate anxiety levels are important in initiating alcohol drinking episodes (Cornelius, Bukstein, Salloum, & Clark, 2003). Other studies have shown a positive correlation between anxiety levels and alcohol consumption (Bowers, Sabongui & Amit, 1997). A proposed model suggests that a genetic predisposition to either high anxiety levels or anxiety during ethanol withdrawal results in higher alcohol intake (Pandey, 2003). A related hypothesis to the self-medication paradigm is the tension-reduction hypothesis, also known as the stress-reduction hypothesis. In short, this theory posits that people drink alcohol because it reduces tension (Leonard & Blane, 1999; Cappell & Herman, 1972), tension in this case being anxiety and stress. This hypothesis is similar to Hull's drive-reduction hypothesis (Bouton, 2007) where the drive is the aversive state of anxiety and reduction in anxiety playing the part of the reinforcer. If there is no means of removing the cause of tension then a relief from this tension would be sought. In this case, alcohol would be the substance that provides such relief. The original formulation of the hypothesis was met with much criticism since the general consensus was that withdrawal

was viewed as a physical syndrome and that alcoholics increased their intake to alleviate them. However, an early study by Hershon (1977) examined the extent to which physical withdrawal symptoms provoked drinking in male alcoholics. It was found that less than 25% of the patients examined reported that they continued to drink in order to alleviate physical withdrawal symptoms. However, in the same study over 80% of the participants reported drinking alcohol to alleviate anxiety and depressed mood. Annis, Sklar, & Moser (1998) also reported that male and female alcoholics report negative affective states as the most common reason for alcohol intake and relapse. This is further supported by the fact that in humans, physical withdrawal symptoms last for 12-72 hours after the last drink (Mello & Mendelson, 1972), but abstinent alcoholics report cravings for months after withdrawal (Roelofs, 1985). The view now is that tension refers to the negative affect and not the physical symptoms. Further support for the tension-reduction hypothesis of alcohol intake comes from studies where stress and anxiety have been reported to increase alcohol craving and consumption. One study examined the reasons for drinking and situational factors for alcohol consumption (Abbey, Scott & Smith, 1993). In an interview conducted with 781 randomly selected Michigan drinkers, it was found that there was a significant interaction between drinking to cope with stress and perceived stress. Billingham, Parrillo, & Gross (1993) also found the same interaction in college students.

As previously stated, the transition from positive reinforcement to negative reinforcement is believed to be one of the main causes behind relapse and alcohol abuse and dependence. Exactly how this transition occurs is not well understood, however tolerance to the positive reinforcing effects are believed to play a role. Withdrawal

symptoms and the negative affect that accompanies it seem to play a part in the recurring episodes of alcohol drinking, however, how these two variables differ in dependent individuals and occasional users is not fully understood. Solomon's (1980) opponent-process theory has been applied in the context of addictive behavior, including alcohol dependence (Fig. 1.1). In this model, two processes are defined: the a-process which includes affective or hedonic habituation (tolerance) and the b-process which includes affective or hedonic withdrawal (abstinence). The a-process occurs shortly after the presentation of the stimulus and correlates closely with the intensity, quality and duration of the reinforcer. This a-process shows tolerance. The b-process, which appears after the a-process has terminated, has a slow onset, is slow to build up to an asymptote, and slow to decay. Unlike the a-process, the b-process gets larger with each repeated exposure (Solomon & Corbit, 1974; Solomon, 1980). The dynamics of the opponent-process theory proposes that the same behavior can be reinforced by entirely different motives. This theory can be applied to the drug-taking paradigm and more specifically, the alcohol abuse and dependence paradigm. The initial acute effect of alcohol (the a-process) is hypothesized to be opposed by the b-process, compensatory homeostatic changes in numerous brain systems. Affective states, either pleasant or aversive, are hypothesized to be opposed by centrally mediated mechanisms that reduce the intensity of the initial affective state (Koob & Le Moal, 2008). In the alcohol scenario, this means that the initial euphoria (a-process) is counteracted by the subsequent negative affect (b-process). With each successive administration, tolerance develops to the euphoria (a-process gets smaller), but the negative affect which appears after the effects of alcohol wear off (during withdrawal) get intensified.

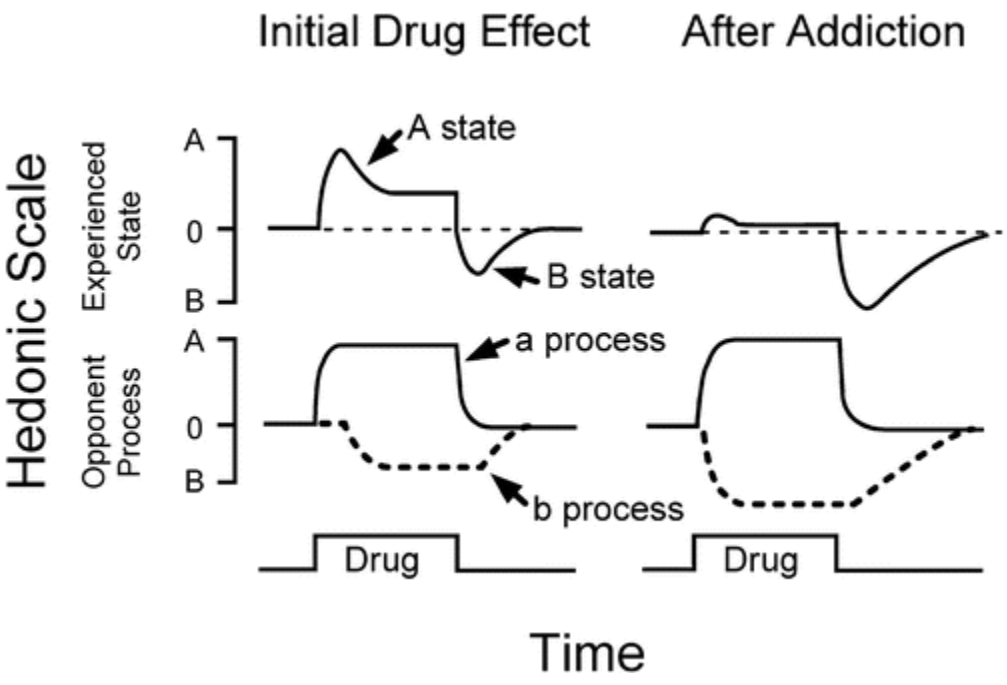

Figure 1.1 The Opponent Process Theory
Repeated drug exposure increases the intensity and the magnitude of the b-process. In our example, the b-process represents the negative affective state. Adapted from Robinson & Berridge, 2003.

Although the opponent-process theory provides a plausible model for the occurrence of alcohol dependence and its relationship to stress and anxiety due to withdrawal, current research has focused on finding a neurobiological equivalent. Koob & Le Moal (2001; 2008) have proposed an allostatic model of brain motivational systems to account for the persistent changes associated with dependence and addiction (Fig. 1.2). Allostasis refers to the regulation of physiological systems outside the normal homeostatic range (Valdez & Koob, 2004). The body normally maintains internal parameters necessary for survival, near a determined set-point through a process termed homeostasis (Sterlin & Eyer, 1981). Through homeostatic mechanisms, physiological systems are maintained within a range optimal for survival (McEwen, 2000). If these parameters are disturbed, the organism must be able to correct these in order to survive.

Chronic exposure to alcohol and subsequent stress and anxiety places demands on an organism to the point where it is unable to maintain a normal homeostatic range. In allostasis, the body varies the parameters of its physiological systems to adapt to any perceived or anticipated environmental demands. If such state continues, it can lead to an allostatic load, defined as the changes the body must enlist to face incoming environmental challenges (McEwen, 2000; Koob et al., 2008). If the allostatic load continues, returning to a homeostatic range becomes increasingly difficult. In other words, with increased allostatic load elicited by chronic alcohol abuse, the physiological systems are maintained at a level that is outside the homeostatic range. In theory, this new set point is appropriate to the perceived conditions that are endured yet they may be within a range that can lead to pathological behavior (Valdez & Koob, 2004).

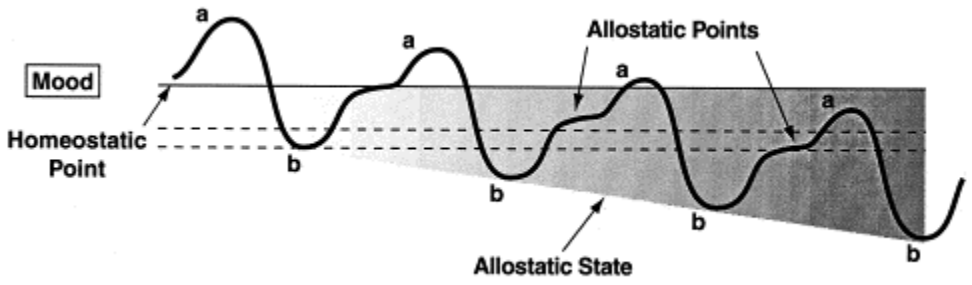

Figure 1.2 Allostatic Load
The changes in the affective stimulus (state) in an individual with repeated frequent drug or alcohol use that may represent a transition to an allostatic state in the brain reward systems and, by extrapolation, a transition to addiction. Note that the apparent b-process never returns to the original homeostatic level before drug-taking begins again, thus creating a greater and greater allostatic state in the brain reward system. In other words, here the counteradaptive opponent-process (b-process) does not balance the activational process (a-process) but in fact shows a residual hysteresis. Adapted from Koob & LeMoal, 2001.

This allostatic model of tolerance and dependence accounts for the transition from casual alcohol drinker to alcohol dependent, as well as the user's transition from alcohol

as positive reinforcement to negative reinforcement. Under this model, when alcohol is consumed a euphoric, positive state is experienced followed by a negative affective state characterized by anxiety and stress experienced during withdrawal. Following the negative affect, the mood of the user returns to homeostatic state. If the user experiences repeated exposure to alcohol, the positive mood diminishes due to tolerance, whereas the anxiety and stress experienced during withdrawal increases due to sensitization. The user moves toward a dependent state and an allostatic load is endured due to the increasingly powerful negative affective state experienced. If this cycle repeats itself, eventually the mood state is unable to return to a homeostatic range and becomes dysregulated (Valdez & Koob, 2004; Koob, 2004; Koob & Le Moal, 2008). By acquiring this new state of negative affect, the user begins to drink more alcohol in an attempt to return to normal homeostatic levels. At this point, alcohol is no longer consumed for the positive relaxing and euphoric effects but rather to alleviate the negative affect brought upon by the repeated allostatic load endured during the first instances of alcohol consumption. Although the simplicity of this model makes it an attractive explanation for alcoholism, the transition from positive to negative reinforcement and the subsequent transition from abuse to dependence is not clear cut. Clinical studies on alcoholics who have been drinking for years prior to participation makes it difficult to determine a precise period of exposure and blood alcohol level needed for someone to become dependent. One hypothesis is that the transition to dependence occurs following long periods of alcohol intake that lead to alcohol levels sufficient to produce intoxication (Valdez & Koob, 2004). This problem can be circumvented by animal models, which demonstrate that dependence can occur in weeks if animals are exposed to high levels of ethanol. For

instance, in an experiment conducted by Macey, Schulteis, Heinrich, & Koob (1996) rats were maintained on an ethanol drinking diet for about 4 weeks or exposed to ethanol vapor for about 2 weeks. These rats showed blood ethanol concentrations ranging from 100 to 200 mg/dL and showed signs of physical withdrawal. Other studies (Hwang, Stewart, Zhang, Lumeng, & Li, 2004; Thorsell, Slawecki, & Ehlers, 2005) show similar patterns if animals are kept under similar conditions. It seems that chronic exposure to alcohol at levels sufficient to produce intoxication may lead to homeostatic disruption and produce an allostatic load on stress systems (Valdez & Koob, 2004).

The opposing, counteradaptive processes are believed to be modulated by two other processes: within- and between-system neuradaptations (Koob & Bloom, 1988). The within-systems adaptation is a molecular or cellular change within a reward circuit that accommodates the over activity of hedonic processing (the a-process) associated with addiction. This accommodation results in a decrease in reward function, which manifests itself as the b-process. Between-systems adaptation describes the functional changes in neurochemical systems (usually anti-reward systems) other than those involved in the positive rewarding effect of drugs (Koob & Bloom, 1988). These systems are recruited or dysregulated by chronic activation of the reward system by the drug, which has the effect of opposing the original hedonic action of the drug, limiting the reward function.

The systems that are involved in this dysregulation in alcohol dependent individuals are still not fully understood. Current research has pointed out that the amygdala, a collection of nuclei involved in emotional processing, reward, and learning (LeDoux, 2007) may be involved in the development and maintenance of alcoholism. For

instance, exposure to ethanol induces craving in alcoholics that is associated with amygdala activation which may represent aspects of emotion, motivation, and memory in cue-induced craving (Schneider et al., 2001). It has also been found that alcoholics tend to exhibit a small reduction in amygdala volume (Wrase et al., 2008). Animal studies have also supported the idea that the amygdala may be related to alcoholism. Acute ethanol exposure inhibits total spontaneous neural activity in male Sprague Dawley rats (Perra, Pillolla, Luchicchi & Pistis, 2008), induces changes in gene expression (Pandey, Zhang, Roy, & Misra, 2006) and enhances GABAergic transmission in the amygdala (Roberto, Madamba, Moore, Tallent & Siggins, 2003). Other findings have pointed out that the actions within the amygdala related to alcoholism may be more specific and may involve other neuroregulatory systems. Within the context of this topic, it seems that the amygdala may be involved in regulating not only the maintenance of alcohol dependence but also the negative affective states (anxiety and stress) that are responsible for maintaining alcohol intake behavior in humans. Two neuromodulators implicated in the regulation of amygdala activity during anxiety and stress are neuropeptide Y (NPY) and corticotropin-releasing hormone (CRH) (Funk, O'Dell, Crawford, & Koob, 2006; Gilpin Misra, & Koob, 2008; Gilpin, Stewart, Badia-Elder, 2008; Hwang et al., 2004; Thorsell et al., 2005). NPY has been implicated in decreasing anxiety levels in rats that showed anxiety-like behaviors (Gilpin, Stewart, Murphy, Li & Badia-Elder, 2003; Cippitelli et al., 2010) and as it has previously been stated, there is a strong correlation between anxiety and alcohol drinking behavior. It has also been shown that central administration of NPY attenuates alcohol self-administration in dependent rats (Pandey, 2003). CRH levels are strongly correlated with increased anxiety in rats (Hwang et al., 2004; Richardson, Lee,

O'Dell, Koob, & Rivier, 2008) and increased drinking behavior. Both neuroregulators seem to exert their effect by acting in the amygdala, more specifically the central nucleus of the amygdala (Wand, 2005; Koob, 2008; Thiele, Koh, & Pedrazzini, 2002) which is believed to play a role in modulating anxiety and stress behaviors.

The purpose of the present paper is three-fold:

1. To introduce the two proposed neuromodulators believed to play a role in alcohol drinking behavior, NPY and CRH, and provide a detailed outline of the current findings relating to the physiology of these and their involvement in brain circuitry relating to reward.
2. To examine the link between anxious behavior and alcohol drinking and how both NPY and CRH provide a possible mechanism to regulate both types of behaviors by looking at the scientific literature on the topic.
3. To present the possible molecular mechanisms by which CRH, NPY, and ethanol work to regulate anxiety. It is the hope that this paper will provide a clear and concise outline of the current literature on the topic of NPY and CRH and its relationship to anxiety and alcoholism.

CHAPTER TWO

Neuropeptide Y and the Amygdala

The Amygdala: The Locus of Emotion

What follows is an overview of the outstanding anatomical features of the amygdala critical for the central discussion of this paper. This information is available in greater detail in a review by Sah, Faber, Lopez de Armentia, & Power (2003) and, unless otherwise noted, the information presented here pertains to that review.

The amygdala, or amygdaloid complex, is an almond-shaped structure located deep within the medial temporal lobe. Consisting of about 13 different nuclei and further divided into subdivisions based on cytoarchitectonics, histochemistry, and the different connections they make, the amygdala has been suggested to play a role in the regulation of emotion. Indeed, as a part of the limbic system whose function seems to be the regulation of emotion, the amygdala has been labeled the locus of fear conditioning, as well as the core structure in disorders such as anxiety and depression.

The amygdalar nuclei can be divided into three broad groups

1) the deep or basolateral group – includes the lateral nucleus, the basal nucleus, and accessory basal nucleus
2) the superficial or cortical-like group, which includes the cortical nucleus and nucleus of the lateral tract
3) the centromedial group, composed of the medial and central nuclei

Although the amygdala as a whole has been implicated as being important in anxiety and depressive disorders, this overview will focus on the structures that

play a critical role in the present discussion: the central nucleus, the basolateral nucleus, and the medial nucleus.

The basolateral nucleus (BLA) is comprised of the lateral nucleus and the basal nucleus. Along with the accessory basal nucleus, these are known as the basolateral complex, or the deep nuclei. The lateral nucleus is located dorsally in the amygdala where it lies alongside the basal nucleus ventrally. It is bordered laterally by the external capsule and medially by the central nucleus of the amygdala. The basal nucleus is located ventral to the lateral nucleus.

The CeA and the MeA are both part of the centromedial nuclear group, which is found in the dorsal medial portion of the amygdaloid complex, along with the bed nucleus of the stria terminalis (BNST). It is worthy to note that the BNST is considered to be part of the extended amygdala, which is thought to be critical to the addiction cycle (Koob, 2003). The CeA is located dorsomedially in the rostral part of the amygdala, surrounded laterally by the basolateral complex, dorsally by the globus pallidus of the basal ganglia, and medially by the stria terminalis. The CeA is furthered divided into four distinct divisions: the capsular subdivision (CeC), the lateral subdivision (CeL), intermediate subdivision (CeI), and the medial subdivision. The MeA is found near the surface, bounded medially by the optic tract. It also has four subdivisions: rostral, central (dorsal and ventral), and caudal.

Given its role as the locus of anxious behavior, the amygdala is expected to receive, integrate, and provide connections to other brain areas. The amygdala receives sensory information from all sensory modalities. Furthermore, these sensory inputs target structures in the amygdaloid complex at all levels (Figure 2.1).

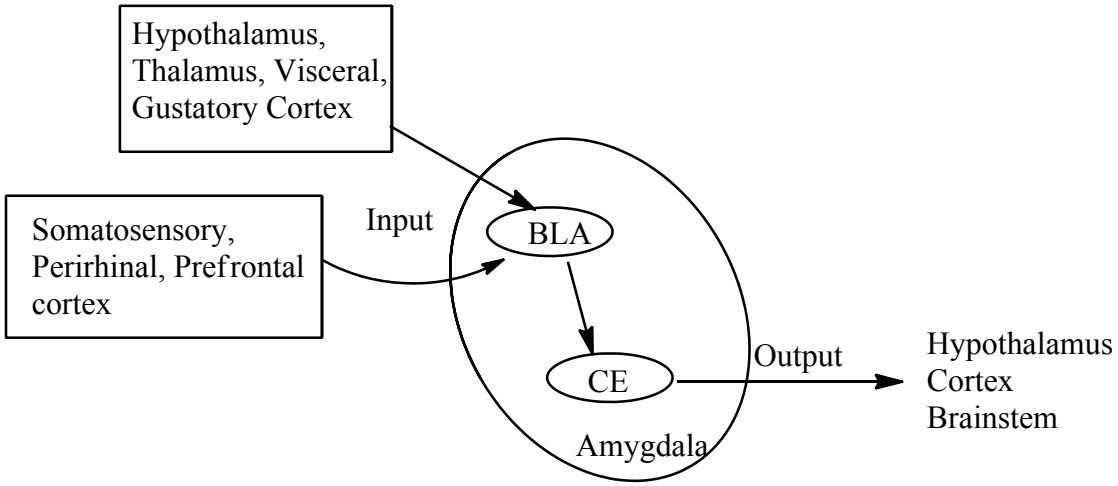

Figure 2.1 Overview of amygdalar inputs and outputs.

As seen in the above figure, the major outputs for the CeA include the hypothalamus, the cortex, and the brainstem. Fear conditioning responses in rats is characterized by freezing, release of stress hormones, and changes in blood pressure and heart rate elicited by the activation of the autonomic nervous system. Not surprisingly, the CeA can induce this response by stimulating neurons in the brainstem that control the autonomic system and by stimulating hypothalamic nuclei that modulate these responses. For instance, the CeA has direct projections with the following structures: the periaqueductal gray, which leads to startle, analgesia, and cardiovascular changes; the parabrachial nucleus, involved in pain pathways; and the nucleus of the solitary tract. CeA connections to the hypothalamus have an influence in the coordination of reproductive and defensive behaviors.

The lateral/BLA is the central component of the brain's fear/anxiety circuit (Silberman et al., 2009) and as such, is the primary input nuclei of the amygdala. For instance, the BLA receives extensive input from sensory/limbic/insular cortex and thalamic nuclei which in turn provides excitatory input to the CeA (Nose, Higashi,

Inokuchi, & Nishi, 1991) and the nucleus accumbens (North, Williams Surprenant, & Christie, 1987) with further reciprocal connections with the medial prefrontal and orbitofrontal cortex. The BLA consist primarily of glutamatergic pyramidal neurons (roughly 90% of all cells in BLA), which provide the main excitatory input to the CeA as well as many other brain structures. Given this information, the BLA is in a position to serve as the major input for sensory information into the amygdala and it is involved in establishing the emotional salience of environmental stimuli. The role the BLA plays in alcoholism will be discussed later.

General Characteristics of Neuropeptide Y

First isolated from a pig brain by Tatemoto and colleagues in 1982 (Tatemoto, Carlquist, & Mutt, 1982; see Eva, Serra, Mele, Panzica, & Oberto, 2006) NPY is a 36 amino acid peptide that is highly conserved through many species, including rats, mice, and birds (Allen et al., 1983), resulting from the cleavage of its 97-amino acid precursor, preproNPY (Gilpin et al., 2003). Human NPY (hNPY) and porcine NPY are almost structurally identical except for residue 17, where humans show the amino acid methionine while the porcine NPY shows the amino acid leucine (Jacques, Dumont, Fournier, & Quirion, 1997; Table 2.1). Such strong sequence conservation of the NPY structure across species suggests that it serves an important function. Along with polypeptide YY, the pancreatic polypeptides and seminalplasmin, NPY belongs to a family exhibiting the pancreatic polypeptide fold, a proline helix folded into an alpha-helix with a carboxy-terminal tyrosine (Stephen, 1996). The gene encoding for the NPY precursor is located on chromosome 4 (Carr et al., 1998)

Table 2.1. Amino acid sequences (in one letter notation) of avian pancreatic polypeptide (APP), rat and porcine NPY, peptide YY (PYY), and bovine pancreatic polypeptide (BPP); adapted from Allen et al., 1987

APP	G P S Q P T Y P G D D A P V E D L I R F Y D N L Q Q Y L N V V T R H R Y
Rat NPY	Y P S K P D N P G E D A P A E D M A R Y Y S A L R H Y I N L I T R Q R Y
Porcine NPY	Y P S K P D N P G E D A P A E D L A R Y Y S A L R H Y I N L I T R Q R Y
PYY	Y P A K P E A P G E D A S P E E L S R Y Y A S L R H Y L N L V T R Q R Y
BPP	A P L E P E Y P G D N A T P E Q M A Q Y A A E L R R Y I N M L T R P R Y

Early studies in the physiology of NPY have demonstrated that it is the most abundant and widely distributed neuropeptide in the mammalian central nervous system, preferentially expressed in interneurons (Chronwall, DiMaggio, Massari, Pickel, Ruggiero, & O'Donohue, 1985) High levels of NPY like-immunoreactivity material are found in areas such as the hypothalamus, the septum, nucleus accumbens, the periaqueductal gray, and the locus coeruleus while moderate levels are found in the amygdala, the hippocampus, cerebral cortex, basal ganglia, and the thalamus (Allen et al., 1983).

Although the NPY mRNA is present throughout the central nervous system, there are at least four cell groups within the brain where neurons containing NPY mRNA are in high abundance (reviewed in Kask, Harro, von Horsten Redrobe, Dumont, & Quirion, 2002). The first of these cell groups is found in the brainstem, specifically in the area A6 (also known as the locus coeruleus). The second group is found in the arcuate nucleus of the hypothalamus, where these neurons send projections to the paraventricular nucleus of the hypothalamus and other regions of the brain. In fact, the vast majority of NPY derives from neurons located within the arcuate nucleus (Chronwall, 1985; Chronwall et al., 1985). The third group is found in an area overlapping with, and extending from the

septohippocampal nucleus, while the fourth group is found in the nucleus of the solitary tract and the ventrolateral medulla.

The distribution of NPY mRNA suggests that it is involved in a variety of basic physiological functions. For instance, it has been observed that intracerebroventricular (ICV) administration of NPY produces effects such as the production of anti-convulsant effects (Vezzani, Sperk, & Colmers, 1999), modulation of cognition (Redrobe, Dumont, St-Pierre, & Quirion, 1999), and inhibition of neuronal excitability (Colmers & Bleakman, 1994). It has been found that NPY is critical in the regulation of cerebrocortical and hippocampal excitability (Bison & Crews, 2003) and that mice lacking NPY (via knockout technique) have a higher propensity for seizures (Stephens, 1996). Other lines of evidence show that NPY is involved in the modulation of limbic seizure activity since the use of Y_1-R antagonists (see later) reduces seizure duration and frequency while Y_1-R agonists produce an opposite effect (Benmaamar, Pham-Le, Marescaux, Pedrazzini & Depaulis, 2003 Vezzani, Rizzi, Conti, & Samanin, 2000). Furthermore, central infusion of NPY reduces cerebroexcitability and NPY -/- mutant mice, those that present lower levels of NPY, are more susceptible to seizures induced by a gamma-aminobutyric acid (GABA) receptor antagonist as opposed to wild-type mice (Vezzani et al., 1999).

Within the hippocampus and the cortex, NPY is mostly co-localized with the GABA neurotransmitter in interneurons; however Oberto, Panzica, Altruda & Eva (2001) have shown that GABA and NPY co-exist in many cortical and deep brain structures. Studies by Obernier, Bouldin, & Crews (2002) found that cerebrocortical excitability is altered during the development of ethanol tolerance and dependence, with withdrawal being associated with enhanced cerebrocortical excitability. Grant & Lovinger (1995) as

well as Harris & Buck (1990) have also demonstrated that during ethanol withdrawal, there is a decrease in GABA function that leads to neuronal hyperexcitability. Bijak (2000) suggested that NPY reduces hippocampal excitability by presynaptically inhibiting the release of glutamate, and a similar mechanism may occur in the cerebral cortex.

NPY also seems to play a role in the regulation of circadian rhythms, memory, and cardiovascular regulation via vasoconstriction and potentiation of nor-epinephrine induced vasoconstriction (Bison & Crews, 2003; Sheriff, Qureshy, Chance, Kasckow, & Balasubramaniam, 2002). However, one of the most noted effects of ICV administration of NPY is the stimulation of food intake (Stephen, 1996). Indeed, NPY is perhaps the most powerful orexigenic agent that has been isolated and NPY neurons have been found to heavily innervate the perifornical hypothalamus, an area thought to be involved in the regulation of feeding behavior (Sheriff et al., 1997). Early studies also demonstrated that infusion of NPY into the paraventricular hypothalamus of satiated rats produced a dose-dependent increase in food intake (Stanley & Leibowitz, 1985) with recent findings that NPY regulates feeding behavior via the orexin system (Toshinai et al., 2010). Mice lacking the Y_1 receptor tend to develop late onset obesity, increase in body fat mass, and hyperinsulinemia (Kushi et al., 1998), indicating the importance of NPY in feeding behavior and metabolism. Moreover, elevated mRNA NPY levels have been found in obese, hyperphagic, diabetic rats (Beck, Burlet, Nicolas, & Burlet, 1990; see Sheriff et al., 1997).

Receptors Involved in NPY Function

Five different receptors mediate the effect of NPY within the brain (Jacques et al., 1997) with receptors Y_1, Y_2, and Y_5 being abundant in rodent brains (reviewed in Schroeder, Olive, Koenig, & Hodge, 2003).The anatomical distribution of these receptors was observed using radioligands such as [^{3}H]NPY, [^{125}I] Bolton-Hunter NPY, [^{125}I][Leu^{31}, Pro^{34}]PPY (Eva et al., 2006; Jacques et al., 1997). The first receptor to be cloned is the NPY Y_1 receptor, found in high densities within the frontal cortex of the rat, and to a lesser extent within the lateral dorsal septum, the dorsal hippocampus, the nucleus of the solitary tract, area postrema, and the amygdala (Kask, Nguyen, Pabst, & Horsten, 2001; Thiele et al., 2002; Badia-Elder, Gilpin, & Stewart, 2007). Within the hippocampus, there are high amounts of Y_1 receptors within the dentate gyrus (Pandey, Roy, & Zhang, 2003) and within the amygdala, we find high densities of Y_1 receptors within the central nucleus of the amygdala and the medial amygdala, however there seems to be no receptors within the basolateral amygdala (Pandey et al., 2003). Although these are the areas with high Y_1 receptor densities, the receptor is also found in other areas in the brain, including the lateral septum, nucleus accumbens, bed nucleus of the stria terminalis, the pernifornical area, arcuate nucleus, and the ventral tegmental area (Kask et al., 2002, Gilpin et al., 2008a; Wolak et al., 2003; Larsen, Sheikh, Jakobsen, Schwartz, & Mikkelsen, 1993).

Other receptors are also in high densities in several structures within the rat brain. For instance, the lateral septum, lateral dorsal septum, and the area postrema present high densities of the Y_2-receptors. Of interest is the dorsal hippocampus, where Y_2-receptors

suppress hippocampal glutamatergic transmission through presynaptic mechanisms (McQuiston & Colmers, 1996; Greber, Schwarzer, & Sperk, 1994). If a similar mechanism works within the CeA, the activation of presynaptic Y_2 receptors will lead to decreased glutamate release. Furthermore, there seems to be a greater distribution of Y_2-receptors than Y_1-receptors within the rat brain. Y_4-receptors are only modestly found, with high densities in the medial pre-optic area, the paraventricular nucleus (PVN) of the hypoathamus, the nucleus of the solitary tract and area postrema. Y_5-receptors are difficult to detect, with moderate levels located in the area postrema, the lateral septum, the PVN, and the nucleus of the solitary tract.

Molecular Mechanisms Following Receptor Activation

Much of what is known about the other receptors ($Y_2 – y_6$) comes from studies involving the Y_1 receptor and respective agonists and antagonist. The variability between different types of receptors stems from the different amino acid make up and distribution of receptors within the central nervous system. All receptors belong to the seven transmembrane G protein-coupled rhodopsin superfamily (Bard, Walker, Branchek, & Weinshank, 1995; Gerald et al., 1995; Gerald et al., 1996), with G proteins being heterotrimeric (Thiele, Marsh, Marie, Bernstein, & Palmiter, 1998). Three different molecular mechanisms follow receptor activation:

(1) Inhibition of adenylate cyclase via the pertussis toxin sensitive GTP binding protein G_i/G_o (Herzog et al., 1992; Sheriff, Chance, Fischer, & Balasubramaniam, 1997; Thiele et al., 2002). This leads to a decrease in the levels of cyclic adenosine monophosphate (cAMP), an important second-messenger molecule in cells. One of the targets of cAMP is the molecule cAMP dependent protein kinase

(PKA), which relays the message on to other molecules via phosphorylation (addition of phosphate groups) those molecules. Of interest is the target cAMP-response element binding protein (CREB). In its normal configuration, CREB is bound to its binding site in DNA, called the cAMP response element (CRE), either as a homodimer or bound to another, related transcription factor (Purves et al., 2008) in site 5'-TGACGTCA-3' (Sheriff et al., 2002). If a cell is inactive and its CREB molecule is not phosphorylated, there is little or no transcriptional activity. Phosphorylation of CREB on its Ser-133 by PKA recruits the adapter protein CREB-binding protein (CBP) which interacts with the basal transcription complex and modifies histones to enhance the efficiency of transcription.

(2) Mobilization of intracellular Ca^{2+} (Herzog et al., 1992) is another effect of Y_1-R activation. This mobilization is a consequence of the phosphoinositide pathway, which begins by the activation of phopholipase C (PLC) by G_q (Herzog et al., 1992; Purves et al., 2008). PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-trisphospate (IP_3), the latter of which acts on IP_3 receptors within the endoplasmic reticulum to allow intracellular Ca^{2+} mobilization (Herzog et al, 1992; Purves et al., 2008). This Ca^{2+} mobilization is thought lead to increased levels of calcium-calmodulin protein kinase II (CaMKII) (Pandey, 2003).

(3) A third mechanism, although not fully understood, is the mitogen-activated protein kinase (MAPK) pathway (Mannon & Mele, 2000; Nie & Selbie, 1998) which depends on the phosphoinositidide 3-kinase (PI-3).

In essence, activation of Y_1-R leads to the decrease of cAMP, and thereby decreases the amount of phosphorylated CREB (pCREB) present in cells. However, this does not seem to be the case. For instance, studies by Sheriff et al. (1997, 1998, 2002) have shown that NPY infusion into the hypothalamus of rats increased CREB phosphorylation. Similarly, studies by Pandey et al., (2005a) and Pandey, Zhang, Roy, & Zu (2005) have demonstrated that direct infusion of NPY into the central nucleus of the amygdala also leads to increased levels of phosphorylated CREB. The conflicting results from the studies above can be easily resolved by the fact that CREB-activating pathways tend to converge. In other words, not only is the PKA pathway able to phosphorylate Ser-133 in CREB to induce transcription, but also the family of calcium-calmodulin protein kinase (CaMK), more specifically CaMKII and CaMKIV, can activate CREB (Pandey et al., 2005; Sheriff et al., 2002). It seems that phosphorylation of CREB due to the activation of Y_1-R is entirely due to the family of CaM kinases. For instance, central administration of NPY into the CeA of rats increased protein levels of CaMKIV but not of PKA (Zhang et al., 2010; Figure 2.2). Studies by Sheriff et al. (2002) shed some light to the nature of this mechanism. Direct administration of the CaMK II inhibitor, KN-93, lead to a 32% inhibition of CREB phosphorylation in the presence of the Y_1-R agonist [Leu^{31}, Pro^{34}]-NPY

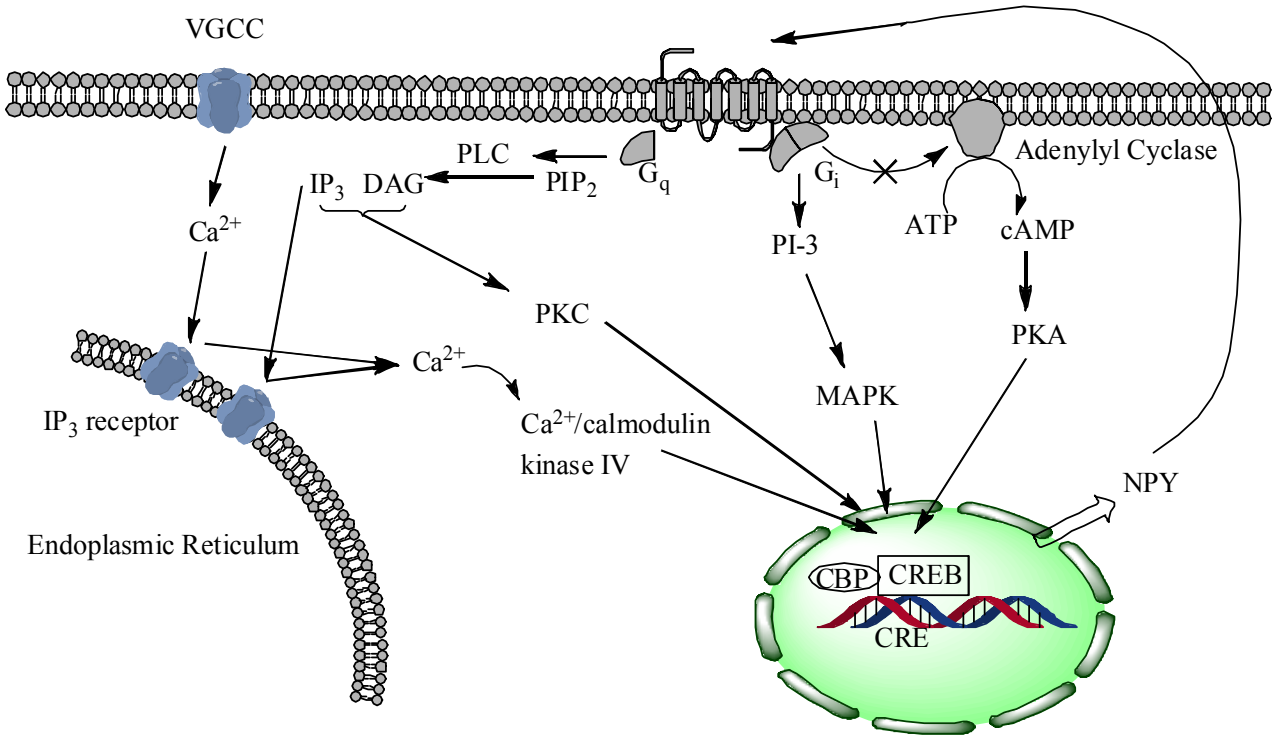

Figure 2.2 Overview of pathways activated by NPY
These signaling pathways lead to the synthesis of NPY via the phosphorylation of CREB (see text for details). Note that NAPK can also be activated by the TrkB (tyrosine kinase) pathway (not shown). PLC - phospholipase C; IP_3 - inositol trisphosphate; DAG - diacylglycerol; VGCC - voltage gated calcium channel, PI-3 phosphoinositide-3-kinase

These studies suggest that the phosphorylation of CREB, when Y_1-R is activated by either NPY or another agonist, is due to the CaMKII pathway. This suggests some degree of redundancy in the phosphorylation of CREB, but opposing mechanisms by which this is achieved: activation of the Y_1-R leads to the inhibition of adenylate cyclase whose pathway leads to the phosphorylation of CREB, however it also leads to the mobilization of internal Ca^{2+} which leads to the subsequent activation of CaMK II or CaMK IV. One reason as to why there is such redundancy most likely stems from the

target gene of this pathway, which codes for the production of the NPY peptide (Heilig & Koob, 2007; Zhang et al. 2010). In a hypothetical situation, if a decrease in endogenous NPY results in a decrease in intracellular CaM kinases, it would be possible to re-establish a homeostatic equilibrium in endogenous NPY levels if another pathway were to be activated. Inactivation of the CaMK pathway leads to decreased levels of pCREB, but pCREB levels remain stable due to the activation of the PKA pathway. If endogenous NPY levels within the brain are lower than baseline levels, one can expect a marked decrease in pCREB and changes in gene transcription. More specifically, lower NPY levels within the amygdala, given that NPY works as an anxiolytic, can lead to dysregulation of the above two pathways and anxious and stress behavior.

NPY as Anxiolytic and As Modulator of Alcohol Intake Behavior

As mentioned in Chapter 1, the negative reinforcing effects of alcohol withdrawal are believed to play a role in the continuing consumption of alcohol. Recently, Valdez and Koob (2004) have suggested that these negative reinforcing effects may be mediated by NPY brain systems involved in regulating anxiety-like behavior. These tend to be more predominant in the alcohol-dependent organism and are driven by the negative affective state that becomes apparent upon absence of the drug. Furthermore, it seems that the negative affective state that drives ethanol consumption has its locus on the amygdala (Koob, 2003) and studies by Heilig et al. (1993) and Sajdyk, Schober, & Gehlert (2002) have suggested that NPY has anxiolytic (anxiety-relieving) effects that are mediated by the amygdala. If such an assumption is correct, it is reasonable to hypothesize that NPY activity in the amygdala is heavily involved in the regulation of anxiety and alcohol intake behavior.

During the past two decades, the role of NPY in modulating anxiety behavior has emerged. It has been shown that central administration of NPY is anxiolytic in the elevated plus maze (EPM) test of anxiety (Primeaux, Wilson, Cusick, York, & Wilson, 2005). NPY increases the preference for the open arms of the maze. Earlier studies by Broqua, Wettstein, Rocher, Gauthier-Martin, Junien (1995) and Heilig et al. (1993) also demonstrate decreased anxiety following central administration of NPY in the EPM and the open field exploration test. Interestingly, NPY has been used to show a relationship that it has with ethanol intake behavior (Thiele et al., 1998). NPY -/- mice, produced by gene targeting, showed reduced levels of NPY immunoreactivity throughout the many brain structures that normally express NPY, including the amygdala. Although there is a marked reduction of NPY levels in these mice, they grow and reproduce at normal rates and showed no abnormalities in food intake or body weight. NPY -/- mutant mice consumed twice as much of the 6% ethanol solution and about 30-50% more of the two other solutions of higher concentration when compared to control mice. These mice also showed higher preference to the ethanol over water during access to the 6% and 10% solutions when compared to wild type mice (Thiele et al., 1998). It seems that this preference for ethanol by the mutant mice was not dependent on flavor, as there were no significant differences in the preference for sucrose and quinine solutions when compared to water.

The previous studies mentioned indicate that central administration of NPY does attenuate ethanol intake behavior. Instead, NPY-mediated decreases in ethanol consumption are dependent on the subject displaying high levels of anxiety-like behavior or being bred for high alcohol preference. For instance, in one study, Wistar rats were

implanted with cortical recording electrodes and cannulae above the CeA nuclei (Katner, Slawecki, & Ehlers, 2002). After using a sucrose-substitution procedure to establish ethanol self-administration, NPY (0-250pmol/0.5µl) was infused into the amygdala before drinking sessions. During these sessions, 10% ethanol (10E), 2% sucrose (2S), or food was available with consumption, locomotor activity, and cortical electroencephalography (EEG) activity being monitored concurrently. NPY had no effect on the intake of 10E, 2S or food, nor on the cortical EEG or locomotor activity. There were, however, distinct changes in the EEG associated with ethanol or sucrose consumptions. The point to note, however, is that this study only examined wild-type rats whereas the previous studies examined used rats that were bred for high alcohol preference and wild-type rats. The consensus seems to be that infused NPY only attenuates ethanol consumption in alcohol preferring (P) rats but has no effect in non-alcohol preferring (NP) rats.

Since other studies showed that NPY is involved in potentiating the sedative/hypnotic effects of several drugs, including ethanol, Thiele et al., (1998) compared the sedative effects of ethanol in NPY -/- and wild-type mice. The NPY -/- mutant mice were found to be resistant to the sedative effects of ethanol, regaining their righting reflex in the aerial righting reflex test about 15 minutes sooner than the wild-type mice. In transgenic mice that overexpress a marked NPY gene (NPY-OX; Thiele et al., 1998), there was about five times more NPY transgene mRNA than endogenous mRNA in these mice, and NPY-OX mice drank less ethanol at all concentrations tested and had lower relative preference for ethanol compared with wild-type littermates. NPY-OX mice

were also more sensitive to the ethanol induce sedation, requiring about 20 more minutes to regain their righting reflex when compared to wild type mice.

NPY has also been shown to modulate sedation in different types of anesthetics, namely avertin, pentobarbital ($GABA_A$ agonist), and ketalar (NMDA antagonist; Naveilhan et al., 2001). The three anesthetics induced a loss of righting reflex, with their effects being increased following central NPY administration, with its effect on avertin and ketalar being more potent when compared to pentobarbital (Naveilhan et al., 2001).The Y_1/Y_5 agonist, [$Leu^{31}Pro^{34}$]-NPY was found to have a similar effect as NPY, and Y1 -/- mice injected with either anesthetic did not show NPY potentiated effects in sedation (Naveilhan et al., 2001). Taken together, these data show a bidirectional relationship between NPY levels and ethanol intake and that this effect is mediated by the Y_1-R. When NPY levels are low, there is an increase in ethanol intake, and conversely, high NPY levels decrease ethanol intake.

However, the fact that NPY potentiates the sedative/hypnotic effects of ethanol (Thiele et al., 1998) raises the following question: does administration of NPY alone deliver this effect? In other words, can we also expect a sedative/hypnotic effect from NPY alone? Gilpin et al. (2004) have demonstrated that administration of NPY decreases motor activity in unselected rats, and P and NP rats. However, these reductions in motor activity are subtle. For instance, NPY reduced vertical activity (such as rearing) but not horizontal activity (such as ambulation) in both P and NP rats (Gilpin et al. 2004). Furthermore, the effects are not debilitating since they do not prevent the increases in feeding seen with the same NPY treatment. One thing to note is that ethanol and NPY seem to have additive sedative effects, as evident on the P1 and N3 components of event-

related potentials in rats and the fact that the event-related potentials seen in NPY and ethanol are nearly identical (Ehlers, Somes, & Cloutier, 1998). It is possible that in self administration studies, P or high-alcohol drinking (HAD) rats may consume sufficient amounts of ethanol to experience additive sedative effects of NPY and ethanol and thus, terminate drinking at smaller ethanol doses. Rats that are not bred for ethanol drinking may not consume sufficient amounts of ethanol to be affected by this interaction.

Although the studies above do seem to indicate a relationship between NPY and ethanol intake, they do not specifically address the effect of NPY within the amygdala and its relationship to alcohol intake. Lesioning the central nucleus of the amygdala (CeA) lead to significant decreases in anxiety levels and ethanol intake relative to non-lesion controls (Moller, Wiklund, Sommer, Thorsell, & Heilig, 1997). Note, however, that this effect appears to be specific to the CeA, as lesions to the basolateral amygdala (BLA) do not alter ethanol intake. Interestingly, studies by Hwang, Zhang, Ehlers, Lumeng, & Li (1999) have demonstrated that alcohol preferring rats have elevated NPY levels in the PVN of the hypothalamus and in their arcuate nucleus, while NPY levels are reduced in the CeA, suggesting an inverse relationship between NPY levels and CeA. Furthermore, an increase in ethanol self-administration following NPY administration directly into the PVN has been reported, however given that ethanol contains a modest amount of calories this effect may stem from the orexigenic effects of NPY rather than to its anxiolytic effects (Gilpin et al., 2004).

Other studies have looked at the predictive role of anxiety in ethanol self administration and the relationship of NPY in the CeA (Primeaux, Wilson, Bray, York, & Wilson, 2006). Briefly, rats were divided into either an anxious or non-anxious group

based on their behavior in the elevated plus maze. Following testing periods, rats were allowed to consume increasing concentrations of ethanol (2, 4, and 6%) in a 2-bottle choice procedure over a 31-day period. Following 20-day access to the 6% ethanol solution, rats underwent gene transfer surgery with replication defective-recombinant herpes simplex 1 vectors encoding prepro-NPY, an antisense NPY RNA, or LacZ (serving as control) into the CeA of rats. Anxious rats had high preference for the 4% and 6% ethanol solutions. Bilateral injections into the CeA with the NPY antisense vector increased preference for the 6% solution. Furthermore, the vector encoding NPY decreased preference for the 6% solution in anxious rats. One critical point is that this effect was seen only in the anxious group. Herpes simplex viral mediated alterations in CeA NPY expression did not alter ethanol preference in non-anxious rats. These data suggest that elevated NPY mRNA and protein levels within the CeA work to modulate anxiety and ethanol intake behavior. However this effect is observed only in those rats that have reduced NPY levels already (i.e. anxious rats). This suggests that administration of NPY into the CeA only works in rats that have been bred for alcohol preference (P rats) or those that have HAD rats. These rats also tend to be the ones that display high anxiety levels. For instance, it has been demonstrated that intra-brain infusions of NPY attenuated ethanol intake and that over-expression of NPY within the amygdala suppresses ethanol intake in rats with high anxiety-like behavior and P rats (Badia-Elder, Stewart, Powrozek, Murphy, & Li, 2003). Furthermore, it seems that this effect is limited to those rats displaying high anxiety-like behaviors, as equal infusions of NPY did not suppress ethanol intake in rats with low anxiety-like behavior. In another study, ethanol withdrawn NPY -/- mice showed less time spent in the open arm of the EPM when

compared to ethanol withdrawn NPY +/+ mice when compared to NPY -/- and NPY +/+ mice that did not consume ethanol (Sparta, Free, Knapp, Breese, & Thiele 2007), further showing NPY working as an anxiolytic, this time following ethanol withdrawal which is known as a behavioral stressor. If repeated bouts of ethanol drinking and withdrawal occur, these induce neuroadaptational changes in the individual that leads to enhanced stress reactivity and ethanol intake. This is referred to as the "kindling"/stress model of alcoholism (Breese, Overstreet, & Knapp, 2005), which is similar to the kindling induced in various epilepsy animal models. It has been shown previously that NPY infusion blunts the effects of abstinence on alcohol preference (Gilpin et al., 2003) and increased alcohol intake following deprivation cycles (Gilpin, Stewart, Murphy, & Badia-Elder, 2005), although NPY reductions in ethanol intake are not affected by restraint stress (Bertholmey, Henderson, Badia-Elder, & Stewart, 2011) suggesting that NPY induces its actions via pathways that mediate anxiety.

Exogenous stressors also tend to increase ethanol intake behavior in P rats. For instance, yohimbine, an alkaloid found within the plant *Pausinystalia yohimbe,* reinstates ethanol seeking behavior in P rats following periods of prolonged abstinence (Cippitelli et al. 2010). Note, however, that yohimbine exerts its stressing effect on P rats only if administered within the CeA or the nucleus accumbens (NAc), but not in the BLA. Yohimbine induces neuronal activation within these structures, which is believed to be involved in the induction of anxiety-like behaviors. The mechanism by which yohimbine is believed to work involves disinhibition of central norepinephrine signaling (Aghajanian & Vandermaelen, 1982) which produces peripheral sympathomimetic effects and leads to activation of the hypothalamic-pituitary-adrenal (HPA) axis (Charney,

Woods, & Heninger, 1989). In humans, this leads to feelings of anxiety and panic attacks as well as rapid heart rate, high blood pressure, overstimulation, insomnia and/or sleeplessness (Charney et al., 1989). This increase in anxiety leads to a rise in ethanol intake in alcohol preferring (P) rats, but not in non-alcohol preferring (NP) rats as previously mentioned. Interestingly, this rise in anxiety-like behaviors in P rats can be alleviated by administering NPY directly into the CeA. If this is the case, NPY might be expected to inhibit neuronal activation within the CeA or NAc, both of which are activated by yohimbine. Furthermore, the yohimbine-induced reinstatement of ethanol intake behavior is blocked by the corticotropin releasing hormone (CRH) – 1 receptor antagonist, antalarmin, and the glucocorticoid receptor antagonist, mifepristone (Simms, Haass-Koffler, Biton-Onon, Li, & Bartlett, 2011). These results seem to indicate that the HPA axis, especially CRH, plays a role in the modulation of ethanol drinking behavior. This involvement is described in detail in Chapter 3.

NPY-induced reductions in ethanol intake have also been shown in the rhesus macaque (Lindell et al., 2010). Rhesus NPY (rhNPY) and regulatory regions for variations were screened, and the functionality of a nucleotide polymorphism (SNP; rhNPY – 1002 T > G) was observed. In particular, this polymorphism was located in a region that is orthologous to one important for the regulation of human NPY promoter activity (reviewed in Lindell et al., 2010). Of the two alleles (G or T), the G allele resulted in decreased levels of NPY within the amygdala, and peer-reared animals (a model for early stress) carrying the G allele (T/G or G/G) had lower CSF NPY levels than peer-reared T/T animals). Behavioral measures were also used to collect data, with three factors, those being separation anxiety, arousal, and behavioral pathology, being

measured. Peer-reared infants had higher levels of arousal than mother-reared infants, and those homozygous for the G allele had higher arousal scores than the T allele homozygous macaques. Peer-reared T/T macaques had lower levels of arousal than those with the G-allele, either T/G or G/G. This suggests that NPY within the rhesus macaque mediates behavioral anxiety, with the G-allele variation reducing the efficiency of NPY to work as an anxiolytic. Indeed, it was found that only the carriers of the G allele consumed higher levels of ethanol than their mother-reared monkey counterparts (Lindell et al., 2010), indicating the importance of NPY in modulating ethanol drinking behavior.

It has been suggested that ethanol withdrawal leads to lower NPY levels which causes elevated anxiety-like behavior in rats. Gilpin et al. (2003) ran two separate experiments. In experiment 1, female P rats were given 6 weeks of continuous access to ethanol (8% w/v) and water. Ethanol was then removed for a period of 2 weeks. During this period, rats were implanted with cannula into the lateral ventricle. Following the period of ethanol abstinence and prior to ethanol reinstatement, rats were given a single infusion of either aCSF or μ10g of NPY. A second experiment was run in parallel with experiment 1, with the exception that rats did not undergo a period of ethanol abstinence. The data demonstrated that following 2 weeks of imposed ethanol abstinence, NPY suppressed ethanol intake through the second day of post infusion (i.e. total of 3 days). Results from experiment 2 showed that NPY suppressed ethanol intake to a lesser extent and the effect lasted only 24 hours (Gilpin et al., 2003).

Elevated NPY levels during withdrawal, especially within the CeA, are responsible for the feelings of anxiety felt when an alcoholic undergoes periods of imposed abstinence. Furthermore, it has been found that the phosphorylation status of

CREB is decreased in several cortical and amygdaloid structures during ethanol withdrawal after chronic ethanol intake (Pandey, Roy, & Mittal, 2001; Pandey, Saito, Yoshimura, Sohma, & Gotz, 2001). This suggests that decreased pCREB may lead to decrease expression of NPY in cortical and amygdaloid structures during ethanol withdrawal. A 2002 study by Roy & Pandey (2002) investigates the possible involvement of NPY in the neuroadaptational mechanism to chronic ethanol exposure and its withdrawal. Ethanol withdrawal but not ethanol treatment produced significant reduction in NPY levels throughout the cortex, but of interest, in the MeA and CeA. One thing to note is that neuronal hyperexcitability is one of the major consequences of ethanol withdrawal after chronic ethanol intake. It is believed that this hyperexcitability leads to the development of several withdrawal symptoms (Koob & Bloom, 1988).

Endogenous NPY is important for the modulation of anxiety and subsequent ethanol intake. Genetic predispositions to anxious behavior leads to a genetic vulnerability to increased ethanol drinking, as demonstrated with P and HAD rats. Furthermore, the withdrawal symptoms seen in normal alcoholics following a period of abstinence are similar to those seen in animal models following decreased levels of NPY, Interestingly, as mentioned before, a hyperexcitable state within the CeA is seen, which leads to increased anxiety, and a risk of convulsions and tremors (Pandey, 2003). Both NPY and ethanol have similar electrophysiological profiles, which take into account the "tension-reduction" hypothesis: a genetic predisposition for lower NPY leads to heightened anxiety and therefore, a hyperexcitable state, which is relieved by subsequent ethanol intake. This suggests that NPY plays a critical role in hyperexcitability, being important in the control of neuronal activity in areas such as the amygdala. For instance,

increased levels of NPY and prepro-NPY mRNA were found in the cortical and limbic neurons of rats following acute seizures (White & Gall, 1987; Gruber, Schwarzer, & Sperk 1994). Furthermore, in epileptic rats, there is a significant and long-lasting increase in NPY mRNA following convulsive stimulation (Schwarzer, Williamson, Lothman, Vezzani, & Sperk, 1995), suggesting that NPY may be responsible for an endogenous anticonvulsant mechanism. Y_2 receptors are believed to modulate NPY's anticonvulsant effects, with radioligand-binding being enhanced within the hippocampus and amygdala following kindling-induced seizures (Schwarzer, Kofler, & Sperk, 1998; Gobbi et al., 1998) by reducing Ca^{2+} influx into presynaptic nerve terminals through Ca^{2+} channels (Qian, Colmers, & Saggu, 1997). Recently, recombinant adeno-associated viral vector expressing the NPY gene has been used in rats to decrease spontaneous temporal lobe seizures (Noe et al., 2008), and it has been found that increased NPY in the dentate hilus and retrosplenial cortex decrease the instance of seizure activity in rats (Cardoso, Freitas-de-Costa, Carvalho, & Lukoyanov, 2010). Although these findings concern other areas such as the hippocampus, there is amounting evidence that a similar mechanism plays a role in the hyperexctability of the amygdala. For instance, animals that are subjected to early life stress by handling and maternal separation (HMS) where litters of rat pups are removed from their dam for about 180 minutes a day from postnatal days 2-4, tend to have accelerated acquisition of amygdala kindling (Salzberg et al., 2007). There is also evidence that suggests that depression, among other psychiatric disorders, is associated with early life stresses and represent a risk factor for the development of epilepsy (Hesdorffer, Hauser, Annegers, & Cascino, 2000; Hesdorffer, Hauser, Olafson, Ludvigsson, & Kjartansson, 2006), and that increase emotion output can be driven by

increased activity within the amygdala (Collins & Pare, 2000). Furthermore, early life stress exacerbates mood and cognitive disturbances, which are associated with the development of epilepsy (Jones et al., 2009). A possible mechanism for this excitability is the channelopathy of calcium-activated potassium channels that occurs in the BLA after chronic stress (Rosenkranz, Venheim, & Padival, 2010). Activation of these channels during action potential firing leads to afterhyperpolarization potentials (AHP), with chronic stress greatly inhibiting K_{Ca} channel function by reducing the amplitudes of slow AHP and medium AHP.

Recent studies have demonstrated the role different receptors play in mediating NPY's anxiolytic effects. For instance, knock-out mice lacking the gene to produce the Y_1 –receptor (Y_1 -/-) are more resistant to the sedative effects of ethanol and tend to consume more ethanol than their wild-type counterparts (Thiele et al., 2002). Furthermore, high ethanol intake in Y_1 -/- rats does not depend on the caloric properties of ethanol since that they consumed equal amounts of sucrose and quinole. Moreover, mutant Y_1 -/- mice regained their righting reflex sooner than wild-type rats. This suggests that Y_1-receptors mediate the anxiolytic effects of NPY. NPY seems to have an anxiolytic effect when administered alone which would coincide with the results obtained by Thiele et al. (2002). Given that a lack of Y_1-receptors leads to an increase to the sensitivity of the sedative effects of ethanol, it would seem reasonable to conclude that Y_1-receptors mediate the sedative and anxiolytic effects of NPY.

Both NPY and ethanol affect event related potentials from the cortex and amygdala similarly (Ehlers, Somes, Lumeng, & Li, 1999) and combined administration of both produces additive effects. This suggests that Y_1-R in the amygdala are necessary

for the reinforcing properties of ethanol. Moreover, 70% of the cells exhibiting increased c-fos expression within the CeA after acute ethanol administration are GABAergic (Morales, Criado, Sanna, Henricksen, & Bloom, 1998), and that rewarding effects of ethanol appear to be mediated by $GABA_A$ receptors (Chester & Cunningham, 2002) suggesting that intra-amygdala infusion of Y_1 antagonist influenced GABAergic function within the CeA. NPY Y_1 –R antagonists blocks eating stimulated by $GABA_A$ agonist muscimol (Pu et al., 1999), NPY and GABA are co-localized within the amygdala, and several NPY-producing neurons in the amygdala make contact with GABAergic neurons that are positive for the Y_1-R (Oberto et al., 2001).

Y_2 receptors, functioning as presynaptic auto-receptors on NPY-ergic terminals, inhibit NPY release as a feedback mechanism (King, Widdowson, Doods, & Williams, 1999; 2000). The suppressive actions of NPY signaling on ethanol self-administration might be sensitized in subjects with a history of dependence induced through the long term cycled vapor exposure paradigm. Central NPY-signaling is potentiated using an Y_2 antagonist, which presumably enhances release of endogenous NPY. BII0246 suppresses self-administration of ethanol in ethanol vapor exposed rats with higher potency than in subjects without a history of dependence. For instance, Y_2-antagonism in animals with a history of dependence suppressed operant responding for ethanol (approx. 50%) at a dose of 0.5nmol i.c.v. (Rimondini, Throsell, & Heilig, 2005). This same dose was proven ineffective in subjects without a history of dependence. However, at higher doses of the antagonist, intake of ethanol in Wistar rats without a history of dependence was suppressed. Furthermore, there was a sensitization to the suppressive effect of BIIE0246 on ethanol self-administration in subjects with a history of dependence. It has been

hypothesized that this lack of effect might be due to a compound effect of central NPY administration, to simultaneously stimulate intake of caloric nutrients including ethanol through actions in the hypothalamus, and to reduce motivation to consume ethanol for its rewarding properties through other centers.

Central infusion of NPY directly into the CeA, which acts as an agonist at Y_1-R and an antagonist at Y_2-R, was shown to blunt binge-like ethanol drinking in mice (Sparrow et al., 2012). A similar effect was seen when the Y_1 agonist, [D-His^{26}]-NPY and the Y_2 antagonist BIIE026 were given. The effect was not seen when mice were given the Y_2 agonist NPY_{13-36}. Furthermore, binge-like ethanol drinking reduced NPY and Y_1 receptor immunoreactivity within the CeA, with levels of Y_1 and Y_2 receptors increasing following a period of 24 hours after the last episode of binge-like drinking, however their NPY IR remained significantly reduced. Binge-like drinking also augmented NPY's ability to inhibit GABAergic transmission and demonstrated with electrophysiological recordings. This was demonstrated by reduced paired-pulse ratio (PPR; ratio of the amplitude of the second response to that of the first) of eIPSCs within the CeA. In olfactory bulbectomized (OBX) rats, which display a series of symptoms that mimic several aspects of human depression and anxiety disorders, treatment with the Y_1-agonist [$Leu^{31}Pro^{34}$]-PPY decreased depressive- and anxiogenic-like behaviors and Y_2-receptor antagonist, BIIE0246 decreased the immobility time in the forced-swim test in OBX animals. These results suggest that both Y_1- and Y_2- receptors work in tandem to mediate anxiety-like behaviors and ethanol intake behaviors.

CREB Involvement in Alcoholism: Target Genes

Early studies in NPY targets have demonstrated that CREB activity is decreased in the corpus striatum and the cerebellum of an unselected stock of rats after chronic alcohol exposure (Yang, Horn, Barban, & Wand, 1998). Furthermore, there has been a decrease in CRE-DNA binding activity observed in the cortex of an unselected rat stock during ethanol withdrawal (Pandey, Zhang, Mittal, & Nayar, 1999a). P rats may exhibit greater anxiety than NP rats because of underlying difference in neurocircuitry involving decreased CREB activity that may have a genetic component. For instance, a lower expression of the CREB gene in rats may account for their innately high anxiety-like behavior. One of the things to note, however, is that the early studies did not focus on the amygdala. A series of studies by Pandey and colleagues have demonstrated that there is indeed a difference in CREB activity within the amygdala. For instance, CRE-DNA binding, p-CREB, and total CREB protein levels are decreased in the amygdala of P rats when compared with NP rats (Pandey, Mittal, Lumeng, & Li, 1999b). Moreover, CRE-DNA binding activity within the cortex, hippocampus, and striatum was found to be similar in both strains of rats. PKA activity and protein levels of the α-isoform of the PKA catalytic subunit are similar in the amygdala of P rats and NP rats. These results coincide with the finding of decreased adenylate cyclase activity in platelets and increased expression of $G_s\alpha$ proteins in red blood cells in alcoholics with a positive family history of alcoholism (Menninger, Baron, & Tabakoff, 1998). This suggests that the cAMP second messenger signaling cascade may be important in alcohol dependence.

How CRE-DNA binding activity in the amygdala of P rats is decreased is currently unknown, however some mechanisms have been proposed (Pandey et al.,

1999b). This decrease in CRE-DNA binding may be the result of a decreased expression of CREB protein, which leads to a decrease in p-CREB levels as well as a decrease of cAMP inducible genes in the amygdala. The CeA may be a site at which CRH systems mediate anxiety during ethanol withdrawal after chronic ethanol intake in an unselected stock of rats (Rassnick, Heinrichs, Briton, & Koob, 1993). Furthermore, CREB regulates the expression of the CRH gene, so a decrease in CRE-DNA binding activity may lead to decreased expression of the CRH gene in the amygdala. However, recall that NPY, an anxiolytic, is also regulated by CREB. In other words, CREB regulates the expression of both NPY and CRH and a decrease in CRE-DNA binding activity leads to a decrease in the expression of both genes. The link between CRH and NPY are currently unknown.

Another study attempted to establish a correlation between CREB phosphorylation in the CeA and anxiety, as well as investigating the effects of manipulation of the phosphorylation status of CREB by infusion of the PKA activator Sp-cAMPS, or the inhibitor Rp-cAMPS (reviewed in Pandey, Carr, Heilig, Ilveskoski, & Thiele, 2003b). Ethanol withdrawal significantly reduced CREB phosphorylation and CaMK IV protein levels without modulation of total CREB protein levels within the CeA and MeA, but not within the BLA (Pandey et al., 2003b). Furthermore, chronic ethanol treatment had no effect on CREB, p-CREB, or CaMK IV protein levels within the amygdala and ethanol treatment and withdrawal had no effect on the PKA-Cα protein levels. Moreover, there were no changes in PKA activity or protein levels of PKA-Cα or RII-β subunits in the nuclear extract of the cortex during ethanol treatment or its withdrawal.

In animals withdrawn from their ethanol diet, CaMK IV levels decreased suggesting that CREB phosphorylation must decrease during the withdrawal process. This decrease in CREB phosphorylation appears alongside with the anxiety that is characteristic of ethanol withdrawal. Indeed, it was found that during the ethanol treatment CREB phosphorylation levels were normal. During ethanol withdrawal, infusion of the activator directly into the CeA normalized CREB phosphorylation levels by increasing PKA-Cα protein levels however CaMK IV levels were still low (Pandey et al., 2003b). Furthermore, after infusion of the activator in the CeA there were still reduced levels of CREB phosphorylation in other brain structures, such as the MeA, and the frontal, parietal, and piriform cortical structures. Infusion of the inhibitor directly into the CeA provoked anxiety in normal rats. These results suggest that CREB phosphorylation levels within the CeA are critical in the development of anxiety-like behaviors during ethanol withdrawal.

Other studies have also demonstrated that NPY infusion into the hypothalamus and amygdala increase the levels of phosphorylated CREB (pCREB) in wild type rats, as well as NPY activated CaM kinases in cultured cells (Sheriff et al., 1998, 2002). This suggests that exogenous NPY increases pCREM by CaM kinase-dependent mechanism, as described above. When comparing P rats and NP rats, P rats had lower protein levels of CREB, pCREB, and mRNA and protein levels of NPY in CeA and MeA, but not in BLA. These results indicate that a deficiency in both CREB and NPY in the CeA may be involved in anxiety-like behaviors and alcohol drinking behaviors. Recently, two different hypothesis were examined (Zhang et al., 2010): (1) if NPY infusion into the CeA can attenuate both anxiety-like behaviors, as measured by the light/dark box (LDB)

exploration test, and alcohol intake and (2) if NPY infusion can increase pCREB levels by increasing levels of either CaMK IV or the catalytic α-subunit of PKA, increasing the expression of endogenous NPY in the CeA of P rats. NPY infusion into the CeA produced anxiolytic effects, as measured by the LDB exploration test, and in P rats, a decrease in ethanol intake was observed (Zhang et al., 2010). NPY infusion into CeA also increased the levels of CaMK IV and phosphorylated CREB, as well as increased mRNA and protein levels of NPY. However, NPY infusions did not produce a change in the protein levels of PKA-Cα in the CeA, indicating that NPY receptors work via the CaMK IV pathway.

Early studies (Davis, Rainnie, & Cassell, 1994) have suggested that BLA plays a role in anxiety however more recently (Pandey et al. 2003a) found no differences in the CREB phosphorylation levels during ethanol withdrawal have been found within the BLA. Moreover, decreasing CREB phosphorylation levels with Rp-cAMPs did not produce anxiety behavior suggesting that decrease CREB phosphorylation within the CeA but not the BLA may be a possible molecular mechanism associated with the development of anxiety. Furthermore, infusion of NPY prevents PKA-inhibitor induced increase in of alcohol preference, suggesting that a decrease in NPY lead to alcohol-drinking behavior.

Recently, it was shown that CREB-haplodeficient mice (+/-) have a higher preference for ethanol than wild-type mice (+/+; Pandey, Roy, Zhang, & Xu, 2004). Furthermore, (+/-) mice have about 40% less CREB and CREB phosphorylation protein levels in a variety of brain structures, including the cortex, hippocampus, the amygdala, and the nucleus accumbens when compared to wild-type mice. There is also a decrease in

the expression of CREB target genes such as NPY and BDNF within the brain in (+/-) mice, suggesting that although the CREB-βisoform is slightly up-regulated in the brain of CREB α and δ haplodeficient mice, the protein levels of total CREB and p-CREB and expression of CREB related genes are lower in the brains of (+/-) mice. Moreover, these CREB deficient mice also display more anxiety-like behaviors, with ethanol exposure producing anxiolytic effects and leading to the increase of CREB phosphorylation and NPY in the CeA and MeA but not BLA of wild-type mice. These effects were not seen in the CREB-deficient mice, suggesting that a haplodeficienty of the CREB gene is associated with increased alcohol-drinking behavior.

Ethanol has been found to potentiate $G_s\alpha$ adenylyl cyclase activity (Saito, Lee, & Tabakoff, 1985) and recently, type 7 AC has been reported to be two to three times more sensitive to the effects of ethanol (Yoshimura & Tabakoff, 1995) than the other nine isoforms of adenylyl cyclase. For instance, AC7 can be potentiated by 10-20mM ethanol, an effect which has been found to not be mediated via inhibition of phosphoidestarase activity or an adenosine receptor-mediated event (Yoshimura & Tabakoff, 1999). Furthermore, ethanol potentiates cAMP accumulation independent of receptor effects, but dependent on the effect of AC7 activation (Nelson, Hellevuo, Yoshimura, & Tabakoff, 2003), and forskolin-activated cAMP generation in mice brains was found to alter the development of tolerance to the sedative effects of ethanol (Szabo, Hoffman, & Tabakoff, 1988). Moreover, ethanol dependent individuals displayed decreased G-protein activated AC activity within their platelets despite having been abstinence for a period of time (Tabakoff et al., 1988). Clearly, ethanol not only has effects on GABAergic systems and this activation of AC7 may explain the molecular mechanisms it activates within the CeA.

Potentiated AC7 activity has been found to be mediated by phosphorylation of AC7 via protein kinase C delta (PKCδ; Nelson et al., 2003), indicating that AC7 activity does not depend on typical (Ca^{2+}-mediated) or atyipical (non-Ca^{2+} mediated) PKC. This AC7 effect is though to be mediated via CRH-1 receptors since ethanol has been found to increase plasma adrenocorticotropin and glucocorticoid levels (reviewed in Pronko et al., 2010), suggesting that ethanol increases CRH-1 receptor and $G_s\alpha$ mediated cAMP signaling by promoting AC7 phosphorylation, thereby turning on subsequent signaling pathways. Indeed, it was found that AC7 transgenic (Adcy7huTG) mice expressed higher levels of CRH, adrenocorticotropin, and corticosterone following ethanol injection (Pronko et al., 2010). Subsequent targets of this activated pathway include PKA (Constantinescu, Diamond, & Gordon, 1999; 2002). Interestingly, acute ethanol levels reduces the expression of the protein kinase A inhibitor alpha (PKI-α), which acts as a pseudosubstrate for the catalytic subunit of PKA, inhibiting its action (Knighton et al., 1991). At higher doses, ethanol stimulates the expression of PKI-α (Repuente-Canonigo, Lutjens, van der Stap, & Sanna, 2007), suggesting a compensatory effect. Furthermore, this effect was followed by an increase in the expression of $GABA_A$-β3 and $GAB_AAα1$ subunits within the amygdala. A similar effect was seen in rats that were bred for reduced temporal lobe excitability after seizure induction with kainite (Gilby, Da Silva, & McIntyre, 2005), suggesting an adaptation to increased excitability induced by ethanol intake.

The Role of the GABAergic System in Ethanol Consumption: Relationship with NPY

The involvement of GABAergic transmission in ethanol intake behavior is well known, as ethanol enhances GABAergic transmission (Roberto et al., 2003). As

mentioned above, NPY levels are decreased significantly following withdrawal, especially in brain regions involved in seizure activity and anxious behavior, including the CeA (Roy & Pandey, 2002). Reduced GABAergic function leads to neuronal hyperexcitability, which is believed to play a role in withdrawal symptoms (Harris & Buck, 1990). Recall that ethanol withdrawal is characterized by a central nervous system hyperexcibatility that results in physical and affective signs of dependence. In humans, early stages are characterized by tremor and elevated sympathetic responses including increase in heart rate, blood pressure and body temperature. Such physical signs are accompanied by insomnia, anxiety, anorexia and dysphoria (Koob, 2004). Several studies have implicated GABAergic systems in the effects of ethanol withdrawal, more specifically the anxiogenic-like effects. GABA agonists tend to decrease central nervous system hyperexcitability during withdrawal (Frye, McGowan, & Breese, 1983), while GABA antagonists exacerbate many of the symptoms of withdrawal (Goldstein, 1973), and the partial inverse benzodiazepine agonist RO 15-4513 has been shown to increase the incident of seizures during ethanol withdrawal. Moreover, NPY and GABA seem to be co-localized within a variety of brain structures, including the cortex, amygdala, and hippocampus (Oberto et al., 2001).

GABA is the major inhibitory neurotransmitter in the brain, and it is ultimately derived from glucose metabolism (Deutch & Roth, 2008; in Squire et al., 2008). The main receptor involved in GABA function is the $GABA_A$ receptor (Waxham, 2008; in Squire et al., 2008; Meyer & Quenzer, 2005). $GABA_A$ is an ionotropic heteropentameric complex of about 275 kDa, composed of five different subunits designated α, β, γ, δ, and ε. $GABA_A$ receptor has an ion channel associated with it which is selective for anions,

particularly the Cl^- ion (Waxham, 2008; in Squire et al., 2008). Upon GABA binding, the channel allows the influx of Cl^- which leads to depolarization. GABA is not the only molecule that is able to open up the channel associated with the $GABA_A$ receptor, having additional modulatory sites for benzodiazepines, barbiturates, neurosteroids, and picrotoxin (Meyer & Quenzer, 2005). Both benzodiazepines and barbiturates fall under the category of sedative/hypnotic drugs, along with ethanol. The action of these drugs is to potentiate the effects of GABA on the $GABA_A$ receptor, which leads some of the properties shared by the sedative/hypnotic drugs, including anxiolytic effect, sedating and sleep-inducing effects, and anticonvulsant effects. Ethanol appears to exert its acute effects by opening the channel to allow the Cl^- influx; the chronic effect or repeated ethanol exposure reduces $GABA_A$-mediated Cl^- flux, as well as making animals more sensitive to seizure-inducing doses of the GABA antagonist, bicuculline (Meyer & Quezner, 2004).

Although the role of GABAergic neurotransmission in alcoholism has been examined, alcohol does not seem to have a specific neurotransmitter binding site within the brain. It has been suggested that receptive elements within membranes, and a protein component of neuronal membranes, may provide a site for the action of ethanol (Koob, 2004b). Ethanol has been suggested to act on ligand-gated channels, especially within the GABAergic system and more specifically, $GABA_A$ receptors. An early study (Rassnick et al., 1993) used an operant model of ethanol self-administration. Pretreatment with RO 15-4513, a benzodiazepine inverse agonist, at low doses selectively decreased responding for ethanol but not for water. Furthermore, RO 15-4513 did not affect responding for a saccharin solution, which suggests a specific target action. Additionally,

isopropylbicyclophosphate, a picrotoxinin site ligand, selectively decreased responding for ethanol at very low doses in alcohol-preferring, alcohol non-preferring, and Wistar rats (Rassnick et al., 1993) suggesting that blockade of the $GABA_A$ receptor can block motivation for responding for ethanol.

Microinjection into the CeA of the $GABA_A$ receptor antagonist SR 955332 significantly reduced responses on the ethanol lever without altering responses on the water lever on Wistar rats trained to respond for 10% ethanol solution in a two-lever, free-choice operant task (Hyytia & Koob, 1995). In addition, ibotenic acid lesions of the CeA were able to reduce ethanol consumption in Sprague-Dawley rats without affecting total fluid intake (Moller et al., 1997). These results suggest that activation of $GABA_A$ receptors within the CeA is able to mediate ethanol self-administration. Another study found that microinjection of muscimol, a $GABA_A$ agonist, decrease operant responding for ethanol in dependent rats but not in non-dependent rats, a finding strikingly similar to that found upon microinjection of NPY directly into the CeA (Roberts, Cole, & Koob, 1996).

GABA is often coexpressed with NPY/Agouti-Related Protein in ARC neurons (Pu et al., 1999), projecting into the PVN where they act to inhibit CRH neurons and to enhance feeding (Pu et al., 1999). Furthermore, within the hippocampus and the neocortex, NPY is made by neurons that almost all express GABA (Jinno & Kosaka, 2003), with several studies indicating that such neurons may be involved in the response to epileptogenesis. For instance, NPY was found to increase GABAergic neurotransmission onto pyramidal neurons and to decrease inhibition on GABAergic interneurons (Bacci et al., 2002). NPY actions within cortical neurons decrease

excitability within these circuits, leading to the potentiation of NPY and GABA inhibitory responses. These results demonstrate a possible interaction between the NPY and GABA systems within the brain. More specifically, it has been demonstrated an interaction between GABAergic and NPY-Y_1R mediated transmission which showed that anxiolytic benzodiazepines, such as diazepam, block the anxiogenic effect of Y_1 -R antagonist (Kask et al., 1996). Naveilhan et al. (2001) showed that NPY can induce its sedative effects via the GABAergic system by interacting with the Y_1-R in the posterior hypothalamus. Modulation of the $GABA_A$ receptor complex induces changes in the expression of NPY and NPY mRNA in various regions, including the CeA and it has been found that treatment with benzodiazepines affects the NPY immunoreactivity in the amygdala, cerebral cortex, and locus coeruleus in rats (Krysiak, Obuchowicz, & Herman 1999). Eva et al. (2006) showed that prolonged treatment with positive (diazepam and abecarnil) or negative modulators (FG7142) of $GABA_A$ receptor function induces a significant increase (in the case of a positive modulator) or decrease (for a negative modulator) of the Y_1-R gene expression within the MeA, suggesting that the NPY-Y_1 receptor mediated transmission and GABA-ergic system are closely coupled. Moreover, NPY and GABA may functionally interact in the regulation of anxiety behavior. For instance studies by Kask, Nguyen, Pabst, & von Horsten (2001) and Thorsell et al. (2000) demonstrated that NPY-Y_1R mediated transmission elicits behavioral effects that are identical to those produced by $GABA_A$ receptor activation, suggesting that changes in the function of $GABA_A$ receptors elicit compensatory responses in the firing rate of NPY containing neurons. $GABA_A$ receptors might also be responsible for the changes in Y_1-receptor gene transcriptional activity.

CHAPTER THREE

Corticotropin Releasing Hormone

Anxious behavior is one of the key symptoms of ethanol withdrawal and as mentioned in Chapter 2, NPY levels within the amygdala have been implicated in playing a role in such behavior. With respect to general stress and anxious behavior, NPY is not the only neuromodulator believed to play a role. Corticotropin-releasing hormone (CRH), also known as corticotropin-releasing factor (CRF), is the major neuromodulator of the hypothalamic-pituitary-adrenal axis (Vale, Spiess, & Rivier, 1981). CRH is a 41 amino acid peptide (Table 3.1) expressed in the paraventricular nucleus (PVN) of the hypothalamus (as part of the HPA axis), with highest levels being present in the medial parvocellular part of the PVN. This area is important with regards to stress regulation, as it summates inputs from various brain regions including the brain stem, midbrain/pons, the limbic system, and the circumventricular organs (Gore, 2008). There are also high densities also found within the CeA, the BNST, and the brainstem (Heilig & Koob, 2007; Gore, 2008). CRH is cleaved at a pair of dibasic amino acids from a larger, 191-amino acid precursor molecule (pre-proCRH) by the action of endopeptidases (Morrison et al., 1995). The CRH amino-acid sequence was first discovered by Vale et al. (1981) in the sheep brain. The rat and human peptides are identical and differ from the ovine sequence only by 7 amino acids (See Table 3.1; Chrousos et al., 1992). CRH, along with urocortin 1 (UCN1), urocortin 2 (UCN2) and urocortin 3 (UNC3) (Vaughan et al., 1995; Reyes et al., 2001; Lewis et al., 2001) make up the members of the CRH peptide family. Urotensin

I and frog sauvagine (Reyes et al., 2001; Lewis et al., 2001) have also been found to have high affinities for CRH receptors.

Table 3.1. Amino acid sequences of ovine, and rat CRH peptide; adapted from Vale et al., 1981 and Chrousos et al., 1985

Ovine CRH	SQEPPISLDLTFHLLREVLEMTKADQLAQQAHSNRKLLDIA
Rat CRH	SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII

The HPA axis is responsible for preparing the body in response to stressors, which include social and emotional stressors. Figure 3.1 shows a general schematic of the HPA axis. Activation of the HPA axis leads to a rise in the concentration of glucocorticoids, which hare are important in the stress response, playing a role in the regulation of glycogen utilization and storage, exerting effects on the cardiovascular system to elevate heart rate and blood pressure, and activate the sympathetic branch of the autonomic system (Gore, 2008; Purves et al., 2008).

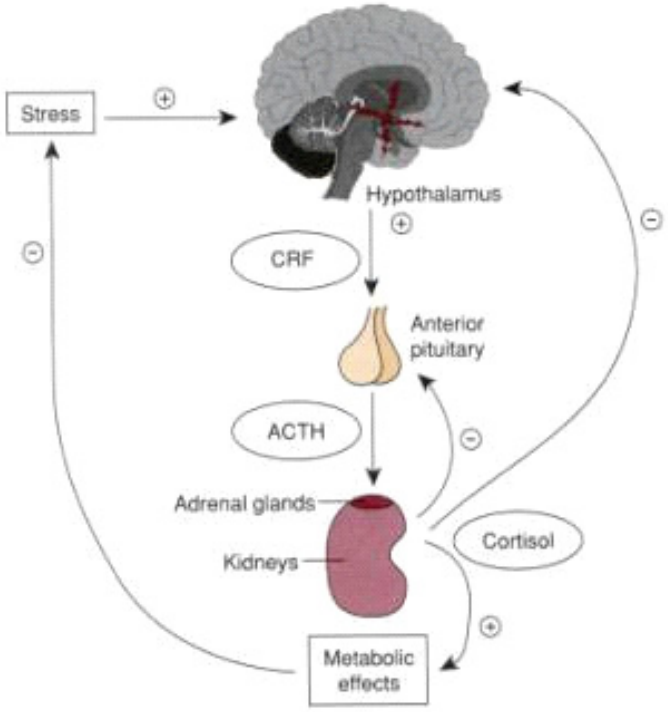

Figure 3.1 Schematic of the hypothalamic-pituitary-adrenal axis.

The functions of CRH within the brain are vast. For instance, central administration of CRH increases neuronal firing with the locus coeruleus (Valentino, Page, Van Bockstaele, & Aston-Jones, 1992) and the hippocampus, in the latter inhibiting the slow after-hyperpolarization (Aldenhoff, Gruol, River, Vale, & Siggins, 1983; Siggins, Gruol, Aldenhoff, & Pittman, 1985). Furthermore, it has been found that ovine CRH can produce tonic chronic seizures within rats after 3-7 hour delays (Ehlers, Henriksen, Wang, Rivier, & Vale, 1983), with studies showing that high CRH levels can produce limbic seizures which resemble kindling. For instance, amygdala-kindled seizures markedly increase CRH and CRH-binding protein levels in various brain regions, including the dentate gyrus of the hippocampus (Smith et al., 1997). These increases were produced almost exclusively in GABAergic interneurons, along with a rise in NPY levels in these same neurons.

Receptors Involved in CRH Function

To date, two types of CRH receptor subtypes have been identified in humans: CRH-1 (415-amino acid residues) and CRH-2 (377-amino acid residues). Both of these are G-protein-coupled receptors, belonging to the class II seven-transmembrane receptor superfamily (Perrin, Donaldson, Chen, Lewis, & Vale, 1993). The two receptors are encoded by two distinct genes, CRH-1 encoded by a gene on chromosome 17 while CRH-2 is encoded by a gene in chromosome 7 (Polymeropoulos, Torres, Yanovski, Chandrasekharappa, & Ledbetter, 1995; Meyer et al., 1997). Both receptor subtypes share 70% homology in their amino acid sequence, yet exhibit distinct pharmacologic profiles and have very distinct central and peripheral distributions. CRH-1 receptors are distributed centrally within the anterior pituitary, amygdala, hippocampus, cerebellum,

lateral hypothalamic nuclei, locus coeruleus, thalamus, and the neocortex (Koob & Heinrich, 1999; Gray, 1993; Gore, 2008), and it is considered to be the only receptor in the locus ceruleus, cortex, thalamus, and striatum. CRH-2 receptors have a distinct central distribution, with high concentrations in the ventromedial hypothalamic nuclei, bed nucleus of the stria terminalis, lateral septum, brainstem, and dorsal raphae, with a reduced concentration within the amygdala (Chalmers, Lovenberg, & De Souza, 1995; Perrin & Vale, 1999; Gore, 2008), and it is strongly associated with appetite suppression (Pelleymounter et al., 2000).

Although there are only two general families of CRH receptors, both genes have the ability of variant splicing to produce different isoforms of each. For instance, the CRH-1 receptor gene has variants R1a, R1b, R1c, R1d, R1e, R1f, R1g and R1h which are produced by alternative splicing of exons 3-6 and 10-13 (Chalmers et al., 1995). The CRH-2 receptor gene only has three variants, encoding CRH-R2a, CRH-R2b, and CRH-R2c isoforms and is produced by the use of alternate 5' exons (Catalano, Kyriakou, Chen, Easton, & Hillhouse, 2003; Johnson et al., 2003).

Binding of an agonist for CRH receptors causes a change in the structural confirmation of the protein, leading to the signal transduction via the activation of the heterotrimeric G-proteins. Different CRH-related peptides have been shown to have different affinities for the two general classes of receptors. Furthermore, the actions or CRH seem to be controlled not only by CRH-receptors, but also by CRH binding protein (CRH-BP) (Potter et al., 1991). CRH-BP is expressed in the outer surface of cell membranes and circulates throughout the bloodstream. Although its exact physiological profile has not been elucidated fully, it has been proposed to act as a modulator of the

HPA axis and other actions by limiting the availability of free ligand (Grammatopoulos & Chrousos, 2002). CRH itself is considered to be specific for CRH-1 receptor, although it does bind to CRH-2 with a lower affinity when compared to the urocortins. Urotensin I and sauvagine also have high affinities for the CRH-1 receptor, but neither UNC 2 nor 3 are able to active it (Grammatopoulos & Chrousos, 2002). UNC 2 and UNC3 both display higher affinities for CRH-2, while UNC 1 has high affinity for both receptors (Gammatopoulos & Chrousos, 2002).

CRH Molecular Mechanisms

Ligand binding on CRH-1 and CHR-2, both of which are coupled to $G_{\alpha s}$ (Perrin & Vale, 1999), activates adenylyl cylcase which induces an increase in cAMP levels (Grammatopoulos, Milton, & Hillhouse, 1994; Heldwein, Redick, Rittenberg, Claycomb, & Stenzel-Poore, 1996). Despite their coupling to adenyly cyclase, in certain tissues CRH cannot activate this pathway and relies in other alternative signaling cascades (Ulisse, Fabbri, Tinajero, & Dufau, 1990; Karteris, Grammatopoulos, Randeva, & Hillhouse, 2000). Furthermore, there is tissue-specific G-protein coupling and activation of alternative signaling cascades (Ulisse et al., 1990; Karteris et al., 2000), with several studies of the CRH transfected in in vitro expression demonstrating multiple G-protein activation with an order of potency $G_s \geq G_o > G_{q/11} > G_{i1/2} > G_z$. However, in vivo, cells have a pattern of G-protein activation unique to each tissue (Grammatopoulos et al., 1999; Karteris et al., 2000). Depending on the tissue, CRH can activate different signaling cascades to produce different responses. This also suggests that the same tissue can switch between different signaling cascades, with some speculation about how this is done. For instance, CRH receptor phosphorylation may underlie a possible mechanism by

which this switch occurs. The receptor sequence has multiple potential phosphorylation sites for signals such as PKA, PKC, and casein kinase.

Activation of CRH-1 via CRH and Unc I stimulates adenylyl cyclase activity (Dautzenberg, Braun, & Hauger, 2001; Grammatopoulos, Randeva, Levine, Katsanou, & Hillhouse, 2000), with other studies indicating that Unc I and sauvagine can activate the p42/p44 MAPK system (Rossant, Pinnock, Hughes, Hall, & McNulty, 1999). The latter effect seems to be exerted primarily via the activation of the G_q- IP_3-PKC pathway. CRH can also activate the MAPK cascade in neurons, albeit in a different manner. CRH relies in the Gs-adenylyl cyclase system, as opposed to the IP_3 - PKC pathway. These pathways mediate the neuroprotective effects of CRH and Ucn (Elliott-Hunt, Kazlauskaite, Wilde, Grammatopoulos, & Hillhouse, 2002; Pedersen, Wan, Zhang, & Mattson, 2002) suggesting that the ability for different CRH-receptor agonist to elicit a response depends on the ability of the receptor-agonist complex to activate a certain second-messenger pathway. Further regulation for CRH effect can occur in two other ways (Gammatopoulos & Chrousos, 2002). First, there is the genetic expression of different types of CRH-receptors, which ensures that cells expressing CRH-1 receptors will not be subjected to the actions of either Unc II or III. Second, CRH receptor splice variants provide flexibility for target tissues to bind and respond differently to the same agonist.

Pisarchik & Slominski, (2001) have suggested that cAMP-activating signaling pathways and PKC serve as potential regulators of CRH-1 receptor splicing patterns. Both these signaling pathways have been implicated in the down regulation of CRH-1 receptor mRNA, especially the CRH-1 receptor within the CeA, which are Gsα coupled. PKA, which is activated by cAMP, acts as the main stimulator of CRH gene transcription.

In other types of cells, including those within the amygdala, the CRH system activates protein kinase C (PKC) pathways (Rossant et al., 1999; Sananbenesi, Fischer, Schrick, Spiess, & Radulovic, 2003) as opposed to the PKA pathway. One significant effect of PKC activation within the body is its ability to affect the number of CRH receptor (Dieterich & De Souza, 1996; Dermitzaki et al., 2005), by increasing the concentration of cytosolic calcium ions in calcium rich and calcium free media. Figure 3.2 summarizes the major molecular mechanisms of CRH receptor activation.

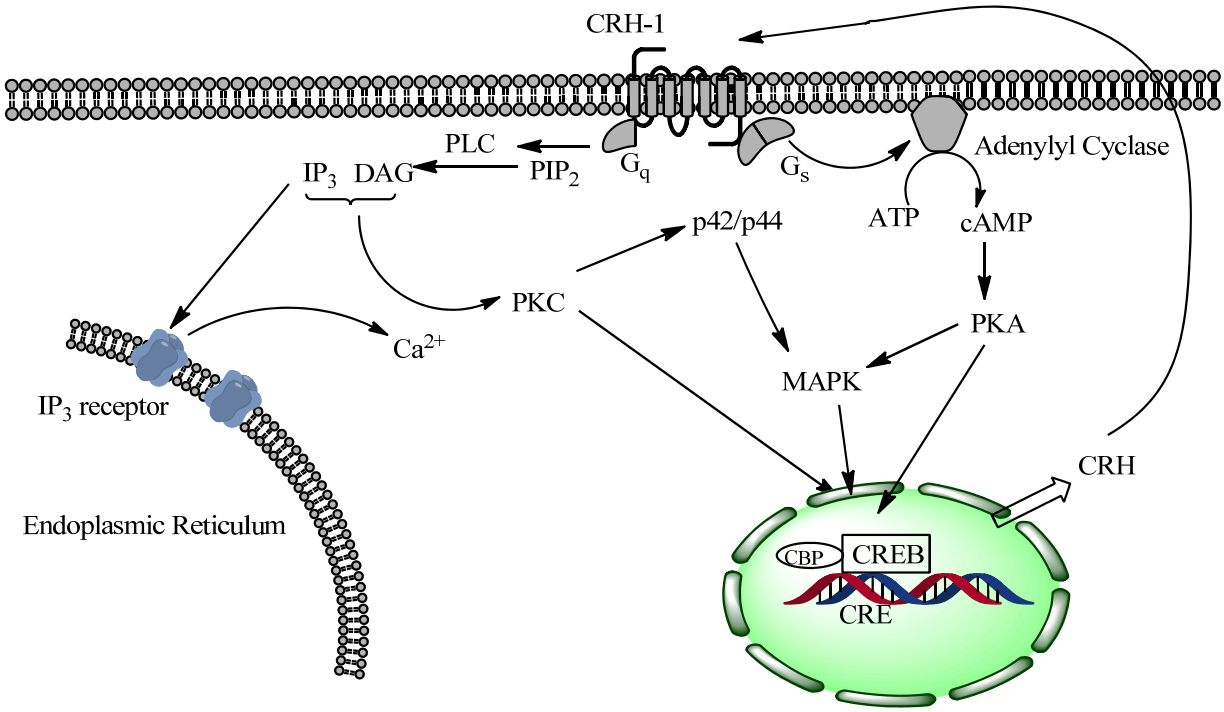

Figure 3.2 Overview of pathways activated by CRH
These signaling pathways lead to the synthesis of CRH via the phosphorylation of CREB (see text for details). PLC - phospholipase C; IP_3 - inositol trisphosphate; DAG - diacylglycerol

Effects of CRH Receptor Activation

Activation of CRH-1 receptor has been implicated in modulating normal responses to stress (Timpl et al., 1998). CRH-1 plays a key role in the HPA axis modulation, as well as memory retention, pain, and analgesia (Hwang et al., 2004). Furthermore, CRH-1 plays a key role in behavioral stress and anxious behavior (Heilig & Koob, 2007) with current research implicating childhood and adolescence activation of CRH-1 being a factor contributing to the development of schizophrenia (Bennett, 2008). Activation of CRH-1 receptors, shown by the hyperactivity of the HPA axis, has been shown to lead to the regression of synapses and a loss of synaptic spines. Furthermore, increases in CRH gene expression within the PVN, BNST, and the CeA lead to greater activation of CRH-1 receptors and increases in anxious behavior as demonstrated by the elevated plus maze test and the light/dark box test (McGill et al., 2006). Additionally, CRH is not required to induce stress-like behavior in animal models. For instance, it has been found that a deficiency in both CRH and Unc I lead to reduced stress-like behavior as seen in the elevated plus maze test (Weninger et al., 1999). Central administration of either CRH or Unc I increased stress-like behavior, suggesting that CRH is not necessary for inducing stress-like behaviors, only CRH-1 receptor activation. Furthermore, CRH-1 knockout mice displayed increased time spent in the light chamber of the light/dark box (Contarino et al., 1999; Timpl et al., 1998), increased time spent in the open arms of the elevated plus maze (Contarino et al., 1999; Smith, Mesiano, Chan, Brown, & Jaffe, 1998) and increased locomotion in the open field test (Timpl et al., 1998). The ability for CRH-1 knockout mice to display reduced anxiety-like behaviors suggests that CRH-1 also has

a role in anxious-like behavior. CRH-2 receptors have been implicated in anxiolytic effects, however many of these effects have been conflicting. For instance, CRH-2 knockout mice either show no change when tested in the elevated plus maze (Coste et al., 2000) decreased open arm activity (Bale et al., 2000) or had sex specific changes, with males having decreased open arm activity (Kishimoto et al., 2000). A similar effect was seen when these mice were tested in the open field test (Coste et al., 2000; Bale et al., 2000; Kishimoto et al., 2000). Recent findings seem to contradict this notion, however. CRH-2 receptor deficient (CRH-2 -/-) mice have been found to lack the dysphoria-like and anhedonia-like states of opiate withdrawal and do not show increase in the activity of brain dynorphin, CRH, and periaqueductal gray circuitry (all of which are major substrates for opiate withdrawal distress; Ingallinesi, Rouibi, Le Moine, Papaleo, & Contarino, 2011). Furthermore, these mice also displayed reduced anxiety-like behaviors. Given that opiate withdrawal works as a strong stressor, these results suggest that CRH-2 receptors work as an anxiolytic.

Recent electrophysiological studies (Fu et al., 2007; Liu et al., 2004) suggest that CRH-2 receptors are localized on glutamatergic neurons originating from the BLA, projecting into the CeA. Electrophysiological experiments using amygdala slices and CRH receptor antagonists and agonists demonstrate that activation of CRH-2 receptors, either presynaptic or post-synaptic, increased glutamate release and enhanced excitatory post synaptic current (EPSC) amplitudes (Liu et al., 2004). Furthermore, CRH-1 receptor activation attenuates this EPSC (Liu et al., 2004). This depolarization of BLA neurons, which project into the CeA, coupled with the channelopathy of K_{Ca} channels within the BLA, provide a basis as to how high levels of CRH can produce the hyperexcitability that

distinguishes anxiety and stress-like behaviors. Interestingly, low concentrations of CRH have been found to preferentially bind to CRH-1 receptors located at terminal GABAergic neurons, which stimulates further CRH release (Lam & Gianoulakis, 2011). This induces a rise in CRH concentration, which now binds to CRH-2 receptors located at the terminals of BLA neurons projecting into the CeA, which enhances glutamatergic neurotransmission (Lam & Gianoulakis, 2011). This explains why in stress situations, hyperexcitability is closely associated with high CRH levels.

One of the central symptoms of alcohol withdrawal is the increase in anxiety levels displayed by the dependent individual. Prolonged dependence is marked by a decrease in mood, elevated anxiety, and increase sensitivity to stress. The negative emotional and anxious state displayed by alcohol dependent individuals during withdrawal can be traced back to a decrease in NPY levels, however it has recently been suggested that CRH may play a bigger role. Indeed, it seems that both systems seem to interact with one another within the CeA to modulate stress and anxious behavior, a cornerstone of alcohol dependence.

Role of CRH in Ethanol Dependence

One of the hallmarks of alcohol withdrawal is increased anxiety, which is mediated by the CRH system. There is a marked increase of CHR release during ethanol withdrawal, and using CRH antagonists directly into the CeA (Pich et al., 1995; Lowery et al., 2010) attenuates anxious behavior, suggesting that the CRH system mediates the negative affect that accompanies the establishment of alcoholism. Following prolonged alcohol intake, depressed mood, elevated anxiety, and increased sensitivity to stress

become predominant. At this point, alcohol is negatively reinforced, consumed to allow an individual to return to a neutral emotional state.

Several lines of evidence points towards a dysregulation of the CRH system as being responsible for the negative affect seen in alcoholism. Mice lacking CRH-1 receptors (Crhr1 -/-) are commonly used as animal models to address this question. Crhr1 -/- mice lacking G protein-coupling domain showed a blunted hormonal stress response (Sillaber et al., 2002; Timpl et al., 1998). No difference in the total fluid consumption was found between the Crhr1 -/- and wild-type mice in a free-choice paradigm, and Crhr1 -/- mice did not differ in the daily intake of alcohol (g/kg/day) at concentrations of 2, 4, and 8% ethanol (v/v). Following 3 weeks of repeated social defeat stress (where rats are subjected to bouts of social defeat by larger and more aggressive rats), an increase in the voluntary alcohol intake in Crhr1 -/- mice was seen whereas wild-type mice ethanol intake levels were not altered (Sillaber et al., 2002). A second period of stress was performed, during which the forced swimming test, a physical stressor, was used. After about 3 weeks, Crhr1 -/- mice started to progressively increase their alcohol intake, which was long lasting and still present 6 months after the second set of stressors. Crhr1 -/- mice that had continuously free access to alcohol over 3 months without receiving any stressor had no changes in alcohol intake over time. This demonstrates that dysregulation of the CRH system is important in mediating ethanol-intake behavior and that alcohol intake behavior can develop not only via genetics, but also by environmental cues which, in this case, was in the presence of stressors. In other words, wild-type mice and Crhr1 -/- mice do not differ from one another in terms of alcohol intake under stress-free conditions,

however following repeated stressors, Crhr1 -/- mice consumed much more ethanol, an effect that persisted throughout their life.

Genetics plays a role in the establishment of excessive alcohol drinking behavior. CRH has been determined to be a gene that contains a CRE consensus sequence within its promoter region (Vamvakopoulos et al., 1990), which is regulated by intracellular cAMP and CRE binding. Foskolin, an activator of PKA, increases binding of CREB to CRE promoter (Wolfl, Martinez, & Majzoub, 1999). Ethanol has been suggested to activate the cAMP second-messenger pathway to increase CRH gene transcription. Both ethanol and forskolin were found to increase CRH secretion, mRNA levels, and gene transcription in CeA and hypothalamic NG108-15 cell cultures (Li, Kang, Lee, & Rivier, 2005). Furthermore, inclusion of adenosine deaminase reduced the promoter response to ethanol, whereas the PKA inhibitor H89 and the cAMP antagonist Rp-cAMP decreased ethanol-induced CRH peptide secretion, gene expression, and transcription (Li et al., 2005) suggesting that ethanol up-regulates CRH expression via the PKA-pathway. In msP rats, there was an up-regulation of the Crhr1 transcript found within several limbic areas including the CeA (Hansson et al., 2006). This was accompanied by a polymorphism of the Crhr1 promoter and an increase in CRH-1 receptor density. Moreover, administration of the CRH-1 receptor antagonist, antalarmin, had no effect on wild-type rats but significantly decreased alcohol seeking in msP rats (Hansson et al., 2006) suggesting that the CRH-1 receptor plays a role in ethanol seeking behavior. Another study analyzed two independent samples in humans, one group of individuals who had little previous exposure to alcohol, and another sample of adults who met DSM-IV criteria of alcohol dependence. The allelic frequencies of 14 polymorphisms of the

CRH1R gene were determined, with two haplotype tagging (ht) single nucleotide polymorphisms (SNPs) determined between haplotypes with a frequency of >0.7%. In the adolescent sample, differences between genotypes were observed in binge drinking and lifetime prevalence of ethanol intake whereas in the adult sample there was an association of CRH1 gene with patterns of alcohol consumption (Treutlein et al., 2006). For instance, six of the 14 SNPs were found to display a binding difference of transcription factors. A more complete analysis of the several different polymorphisms can be found in Treutlein et al. (2006). Needless to say, they provide the first association of CRHR1 gene polymorphisms with patterns of alcohol consumption, suggesting the importance of the CRH system in ethanol intake behavior.

CRH levels were also examined in alcohol preferring (P) rats, non-alcohol preferring (NP) rats, as well as high-alcohol drinking (HAD) and low-alcohol drinking (LAD) rats (Hwang et al., 2004). P rats were found to have lower CRH levels and lower CRH mRNA, as tested by in situ hybridization, within the CeA when compared to NP rats. P rats also exhibited higher levels of anxiety as displayed by the elevated plus maze test. Interestingly, there were no differences in the CRH levels within the CeA of HAD and LAD rats and there was no difference in their levels of anxiety. This suggests that lower CRH within the CeA is associated with increased anxiety in the elevated plus maze, as HAD and LAD rats did not display anxiety-like behaviors and had no changes in their CRH ir levels.

It seems contradictory that lower CRH ir would equate to reduce anxiety-like behavior. One possibility is that this data was taken following acute withdrawal. Following acute withdrawal, ethanol dependent subjects display an increase in ethanol

self-administration as well as an increase in anxious-like behavior as displayed by the elevated plus maze test (Funk et al., 2006). One possibility for this reduced CRH ir is the increase in extracellular CRH within the amygdala of these dependent rats (Merlo Pich et al., 1995) following exocytosis. During withdrawal, there is an increase in CRH release within the CeA which plays a role in mediated the anxiety-like behavior characteristic of ethanol withdrawal, which would coincide with Hwang et al. (2004) and studies from Zorrilla, Valdez, & Weiss (2001), the latter of which demonstrated reduced CRH-like ir following chronic ethanol diet. In the same study (Funk et al., 2006) the CRH antagonist D-Phe-CRH_{12-41} was administered to three different brain areas: CeA, BNST, and NAc. When administered directly into the CeA, there was a marked reduction of ethanol consumption in P rats, but not in NP, HAD, nor LAD rats. Furthermore, this effect was not seen when D-Phe-CRH_{12-41} was administered to either BNST or the NAc, suggesting that the CeA plays a key role modulating ethanol consumption, suggesting that brain CRH-system dysregulation plays a role in increased ethanol consumption. It has been reported that the hypothalamic CRH stress system (Vale et al., 1981) and the extrahyptohalamic CRH system, which regulates the behavioral and autonomic responses to stress (Walker & Davis, 1997) become dysregulated during ethanol dependence and withdrawal. Moreover, acute ethanol has been found to activate the HPA axis (Rivier, Bruhn, & Vale, 1984), while chronic ethanol consumption attenuates HPA axis activity (Zorrilla et al., 2001).

CRH antagonism attenuates the anxiety-like responses brought upon by ethanol withdrawal (Baldwin, Rassnick, Rivier, Koob, & Britton, 1991), which appears to be driven by the activation of CRH signaling within the amygdala (Merlo Pich et al., 1995).

Recently, it was been suggested that prolonged exposure to alcohol causes long-lasting neuroadaptive changes that chronically up-regulate central CRH activity. During acute ethanol withdrawal, there is an increase of CRH within the CeA (Merlo Pich et al., 1995) which is reflected by the decreased tissue levels of CRH. Six weeks after withdrawal, however CRH levels within the CeA increased to supranormal levels (Zorrilla et al., 2001), which has been attributed to increased CRH transcript levels within the CeA (Sommer et al., 2008). Another contributor for the post-dependent phenotype is the upregulation of CRH-1 receptors. This is demonstrated in the alcohol preferring Marchigian-Sardinian Preferring (msP) rats (Ciccocioppo et al., 2006). The msP line of rats demonstrated high alcohol preference and consumption, along with elevated behavioral sensitivity to stress. These rats show upregulation of the transcript encoding the CRH-1 receptor within the CeA, linked to the allele at the Crhr1 locus which encodes the CRH-1 receptor. Administration of the CRH-1 antagonist antalarmin reduced ethanol self-administration to non-dependent levels and blocked the stress-induced reinstatement of ethanol-seeking doses. A similar upregulation of CRH-1 receptors was found in non-selected post-dependent rats (Sommer et al., 2007) which persisted for three months following ethanol exposure, reflecting a long-term neuradaptation.

Intake of ethanol following excessive post-dependent self-administration is vastly different from basal ethanol intake. For instance, post-dependent animals tested two hours into withdrawal had higher rates of ethanol self-administration. CRH-1 selective antagonists such as antalarmin, MJL-1-109-2, and R121919 were able to bring back ethanol self-administration to non-dependent levels in dependent rats. However, these antagonists had no such effects in non-dependent rats (Funk, Zorrilla, Lee, Rice, & Koob,

2007). Note these effects have been found during acute withdrawal (i.e. the first couple of hours following ethanol administration). A similar effect has also been found long after force ethanol exposure (Valdez et al., 2002; Gehlert et al., 2007). D-Phe-CRH_{12-41} administered intracerebroventricularly blocked increased alcohol drinking following acute withdrawal and protracted abstinence (Gehlert et al., 2007).

In situ hybridization identified an up-regulation of the Crhr1 transcript, suggesting a predisposition to high alcohol intake due to genetic make up. Additionally, up-regulated CRH-1 receptor activity, as seen in msP rats, creates a potential for increased negative reinforcement. Alcohol has been suggested to activate CRH-1 receptors (Nie et al., 2004), and excessive consumption of ethanol down-regulates Crhr1 transcript levels. This effect was examined in the msP rat line (Hansson et al., 2007). Within the CeA and MeA as well as the NAc, 2 weeks of ad lib access to alcohol lead to a significant down-regulation of the Crhr1 transcript. This translates as a marked decrease in the CRH-1 receptor density, especially within the CeA, MeA, and NAc. Additionally, using the CRH-1 receptor antagonist antalarmin, up-regulated CRH-1 receptor transmission in msP rats was shown to drive excessive ethanol intake and stress –induced reinstatement of ethanol seeking following extinction. Data by Hansson et al. (2007) demonstrates a down-regulation of CRH-1 receptor expression by alcohol intake to wild-type levels, suggesting the possibility of alcohol acting as a functional antagonist of CRH signaling at CRH-1 receptors. In another study, social interaction was reduced in rats that were treated with CRH for five days while in a control diet followed by an ethanol diet for five days and tested five hours into withdrawal (Overstreet, Knapp, & Breese, 2004). Furthermore, it was found that treatment with the CRH-1 antagonists CRA1000 and CP-154,526

following repeated cycles of ethanol withdrawal blocked the reduced social interaction behavior whereas CRH-2 receptor antagonist administration was without effect (Overstreet et al., 2004).

Binge drinking behavior in C57BL/6J mice has been suggested to involve the CRH system, with antagonist at the CRH-1 receptor and agonist at the CRH-2 receptors attenuating ethanol self-administration. Although CRH activates the rest of the HPA axis to activate the body's stress response, it appears that ethanol drinking behavior occurs independently of the axis and only relies on extrahypothalamic CRH, including CRH receptors within the amygdala. Administration of the CRH-1 receptor antagonist, α-helical CRH9-41, decreased binge-drinking like behavior in C57BL/6J mice relative to a vehicle control (Lowery et al., 2010). Additionally, i.c.v administration of UNC3, which functions at a CRH-2 receptor agonist, produced a similar effect. This suggests that both CRH-1 and CRH-2 receptors produce opposite effects. Mifepristone, the glucocorticoid receptor antagonist produced no effect with regards to ethanol self-administration, while Metyrapone, a corticosterone synthesis inhibitor, reduced ethanol and sucrose consumption. Lastly, injection of CP-154,526, a selective CRH-1 receptor antagonist attenuated binge-like ethanol intake in both normal and adrenolectomized (ADX) mice. This suggests that control of binge-like ethanol consumption relies on CRH-1 and CRH-2 receptors, independently of the HPA axis. In another study, central administration of the CRH-1 receptor antagonists antalarmin, MJL-1-109-2, and R121919 all reduced ethanol self-administration in ethanol dependent rats. This effect was no seen in non-dependent rats, and was restricted only to ethanol and not water (Funk et al., 2007). CRH-2 receptors tend to decrease enhanced anxiety-like behavior and ethanol self-administration

in dependent rats upon activation. Administration of Unc-3, which works as an agonist at CRH-2 receptors, significantly reduced ethanol self-administration in ethanol-dependent rats. Unc-3 also displayed increase ethanol self-administration in non-dependent rats (Sharpe & Phillips, 2009).

Although the HPA axis plays a role in physiological stress, it seems that only behavioral stress is of importance in the establishment of binge-like drinking behavior. This conclusion is based on the following observations: administration of a nonselective CRH-receptor antagonist attenuated binge-like drinking while manipulation of the HPA axis via adrenolectomy did not protect against binge-like ethanol intake (Lowery et al., 2010). Furthermore, pretreatment of both ADX and normal mice with CP-154,526 reduced ethanol intake in the same way in both rats. While binge-like ethanol intake behavior occurs independently of the HPA axis, there seems to be some evidence pointing towards the dangers of binge-pattern ethanol intake during puberty with regards to HPA axis reactivity. Previous studies have indicated that ethanol intake during puberty dysregulated the responsiveness of the HPA axis, manifested by alterations in CRH, arginine vasopressin (AVP), and corticosterone (CORT) levels (Przybycien-Szymanska, Rao, and Pak, 2009; Li et al., 2005). It seems that this effect is long lasting (Przybycien-Szymanska, Mott, Paul, Gillespie, & Pak, 2011).

Recently, it has been suggested that chronic HPA axis dysregulation may play a role in the establishment of alcoholism (Uhart, Oswald, McCaul, Chong, & Wand, 2006), with studies demonstrating that various types of stressors either physical, social, or emotional can increase ethanol self-administration in rodents. For example, stereotaxic implantation of corticosterone micropellets into the CeA resulted in increased CRH

expression within and reduced exploration of the elevated plus maze (Myers, Gibson, Schulkin, Greenwood, & Van-Meerveld, 2005). This effect was eliminated by CRH-1 receptor antagonists. The entire HPA axis, however, is not be involved in mediating the anxious behavior that follows ethanol dependence. For instance, stress via foot shock reinstates ethanol responding, which is reversed by treatment with CRH antagonists, but not adrenalectomy (Lui et al., 2004). A recently synthesized CRH-1 receptor antagonist, 3-(4-chloro-2-morpholin-4-yl-thiazol-5-yl)-8-(1-ethylpropyl)-2,6-dimethyl-imidazo[1,2-b]pyridazine (MTIP) dose dependently reverse the anxiogenic effects of withdrawal from a 3.0-g/kg alcohol dose (Ciccocioppo et al., 2009). This same dose blocked ethanol self-administration in alcohol-dependent Wistar rats and in the msP rat, while having no effect in non-dependent Wistar rats.

CRH and the GABAergic System

Day, Curran, Watson, & Akil (1999) and Veinante, Stoeckel, & Freund-Mercier (1997) both report that CRH is localized and co-synthesized within GABAergic neurons, suggesting that CRH modulates the release of GABA. This suggests a link between both the NPY and the CRH systems via the GABAergic system, which could be critical in finding an effective treatment for alcohol intake behavior.

Ethanol increases GABAergic transmission in various parts of the brain, including the amygdala. Acute ethanol augmented $GABA_A$-receptor mediated IPSPs and IPSCs in CeA neurons (Roberto et al., 2003) and $GABA_A$ antagonism in the CeA attenuates ethanol self-administration (Hyytia & Koob, 1995). Furthermore, activation of $GABA_A$ receptors via the infusion of agonists alters ethanol self-administration in ethanol dependent rats (Roberts et al., 1996). Recently, it has been found that acute ethanol

interacts with GABAergic systems in chronic ethanol-treated (CET) rats (Roberto, Cole, & Koob, 2004). In CeA slices of CET rats, baseline evoked IPSP and IPSC amplitudes increased, with PPF ratios lower in ethanol naïve rats. This suggests increased GABAergic transmission following chronic ethanol treatment. Furthermore, acute ethanol doses significantly enhanced IPSPs and IPSCs in both CET and ethanol-naïve rats, showing lack of tolerance for this particular effect of ethanol. Furthermore, analysis of miniature IPSC frequency suggests increased GABAergic transmission arises from enhanced vesicular release of GABA in presynaptic neurons. Microdialysis data further shows that CET rats present a fourfold increase in baseline GABA dialysate content, and in vivo administration of ethanol produced a dose-dependent increase in GABA release in the CeA dialysate in both CET and naïve rats (Roberto, Madamba, Stouffer, Parsons, & Siggins, 2004).

In one study, immunosensor-based microdialysis probes were used to measure the extracellular levels of CRH within the PVN and in the amygdala of thirty-six female sheep (Cook, 2004). GABA levels within the amygdala were measured as well and venous measurements of cortisol were also included. Following exposure to the stressor, CRH levels increased within the PVN and the amygdala, the latter of which also showed increased levels of GABA. Cortisol levels increased with time. Infusing the CRH antagonist, astressin, prior to the presentation of the stressor had a small effect on the subsequent stress response however, the treatment reduced the responses to repeat stress administered 2 days later. This is compared to the non-treated animals, which had stress responses similar to their first exposure. Another trial examined the effect of 4-

aminopyridine, a neuronal depolarizing agent, into the PVN. This agent induced release of CRH accompanied shortly by a small increase of CRH into the amygdala.

The CeA appears to be an important area involved in the rewarding effects of ethanol, perhaps reinforcing the negative affect associated with ethanol withdrawal with both CRH and GABA playing a crucial role (Nie et al., 2004). For instance, in one study whole-cell recordings were made on CRH-1 KO and wild-type animals. $GABA_A$ inhibitory postsynaptic currents (IPSCs) were isolated by blocking NMDA, non-NMDA, and $GABA_B$ receptors. Infusion of CRH in the CeA slices of wild-type mice enhanced IPSC amplitudes, with increasing concentrations enhancing IPSC amplitudes further (Nie et al., 2004). Ethanol also produced similar effects on IPSCs, however only with concentrations higher than 5mM with the highest enhanced IPSC being recorded when 44mM of ethanol was used. When examining the CRH-1 KO mice, CRH had no significant effect on IPSC amplitudes, as expected. Surprisingly, ethanol did not produce a significant effect on IPSC amplitudes of these mice, however the effect was seen with CRH-2 KO mice. Furthermore, infusion of the CRH receptor antagonist D-Phe-CRH_{12-41} in wild-type mice blocked the effect that ethanol had on IPSC amplitudes (Nie et al., 2004). A similar effect was seen with the infusion of the non-peptide CRH-1 antagonist NIH-3 (LWH-63), and NIH-3 was also able to block the effect produced by CRH. This indicates that the CRH system and CRH-1 are involved in the ethanol enhancement of GABAergic transmission. This effect is seen in CRH-2 KO mice, but not in CRH-1 KO mice suggesting that CRH-1 mediates the effect. Additionally, CRH-1 receptors involved in this effect are most likely located on pre-synaptic terminals of GABA neurons (Nie et al., 2004).

Another study confirms the finding that CRH-1 receptors are involved in GABAergic transmission. Using an in vitro superfusion system, rat amygdalar slices were pretreated with selective CRH-1 or CRH-2 antagonists, which were then labeled with radioactive GABA and transferred to four cylindrical Perspex chambers of the superfusion system (Bagosi, Jaszberenyi, Szabo, & Telegdy, 2008). From this, it was found that CRH and other members of the CRH peptide family, specifically urocortin 1, significantly increased the release of [^{3}H]GABA from slices following electrical stimulation. Neither urocortin 2 nor urocortin 3 were able to produce a similar effect. Furthermore, the actions of CRH and urocortin 1 were blocked by antalarmin, a CRH-1 receptor antagonist, yet were not affected by the CRH-2 receptor antagonist astressin 2B. This suggests that the release of GABA from the amygdala is mediated by CRH and urocortin 1 through the activation of CRH-1 receptors.

How activation of CRH-1 receptors within the amygdala leads to anxiety and ethanol intake is not known, however it appears that CRH-1 stimulates a PKC signaling pathway. Of the most common isozymes of PKC, PKCε is the most commonly isozyme expressed within the CeA (Choi, Wang, Dadgar, Chang, & Messing, 2002) and it is hypothesized that it mediates downstream effectors of CRH-1 receptor activation (Bajo, Cruz, Siggins, Messing, & Roberto, 2008). PKCε -/- mice show reduced anxiety behavior (Hodge et al., 2002) and reduced ethanol intake behavior (Hodge et al., 1999; Olive, Mehmert, Messing, & Hodge, 2000) however until recently, whether or not PKCε was indeed an effector of CRH-1 receptor activation was not known. Bajo et al. (2008) compared the CeA neurons of PKCε -/- rats with those of their PKCε +/+ counterparts. In general, PKCε -/- CeA neurons showed increase GABAergic tone due to enhanced

GABA release. This was performed via evoking pharmacologically isolated $GABA_A$ receptor-mediated IPSPs, with baseline IPSP input-output curves being higher in the CeA neurons of PKCε -/- mice. Furthermore, examining PPF of the IPSPs showed a decrease baseline PPF ratio in the CeA neurons of PKCε -/- mice, suggesting increased GABA release within this line of mice. CRH augments GABAergic transmission via activation of CRH-1 receptors, most likely via the PKC pathway. Superfusion of CRH into CeA slices of PKCε +/+ mice increased the mean amplitude of evoked IPSPs, which was absent in PKCε -/- mice. Similar observations were made when ethanol was superfused into the CeA of rats, indicating that ethanol also induces GABA release via the PKCε route.

The involvement of the CRH system on the GABAergic system following ethanol consumption has recently been validated (Roberto et al., 2010). The electrophysiology of GABAergic transmission of naïve and ethanol-dependent rats within the CeA was evaluated following the infusion of CRH and ethanol. The GABA dialyzate in the CeA after injection of CRH-1 receptor antagonists and ethanol was also evaluated. In naïve rats, superfusion of CRH up to a concentration of 200 nmol/L increased the amplitude of evoked IPSCs, while in ethanol-dependent rats a similar concentration produced a more enhanced effect on the evoked IPSCs, suggesting that in dependent rats there is a greater activation of the GABAergic system following CRH infusion. Given the possibility of the up-regulation of CRH receptors in ethanol dependent rats, this seems plausible. CRH significantly decreased PPF of IPSCs in CeA of naïve rats, suggesting increased GABA release which is modulated by presynaptic CRH-1 receptors (Nie et al., 2004). CRH-1 receptor antagonists, such as antalarmin, LWH-63, and R121919 decreased evoked IPSC

amplitudes and increased the mean PPF of IPSCs. These decreases in IPSC amplitudes were greater in dependent rats than naïve rats, reflecting increased tonic release of endogenous CRH, CRH-1 receptor activation, and possibly CRH-1 receptor up-regulation. CRH-1 antagonists increased PPF of IPSCs and decreased IPSCs, both consistent with decreased GABA release. Within the same study, it was found that dialysate GABA levels were higher in ethanol dependent rats shortly following withdrawal when compared to naïve rats, and these were reduced following reverse-dialysis with R121919. This suggests increased CRH-1 receptor function within ethanol dependent rats, which is demonstrated by increased CRH mRNA levels within the CeA of ethanol-dependent rats.

CHAPTER FOUR

Role of NPY and CRH on Alcohol Dependence

The cyclic nature of alcohol consumption, in which an individual undergoes repeated bouts of ethanol drinking and withdrawal, induces neuroadaptational changes in the individual that lead to enhanced stress reactivity and further ethanol drinking. This is what is referred to as the "kindling"/stress model of alcoholism by Breese et al. (2005), similar to the animal model of kindling induced epilepsy, where repeated stimulation results in a greater propensity for seizures. As in kindling-induced epilepsy, many "stressed" and "anxious" animals display hyperexcitability, with both NPY and ethanol working to depress this altered excitable state. This finding suggests that the self-medication hypothesis has its basis in hyperexcitability; repeated bouts of ethanol drinking and withdrawal causes an increase in neuronal excitability that can be "treated" by either NPY or ethanol. In light of this possibility, the opponent-process theory proposes that repeated ethanol intake, which increases GABAergic activity, is opposed by an increase in neuronal excitability to maintain homeostasis with alcohol dependence being a consequence of such behavior. Repeated episodes of this behavior establish an allostatic load. The resulting excitability is maintained by the chronic stress that accompanies ethanol withdrawal. Stress (in the form of increased CRH levels) puts into play two different mechanisms to maintiain excitability. First, it reduces calcium-dependent potassium (K_{Ca}) channel regulation of action potential via channelopathy (Rosenkranz, Venheim, & Padiva, 2010) within the BLA. K^+ channels allow for the

hyperpolarization following an action potential. Without a hyperpolarization mechanism, the BLA keeps sending input to the CeA in the form of an excitatory signal such as glutamate (see Figure 2.1), which manifests itself as the hyperexcitability seen in the alcohol dependent state. Second, it first activates CRH-1 receptors, which lead to an increase in the transcription of CRH. This rise in CRH allows it to begin binding to CRH-2 receptors located presynaptically within the BLA. This stimulates the release of gluatamate into BLA neurns projecting into the CeA, causing an increase in action potential frequency.

Converging evidence suggests that NPY is important in reducing ethanol intake in P rats and also plays a role in neuronal excitability, as there is marked increase in NPY mRNA following convulsive stimulation (White & Gall, 1987; Gruber, Greber, Rupp, & Sperk, 1994) via activation of Y_1-R. Figure 4.1 proposes a model of how NPY acts within the CeA to produce this anxiolytic effect, starting with an "early" mechanism and followed by a "late" mechanism, somewhat similar to "early" and "late" long-term potentiation. Following the establishment of a hyperexcitable state due to frequent activation of the stress axis, activation of Y_1-R via NPY stimulates a signal cascade that inhibits adenylyl cyclase, but stimulates the phosphoinositidine pathway. The phosphoinositidine pathway stimulates the release of GABA onto a GABAergic output neuron via activation of CaMK IV, reducing hyperexcitability from the CeA. A late mechanism, which follows a similar schematic, differs in that the subsequent activation of CaMK IV causes the phosphorylation of CREB and, ultimately, increases the transcription of NPY mRNA. Y_2-receptor activation leads to the decreased release of NPY by closing Ca^{2+}-channels which allow exocytosis, which suggests that Y_2-receptor

antagonists can also work as a viable treatment for dependence. Furthermore, Y_2-receptor activation leads to a decrease in glutamate release, another mechanism by which NPY can attenuate excitability. This suggests that Y_2-receptor antagonists may present a novel drug target for alcohol dependence.

An opponent-process approach to this model can help illustrate the subject matter. The hyperexcitable state in a dependent individual represents the B-process, which directly opposes the depressive effects (hypoexcitable) of ethanol, the A-process. As an individual shifts toward dependence by the repeated intake of ethanol, the B-process (hyperexcitability) becomes larger in magnitude, while the effects produced by ethanol become smaller. Activation of the NPY system, which ultimately leads to the release of GABA and increases the synthesis of NPY, enhances the magnitude of the A-process by stimulating the further release of GABA, which ultimately reduces the hyperexcitability of the ethanol-dependent individual.

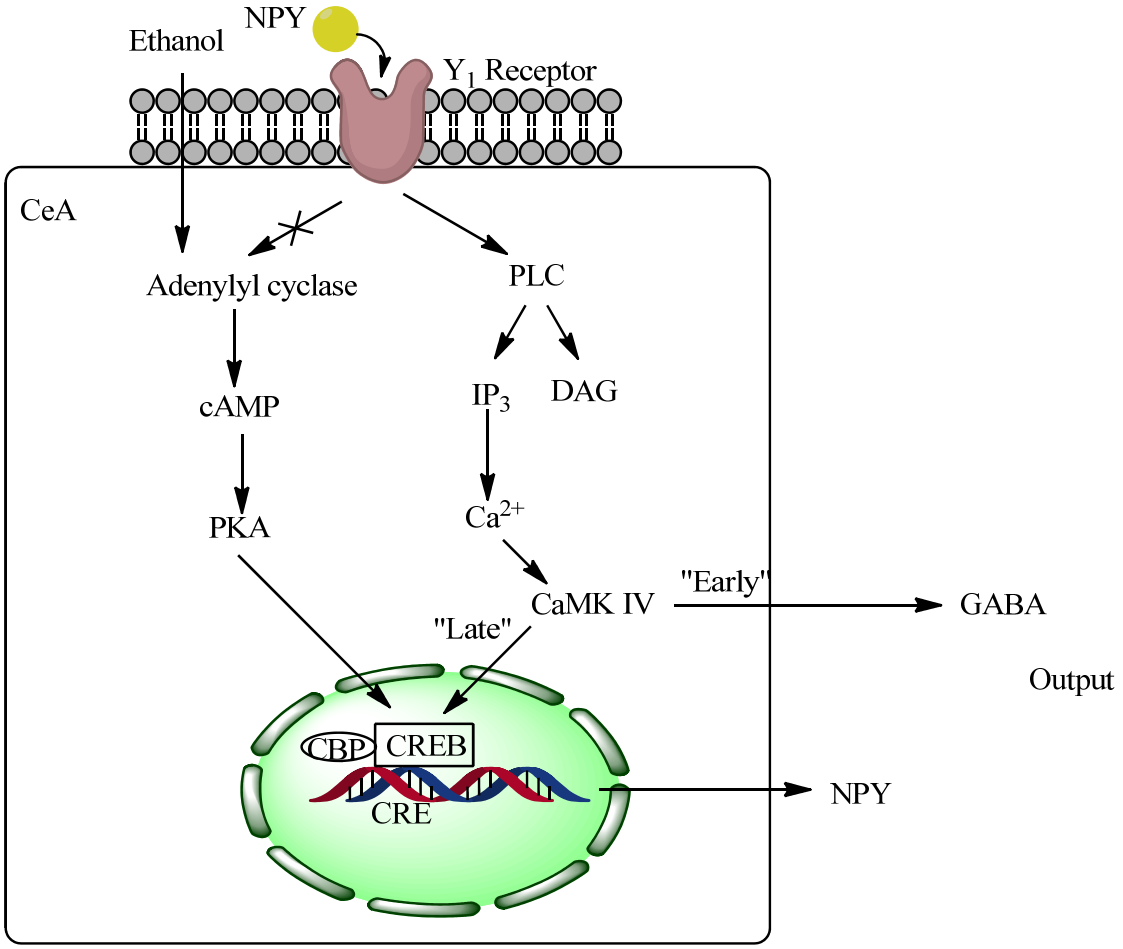

Figure 4.1 Model for NPY's role in anxiety and alcohol dependance
Binding of NPY on Y_1 receptor inhibits adenylyl cyclase and activates the phosphoinositide pathway, which stimulates the release of GABA (short-term mechanism) and increases the transcription of NPY mRNA (long-term mechanism). Ethanol stimulates the activation of adenylyl cylcase via an unknown mechanism, which activates the PKA pathway and increases transcription of NPY mRNA.

This model calls for the establishment of a dependent state, characterized behaviorally by stress and anxiety-like behavior and physiologically by hyperexcitability. Previous studies have shown that NPY infusions are effectve in reducing ethanol intake only in P rats but not in NP rats, coinciding with this model. Furthermore, the model also accounts for the genetic differences seen between P rats and NP rats, including decreased pCREB and CaMK IV, and how increasing pCREB via Sp-cAMPS attenuates ethanol

consumption. Ethanol, which has been found to stimulate $GABA_A$ receptors and adenylyl cyclase activation, also fits this model. The finding that both NPY and ethanol stimulate increased NPY mRNA transcription is also resolved, as they both work by attempting to reduce neuronal excitability. Their additive effects on sedation are also resolved as ethanol works by directly opening GABA receptors and NPY stimulates the release of GABA.

CRH presents an interesting case: it is known to mediate stress and anxiety-behavior, however CRH mRNA levels are increased following acute withdrawal, with CRH transcripts rising to supranormal levels after six weeks of withdrawal (Zorrilla et al., 2001). Furthermore, exposure to stress has been found to increase CRH mRNA (Rosenkranz, Venheim, & Padiva, 2010), high CRH levels can produce limbic seizures (Ehlers et al., 1983), and amygdala-kindled seizures increase CRH protein levels (Smith et al., 1997). The last result is similar to that seen in NPY, suggesting that CRH plays a role in attenuating hyperexcitability. Recently, it has been shown that activation of the CRH-1 receptor within the CeA stimulates the release of GABA via a PKCε dependent mechanism (Bajo et al., 2008), suggesting that CRH does play a role in modulating excitability. This coincides with the finding that following convulsive stimulation, CRH mRNA rises, suggesting that CRH has a protective role following convulsive activity. Moreover, high CRH levels in the BLA accounts for increased excitability following exposure to stress (Bajo et al., 2008). However, as mentioned previously, CRH-1 activation leads to a rise in the transcription of CRH, which increases CRH protein levels and leads to the subsequent activation of CRH-2 receptors. This suggest that acute activation of CRH-1 leads to a decrease in hyperexcitability, while chronic activation of

the receptor and excess CRH levels will begin to cause hyperexcitability via its actions at the presynaptic CRH-2 receptor. This coincides with the findings by Zorrilla et al. (2001) in which withdrawal causes a rise in CRH levels, which may coincide with a rise in anxiety.

Figure 4.2 puts together a model for CRH's involvement in alcohol intake behavior. The early mechanism starts by the activation CRH-1 by either CRH or UNC 1, which stimulates the phosphoinositidine pathway. The cascade leads to the activation of the PKCε isozyme and subsequent release of GABA into the GABAergic efferent neuron of the CeA. In the late mechanism, adenylyl cyclase is activated, which increases intracellular cAMP levels. These events lead to the activation of PKA, and subsequent increase in pCREB and a rise in CRH mRNA transcript. Ethanol, which is found to act on CRH-1 receptors, also activates the PKCε pathway to increase GABAergic transmission. The model proposed for CRH coincides with several findings: (1) increased up-regulation of CRH and CRH-1 receptors in msP rats (Ciccocioppo et al., 2006); (2) enhanced GABAergic transmission following CRH administration and ethanol intake (Nie et al., 2004; Bajo et al., 2008) and (3) enhanced ethanol intake in rats lacking CRH-1 receptors following stress (Sillaber et al., 2002).

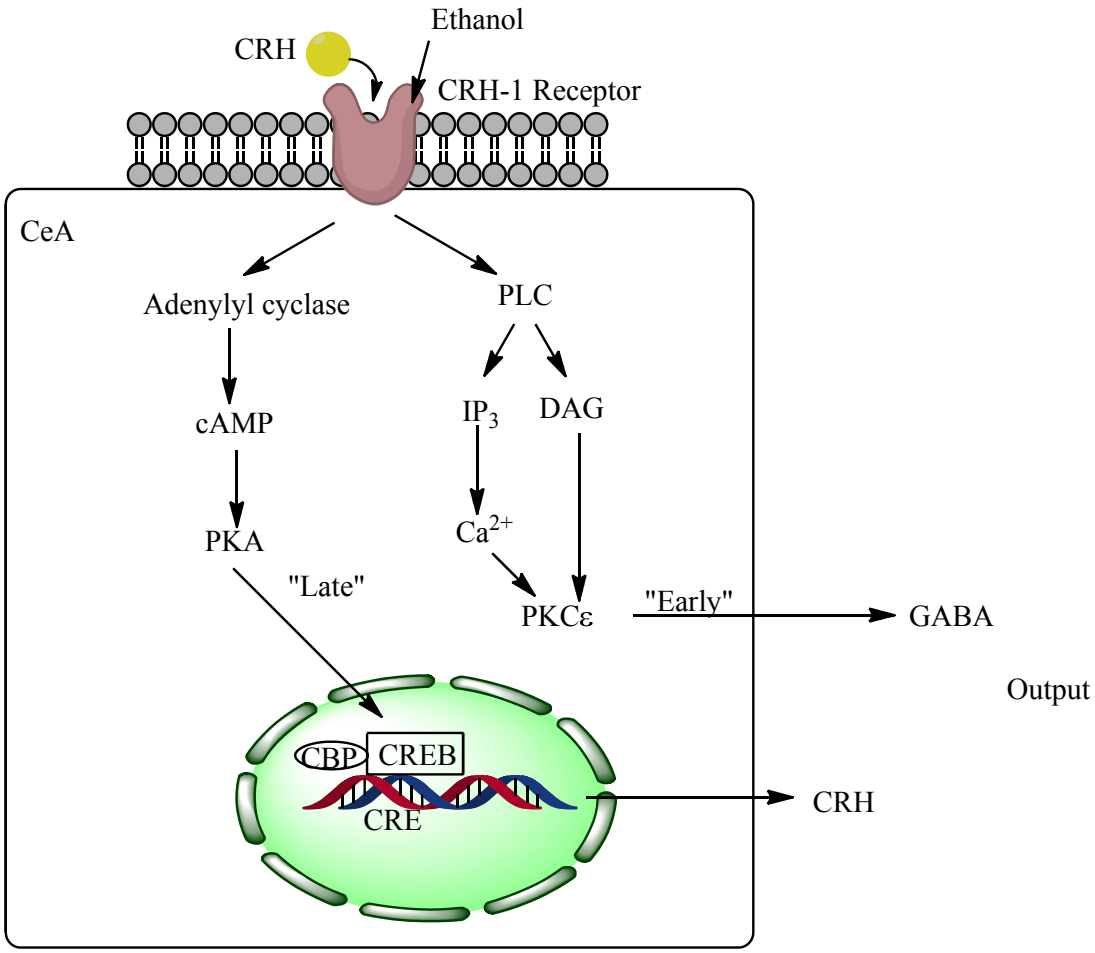

Figure 4.2 Model for CRH's role in anxiety and alcohol dependance
Binding of CRH or urocortin I stimulates the phosphoinositide pathway, which stimulates the release of GABA (short-term mechanism) and stimulates adenylyl cyclase, which activates PKA and increases CRH mRNA transcription (long-term mechanism). Ethanol acts on the CRH-1 receptor to activate the PLC pathway and enhance GABA release.

As with NPY, the effects of CRH receptor activation can be understood by using an opponent-process approach. Following a diminished A-process (reduced excitability) by the depressive effect of ethanol, acute CRH activates CRH-1 receptors leading to the increase in GABA synthesis. This enhances the magnitude of the A-process, which directly opposes the B-process (hyperexcitability) imposed due to withdrawal. A shift toward the B-process occurs due to the cyclic nature of alcohol intake. Frequent

intermittent stress due to repeated bouts of withdrawal and ethanol intake lead to increased levels of CRH, which now bind to pre-synaptic CRH-2 receptors on BLA projection neurons and cause channelopathy of K_{Ca} channels on the BLA. These events induce hyperexcitability on CeA neurons, which corresponds to the increased magnitude of the B-process (Figure 4.3).

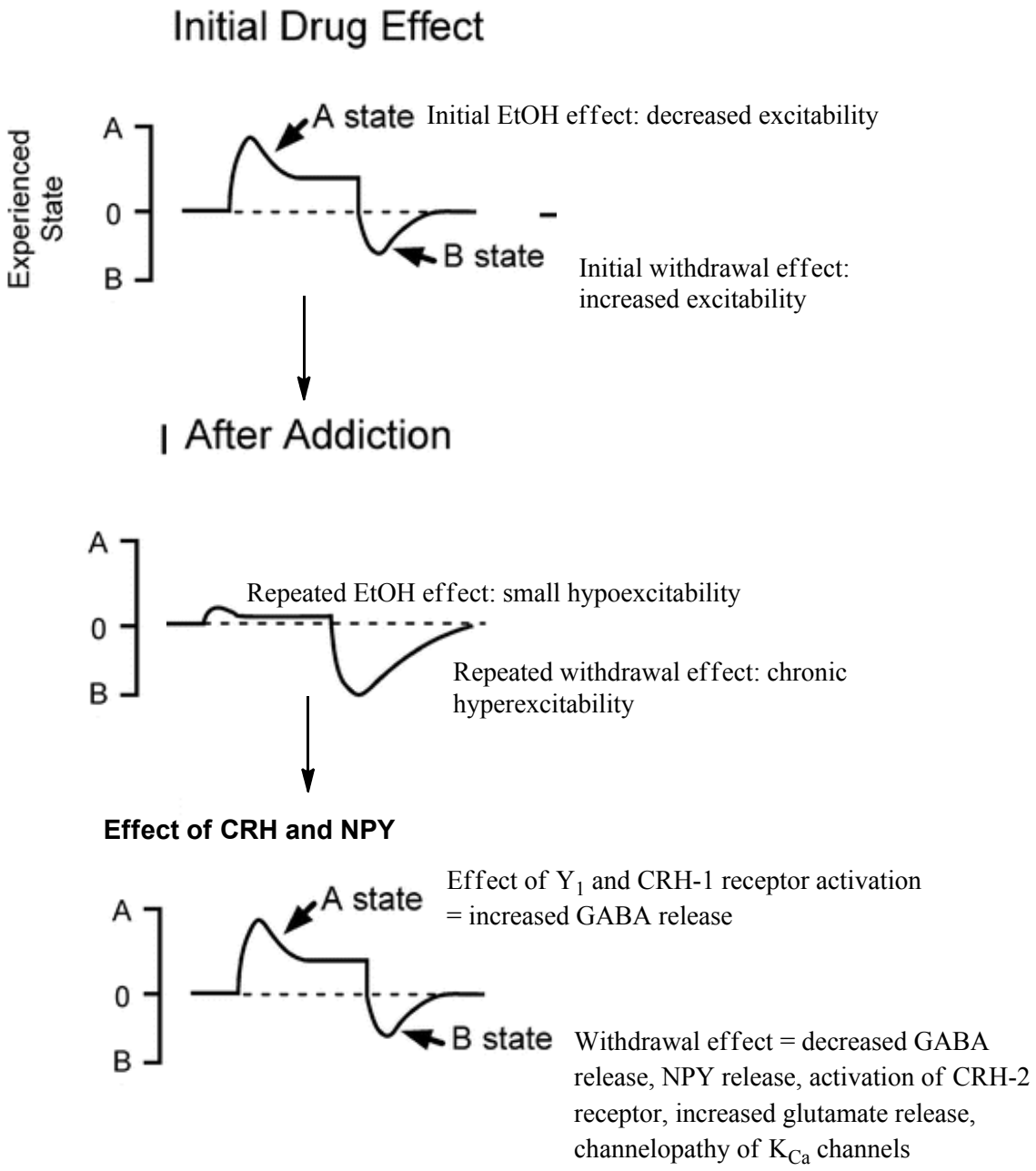

Figure 4.3. Effect of CRH and NPY as it relates to the opponent-process model

Figure 4.4 puts both models together into more cohesive schematic, also taking into consideration the CRH-2 receptor. In general, stress and alcohol withdrawal causes an increase in the firing rate of neurons from the BLA due to channelopathy and increased glutamate release due to CRH-2 receptor activation. This results in a rise in

excitatory input into the CeA, which results in increased excitability. Ethanol, CRH (acting at CRH-1), and NPY all work to modulate this increased activity by releasing GABA into CeA output neurons, resulting in a decrease in firing rate. A rise in CRH levels seen in P rats (Hwang et al., 2004) may serve as a homeostatic mechanism to maintain normal excitability within the CeA to compensate for low NPY levels, though whether high NPY levels suppresses CRH release remains to be seen.

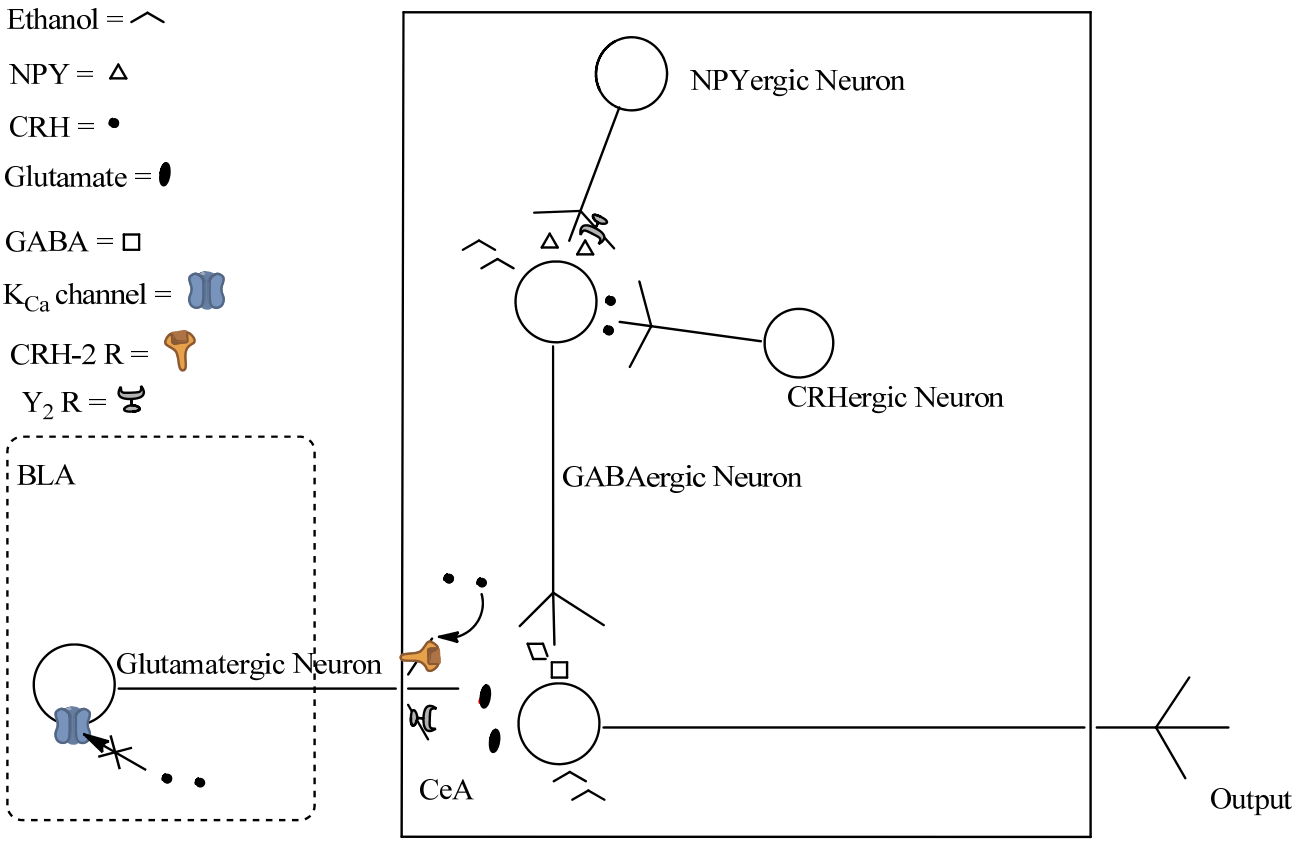

Figure 4.4 General schematic for the roles of CRH and NPY in alcohol dependence
High levels of stress (CRH) induce K_{Ca} channelopathy within the BLA and activate presynaptic CRH-2 receptors to increase glutamate release into the CeA. This causes hyperexcitability within the CeA and excessive activation of output neurons. NPY and CRH (at low levels) release both stimulate the release of GABA into the output neuron, which modulates output from the CeA. Ethanol also stimulates the release of GABA from GABA interneurons via the mechanisms discussed above and stimulates the opening of GABA receptors. NPY can activate Y_2 receptors located presynaptically to either reduce the amount of NPY.

Regardless of the simple nature of the model above, there are several problems with it. It does not address how an individual begins to consume ethanol, nor does it address the process of going from a normal state to a dependent state. This issue can be resolved by the intrinsic rewarding and euphoric properties of ethanol, which lead to an individual to begin repeated bouts of drinking in order to achieve that euphoria. Further bouts of intake lead to a decrease in the magnitude of the euphoric effects and increased magnitude of withdrawal, including hyperexcitability. Another problem deals with the conflicting nature of CRH: low CRH levels activate CRH-1 receptors, leading to GABA release; high CRH levels activate CRH-2 receptors, leading to glutamate release. Futhermore, what type of receptors does glutamate act on after being released from the BLA, either ionotropic or metabotropic, remains to be resolved. Finally, the model does not address the entire issue of alcohol dependency. Given that many other factors, such as other physiological anomalies, environment, and genetics, can contribute to dependency, it seems unlikely that anxiety and stress within the CeA can give us the entire picture.

Future research should focus on the role that glutamate plays in excitability as it relates to the amygdala and anxiety. This will help to consolidate the model and perhaps help in the understanding of anxiety and stress disorders, as well as providing insight into some of the behaviors seen in alcohol dependence. Furthermore, the specific effects of CeA targets following NPY, CRH, and ethanol treatments should be examined as to provide a clearer picture of subsequent brain activity. A complete understanding of all molecular and cellular mechanisms of alcohol dependence will require a survey of the neural circuits involved in this behavior. Nevertheless, obtaining a plausible model opens

the door to other possible treatments for individuals plagued with alcohol dependence and anxiety disorders.

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