

Corticotropin Releasing Hormone (CRH) is a peptide hormone that is responsible for synchronizing endocrine, physiological, neuroendocrine and behavioral responses to stress exposure. The action of the hormone and its receptors activate the hypothalamic-pituitary-adrenal-axis (HPA) to alleviate symptoms of stress. CRH receptor 1^{-/-} mice exhibit symptoms of an impaired hypothalamic-pituitary-adrenal (HPA) axis under basal and stressed conditions. This phenotype is characterized by a blunted response to the initial phase of stress. In an attempt to compensate for this genetic modification, extrahypothalamic regions of the brain (i.e. the hippocampus and amygdala) undergo plastic changes after stressful life events. Alcohol drinking and stress have been associated with one another. After repeated bouts of stress, the animal begins to consume alcohol in an enhanced and delayed fashion, which continues throughout life. The delayed response to increased alcohol intake is caused by an up regulation of the N-methyl-D-aspartate receptor subunit, NR2B. The functionality of the CRH receptor 1 in the presence of stress has been well defined. However, the role of the CRH receptor 2 α and stress in the extrahypothalamic-limbic regions of the brain are poorly understood. CRH-like neuropeptides, Urocortin, Urocortin II, and Urocortin III have recently been cloned and display an increased binding affinity for CRH receptor 2 α . Because these neuropeptides are centrally expressed, their actions may provide insight to understanding the role of CRH receptor 2 α in response to stress. There is evidence that CRH receptor 2 α may be involved in the anxiolytic phases (recovery) of stress. The goal of the work proposed is to assess the role of CRH receptor 2 α and its urocortin ligands in the stress response. This study will target the HPA stress axis, impacts of glutamatergic system, and other regions of the limbic system that may be influenced by stress and stress induced alcohol drinking. In order to gain better understanding of the CRH receptor 2 α , we plan to use the CRH receptor 1^{-/-} and the CRH receptor 1 and 2^{-/-} double null mutant mouse models to draw plausible conclusions about the CRH receptor 2 importance in response to stress and stress induced alcohol drinking.

The CRH receptor 2 has several splice variants, α , β and γ , the α splice variant is centrally located; henceforth the Greek notation will not be used throughout the document.

Specific Aims

Urocortin peptides have been isolated and prefer to bind to CRH receptor 2. Several regions of the brain display predominant expression of CRHR2 and urocortin peptide immuno-reactivity. (1) Investigate the physiological roles of urocortin, urocortin II, III and CRH receptor 2 in response to stress and stress induced alcohol drinking. I will determine if there is a significant difference in mRNA expression levels of urocortin, urocortin II, urocortin III, and CRH receptor 2 distributions in regions of the extra-hypothalamic system.

The CRH receptor 1 mutant mouse model exhibits a blunted (decreased production of the stress hormones, ACTH and corticosterone) response to stress. The model also develops enhanced and delayed consumption of alcohol following repeated stress exposure. Recently a mutant mouse model deficient for both receptors 1 and 2 has been designed, in an attempt to elucidate the functionality and interactions of the two receptors during stress exposure.

(2) To investigate the effects of the CRH receptor 2 and ethanol drinking, I will determine if the double null mutant model will be influenced by stress to consume ethanol. I will determine if there is a significant difference in alcohol preference, intake and time needed to elicit a response to stress-induced alcohol drinking when compared to the CRH receptor 1 mutant.

The CRH receptor 1 mutant mouse exhibits an increased consumption for ethanol because of an up regulation of the NMDA receptor NR2B subunit. These compensatory changes are primarily observed in the hippocampus and nucleus accumbens.

(3) To investigate the effects of ethanol withdrawal and possible conditions of neuro-degeneration within the Hippocampal formation.

I will determine if the animal model will exhibit neuro-degeneration due to neuro-adaptive changes imparted by stress induced alcohol drinking and chronic ethanol intake. I will determine if the GABAergic receptors within septum will be influence by the loss of the CRH receptors and ethanol drinking.

My hypothesis is that while the CRH receptor 1 may be the primary stimulator of stress-induced alcoholism, the CRH receptor 2 and urocortin ligands may modulate stress and stress-induced alcohol drinking, in order to maintain homeostatic conditions.

Significance and Background

The term stress has been applied to many different areas of science, thus the term stressor was coined to redefine the word in its context to biological systems.

Introduced by Han Seyles, stressor is defined as a causative agent, which involves selective pressures from physical and social environment that may threaten or challenge an organism and elicit compensatory response patterns.

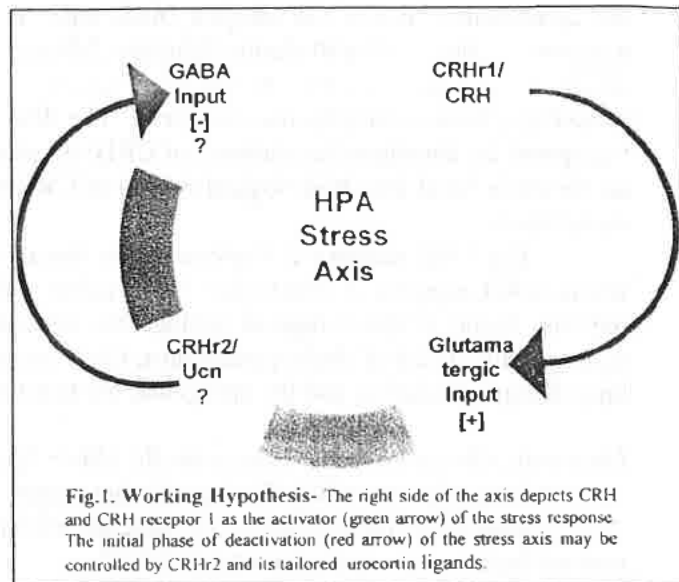
Alcoholism is defined as the continuous or periodic impaired control over drinking; this includes having a preoccupation with alcohol, use of the drug despite adverse consequence, and distortion of thinking (NIAAA, 2002). The disease is characterized by four primary symptoms: craving, loss of control, physical dependence and tolerance. Over the past twenty-five years, the scientific community has provided evidence that genetics and environmental factors serve as strong indicators for the risk of alcoholism³⁰.

Recently, studies have reported that stress induced alcohol drinking is associated with the corticotropin releasing hormone system and the glutamatergic system. Although our knowledge about alcoholism has expanded over the past thirty years, the molecular and cellular influences of the disease are in not as clear. Statistics show that one in thirteen adults is affected by alcohol abuse or alcoholism worldwide; regardless of gender, race, or socioeconomics. Because many individuals use alcohol to cope with stressful life events, investigating the underlying mechanisms and effects of alcoholism may serve as useful.

The Corticotropin Releasing Hormone and its Receptors

Corticotropin releasing hormone (CRH) is a 41- residue peptide that was initially isolated from ovine hypothalamus⁴². The CRH ligand serves as the primary synchronizer of the endocrine, autonomic, behavioral, and immune responses to stress exposure. CRH is secreted from the small nerve cell bodies of the anterior portion of the paraventricular nuclei (PVN) of the hypothalamus, and is distributed to extra-hypothalamic brain regions. Although CRH is the primary mediator of the stress response, it is the action of the hormone and its receptors together, which will determine the magnitude of the physiologic response.

The receptors, CRH receptor 1 and CRH receptor 2 are high affinity G-protein-coupled seven trans-membrane domain receptors (see fig. 2), and are positively coupled to adenylate cyclase. Although these receptors share extreme similarities, factors such as tissue distribution and pharmacological specificity set them apart.



The CRH receptor 1 is highly localized in the brain and the pituitary; it is primarily located in the anterior pituitary, cerebral cortex, hippocampus, nucleus accumbens, amygdala, olfactory bulbs and the cerebellum. When this receptor binds CRH it activates the Hypothalamic-Pituitary-Adrenal (HPA) axis to produce anxiogenic (anxiety-producing) behavioral responses to stress. The data to support the functional importance of CRH receptor 1 in its response to stress is unsurpassable, on the other hand the physiological role(s) of CRH receptor 2 and its response to stress is poorly understood.

The CRH receptor 2 displays a less dense projection in the brain. Interestingly, regions where CRH receptor 2 expression is prevalent, display almost complete dominance in these regions. Some of these regions include the medial and lateral septum, dorsal raphe and the ventromedial nuclei of the hypothalamus. CRH receptor 2 expression has also been located in the hippocampal formation and the amygdala, but to a much lesser degree.

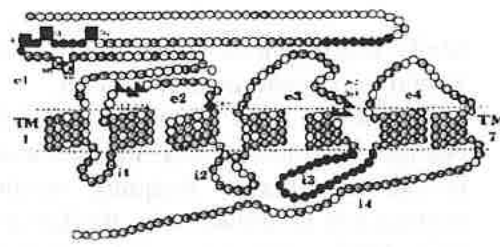


Figure 2. Representation of the CRH Receptor¹⁵

Urocortin, Urocortin II and Urocortin III: Other Ligands that may bind CRH receptor 1 and 2

Recently three CRH-like ligands that possess a distinct preference for the CRH receptor 2 may aid in understanding the function of this receptor. The urocortin ligands have been isolated from human, rat and mouse^{18, 27, 44, 47}.

Urocortin (Ucn) is a 40 amino acid peptide which has the ability to bind to both CRH receptors with a greater affinity than CRH. When compared to CRH, Ucn has a 40 fold greater affinity for CRH receptor 2. Weninger recently reported that Ucn mRNA expression is sequestered to the Edinger-Westphal nucleus (EWN) and the nucleus of Darkschewitsch in mice. The functions of these regions are not fully understood; however they have been implicated to be involved in autonomic pathways of the nervous system⁴⁵.

Ucn II is expressed centrally and peripherally and binds selectively to CRH receptor 2 with little or no appreciation for CRH receptor 1²⁷. This ligand has a sequence length of 38 amino acids and is structurally related to CRH and Ucn. Ucn II expression has been located in several stress-related cell bodies of the central nervous system.

Lastly, Ucn III ligand is 38 amino acids. Like Ucn II, this peptide selectively binds to the CRH receptor 2 with a greater affinity than that of CRH receptor 1. Mouse Ucn III mRNA is expressed in the hypothalamus, amygdala, lateral septum, bed nucleus of stria terminalis (BNST), and the brainstem. This evidence supports the notion that the urocortin peptides and the CRH receptor 2 may be functional in the stress response¹⁸.

Examining the role of the CRH Receptors by Genetic Manipulation

The use of genetically altered mouse models has been a useful tool in understanding the functional importance of the receptors as well as the pathogenesis of stress-related disorders and diseases. These models have provided great benefit in allowing a detailed analysis of the neural circuitry of the brain and its function. It has been shown that deregulation of the CRH/CRH receptor system is associated with an increased vulnerability to alcohol drinking³². The CRH receptor 1 deficient mouse model has been the primary animal model in concluding these findings^{29, 31}. This animal displays a blunted (decreased) hormonal stress response^{9, 41}. The sluggish stress response profile is caused by atrophy of the adrenal cortex, which leads to a reduction in adrenocorticotrophic hormone (ACTH) and corticosterone secretion. This condition has been observed under basal conditions, after stress exposure and acute alcohol withdrawal (via forced drinking paradigm).

Interestingly, the CRH receptor 2 deficient animal model exhibits a behavioral response to stress that is the complete opposite, hypersensitivity to stress^{14, 18}. This report is supportive of

the importance of the CRH receptor 1 in activating the stress response. In addition this finding may support my hypothesis that the CRH receptor 2 is involved in alleviating the HPA stress axis. In other words, if the CRH receptor 1 were an on switch, and in the event that the CRH receptor 2 was absent, the stress response would remain at its peak. Although the CRH receptor 2^{-/-} mouse model has been designed, and behavioral studies have been conducted by several groups; their results contain a variable amount of inconsistency with one another. In regard to the functional role of the receptor, the conclusions are extremely inconclusive. To add to this, most of these studies were conducted solely to question the importance of Ucn and feeding and the changes within the peripheral. Therefore, in an attempt to gain a solid understanding of the CRH receptor system, Bale et al. has recently developed a mouse model that is deficient for both receptors (double null mutant). Reports show that the double null mutant responds to stress much like the CRHr1 mutant. But more interestingly, this animal has an even further depressed response to stress. This suggests that in the absence of the CRH receptor 1, that the CRH receptor 2 may play a dual role in activating as well as deactivating the stress response to some degree. To add to the possibility of the CRH receptor 2 functionality, the urocortin ligands which bind to CRH receptor 2 may be functioning as well. Using this model in comparison with the CRHr1^{-/-} model may provide some insight to the function of the CRH receptor 2 in stress and stress induce alcohol drinking.

Specific Aim 1. Investigate the central expression of urocortin, urocortin II, III mRNA and the s

Several reports have shown that Ucn, Ucn II and Ucn III ligands are expressed in several stress cell bodies. In addition to these findings, researchers have reported that CRH receptor 2 mRNA is co-expressed in these regions. Therefore, this is a primed condition for regional specificity of the urocortin ligands and the CRH receptor 2 to interact with one another during the stress response.

This series of experiments will assess the activity of the urocortin ligands and CRH receptor 2 under basal and stressed conditions. It is believe that Ucn may be the endogenous ligand for CRH receptor 1^{43, 16}; reports have shown that CRH knockout models express significant increases in Ucn expression when stressed. While Ucn may be promiscuous in it's binding to both receptor 1 and 2, Ucn II and III show a more promising profile for interaction with CRH receptor 2 only. Ucn II and III are expressed in many stress cell bodies of the HPA. Some of these regions are the paraventricular, arcuate, and the ventromedial nuclei of the hypothalamus, lateral and medial septum, as well as the locus coeruleus of the brainstem.

Does acute or chronic stress alter measurable parameters of urocortin ligands mRNA expression and CRH receptor 2 concentration?

I will test the animal's (wild type, CRHr1^{-/-} and CRHr1 and 2^{-/-}) response to stress by using methods of restraint, social defeat, Porsolt swim test, and food deprivation. The stressors will be administered in two separate sets of experiments. Experiment A will be performed for acute (1 day) stress trails. Experiment B will be performed for chronic (3 consecutive days) trails. After the trails have been completed, the animals will be allowed to recover, and during this time behavior and home cage activity will be monitored. CRH, Ucn, Ucn II, Ucn III and CRH receptor 2 (for the CRHr1^{-/-}) mRNA expression will be determined by *in situ* hybridization and receptor autoradiography.

Specific Aim 2. Investigate the loss of CRH receptor 2 and Ethanol drinking in CRH double null mutants

Deregulation of the CRH/CRH receptor system induces change in several systems in order to remain homeostatic conditions. The vasopressinergic, glutamatergic, and the amygdaloidal systems^{24, 32} have all been reported to undergo changes. In the interest of the ethanol drinking

experiments, the glutamatergic system is a system of extreme interest, largely because a majority of the synapses in the CNS use the neurotransmitter glutamate, to produce rapid neuronal excitation. Sillaber et al. reported that ethanol sensitive NMDA receptors are up-regulated in mice that lack a functional CRH receptor 1. In turn, when these animals are exposed to repeated stress, they are influenced to consume ethanol more readily than their wild type counterparts. It is important to note that these changes remain persistent throughout life. However, the influence of the CRH receptor 2 was not considered when these studies were conducted. Thus, examining the CRH/CRH receptor and other systems of influence may provide a comprehensive analysis of the feedback systems of the stress response.

Since Bale et al. has reported that the double null mutant is more like the CRH receptor 1 mutant; assessment of the CRH receptor double null mutant under basal and stress induced alcohol drinking conditions will be employed. The goal of these experiments will be to draw conclusions regarding the interactions between CRH receptors 1 and 2. In this series of experiments, the two bottle choice ethanol drinking paradigm will be administered before and after acute and chronic trials of stress. Behavioral analysis will be assessed by Digiscan open-field testing in the presence and absence of stress. Urocortin(s) expression will be assessed at its respective sites by *in situ* hybridization and CRH receptor autoradiography will be performed to analyze receptor expression. Western blot analysis will be performed for NMDA subunit and GABA subunit expression.

A) What is the double null mutant's response to acute alcohol withdrawal?

Because ethanol withdrawal is a severe stressor⁴¹, we must assess the double null mutant's response to acute ethanol withdrawal. Timpl et al. reported that the CRH receptor 1 mutant shows a decrease response to acute ethanol withdrawal (forced drinking paradigm). In this portion of the study, the double null mutant model will be administered ethanol under a forced drinking paradigm. In case the withdrawal from ethanol will serve as the stressor. The animal will be administered the two-bottle choice paradigm for a period of habituation (2 weeks) where ethanol concentration will be increased. I will then change the animals drinking solution to an ethanol only solution for a period of three weeks. After this time, I will withdraw the animal from ethanol; by switching the drinking solution back to water 12 hours before behavioral testing. I will then monitor the animal's levels of anxiety by using an open field test and monitoring home cage behavior via grooming behavior. Crawley et al reports that excessive grooming has been reported as a measure for anxiety. The animal metabolic efficiency for ethanol will be examined by spectrophotometric assays (this assay will be conducted in a second set of animals).

B) Will the double null mutant display a significant difference in ethanol consumption, under stress induced conditions when compared to the CRH receptor 1-/-?

The animals will be allowed to consume ethanol of increasing concentrations via the two-bottle choice drinking paradigm as described in the methods section. I will then induce the restraint, swim defeat, and social defeat stressors in acute and chronic trials. The amount of time needed to establish stress-induced alcohol drinking will be monitored, and fluid intake will be monitored before, during and after stress induction. The animal's home cage behavior while under the influence of ethanol and stress will be monitored. After behavioral analysis the animal's levels of CRH, Ucn, Ucn II and Ucn III mRNA expression will be determined by *in situ* hybridization. NR2B subunit expression and GABAergic subunit expression will be analyzed by western blotting.

Specific Aim 3. Investigate ethanol withdrawal and neurodegenerative changes in Hippocampal tissue.

Sillaber et al. reported that post six months of the stress induction studies that ethanol consumption in the CRH receptor 1 mutant remains constant. This behavior persisted throughout the life of the animal. The long term effects of this observation were not considered. In an attempt

to continue examining the effects alcoholism, withdrawal from alcohol and other drugs of addiction is considered to be a severe stressor. This series of experiments will assess the long term effects of a dysfunctional CRH/CRH receptor system. The condition of withdrawal should to be considered in order to understand long-term consequences of stress induced alcohol drinking. The effects of prolonged ethanol drinking produce adaptational changes to the neural circuitry that may not be reversible once in place.

Sillaber reported that the changes in the NMDA receptor expression is defined as a neuroadaptive change. Several reports have shown that ethanol binds the NMDA receptor and inhibits excitation (i.e. neuronal firing)¹². Because these animals (CRH receptor 1-/-) continue to consume ethanol for an extended period of time, they are now subjects of chronic ethanol consumption. Chronic exposure to ethanol leads to ethanol induced neurodegeneration in wild type mice (see fig. 3). Withdrawal from chronic ethanol exposure is a precursor for neurodegeneration¹². The onset of neurodegeneration due to withdrawal may occur immediately or may develop after repeated periods of withdrawal¹². Treit et al., have demonstrated that the septum is involved in the anxiolytic pathways of the neuronal circuitry. The septum is the interface between the hypothalamus and the hippocampus (see fig.4)²⁵. Within this connection, lie the medial and the lateral septum; which are both regions of the brain that are enriched with CRH receptor 2. Moreover, the medial and lateral septums possess GABA receptors, which work to inhibit excitatory amino acids (i.e. NMDA receptors in the context of normal physiology) (see fig.4). If both of these systems are de-regulated, this lead to an imbalance in excitation, which may cause excitotoxicity to neurons. Therefore, it is feasible to examine the animal's response to withdrawal and to question the fate of neuronal survival. In this study I will withdraw the CRH receptor 1 and the double null mutant from alcohol and assess the animal's levels of stress and possible neurodegeneration.

(A) Will the mutants produce an anxiogenic response when withdrawn from chronic ethanol exposure?

After the animals are withdrawn from alcohol, I will assess the level of anxiety by using the Digiscan open field behavioral analysis test to examine animal's level of anxiety.

(B) Will the mutants exhibit neurodegeneration due to withdrawal from chronic ethanol consumption?

I will study hippocampal slices to examine the absence or presence of neuronal degeneration due to ethanol withdrawal. Hippocampal slices will

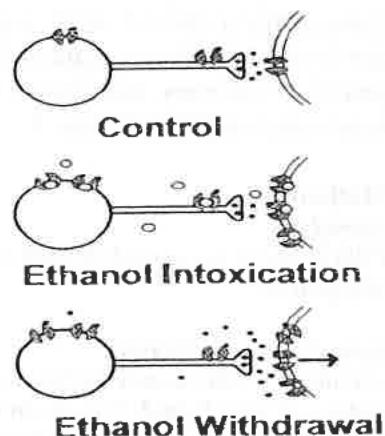


Fig 3. Schematic representation of post synaptic NMDA terminal¹². During EtOH intoxication, EtOH blocks NMDA subunits, and no neuronal firing occurs. During EtOH withdrawal, glutamate binds to subunits and neuronal firing is active

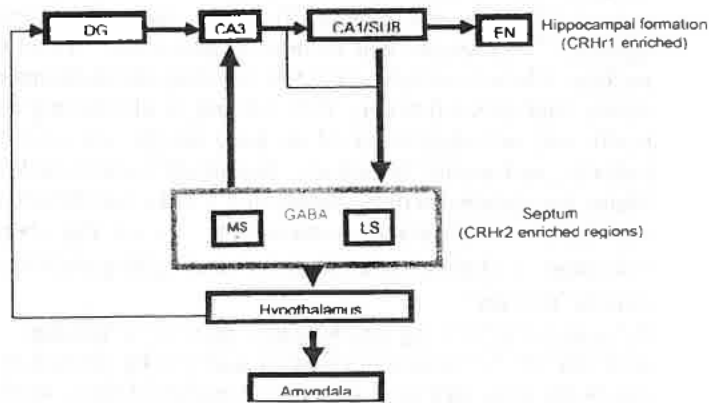


Fig 4. Septo-Hippocampal System^{12, 25, 51}

The septum, which is highly enriched with CRH receptor 2, is the interface between the hippocampus and the hypothalamus. If Glutamate and GABA neurotransmission are improperly (via CRH receptor deletion) balanced, neurotoxicity may occur.

also be examined during stress-induced alcohol drinking and after withdrawal. I will use Flouro-Jade B, a neuronal marker which has a high specificity affinity for neurodegenerative tissue to determine the presence or absence of neurodegeneration.

(C) Will the GABAergic subunits of the medial and lateral septum be influenced?

I will perform protein profiling of the GABAergic receptors in the regions of the medial and lateral septum. I would expect these receptors to be significantly depressed during acute and chronic exposure to alcohol; however, I expect the GABA receptors efficiency to recover to be dramatically impaired under conditions of post withdrawal. Ucn, Ucn II and Ucn III mRNA expression will also be examined. If animals are able to be successfully withdrawn, I will allow a period of recovery and repeat the stress induced studies again in attempt to understand the sensitization effects of ethanol.

Methods

Animals

CRH receptor 1-/- and CRH1/ CRH 2 double mutant littermates will be obtained from the Bale and Timpl laboratories^{32, 5}.

Animal Care and Housing

All animals will be provided food and water freely unless otherwise noted. The animals will be housed under a 12 hour light/dark cycle, in which lights are on at 6:00am and lights are off at 6:00 pm. All animals will be kept in climate controlled environment. Fourteen days prior to the experiments, mice will be placed in individual cages and will be handle on alternate days. All protocols and procedures will be in compliance with guidelines provided by the NIH and the Institutional Animal Care and Use Committee. All experiments will be conducted in F2 generation adult (two to six month old) male control, CRH receptor 1-/-, and CRH receptor double null mutant mice. This age range is in compliance with all subject groups to be tested.

Preliminary Observations

Indication of proper CRH receptor impairment

To ensure that functional impairment of the CRH 1 receptor is successful, pituitary cells from homozygous mutants and from wild type mice will be cultured from adult (2 to 12 month old), stimulated with CRH cAMP induction and ACTH release will be detected by radioimmunoassay was described³⁸. In the case of the CRH receptor 2, impairment will be observed via receptor autoradiography^{3, 40}. The lateral septum (CRH receptor 2 enriched) will be assessed for receptor binding in the mutant, the CRH receptor2 agonist; ¹²⁵I-sauvagine will be used in this assay. Prior to the start of all experiments, all subjects will undergo a battery of behavioral test to screen observations of general health, home cage behavior, sensory ability, and motor function. This will aid in eliminating the possibility of false positives¹⁰. Briefly good health will be indicated based on body weight, temperature, appearance of fur and whiskers, grooming behavior, and nesting behaviors. Neurologic reflexes will be assessed based on eye blink and ear twitch. Motor coordination will be tested on a Basile Accelerated Rotorod, and latency to fail off of the rotorod will serve as indicator of coordination. Home cage behavior with littermates will be monitored via videotape, in which nesting, grooming, and fighting patterns will be monitored.

Stress Studies²

Introduction of Ethanol Drinking by Two Bottle Paradigm

Animals will be housed individually and will be allowed to drink water from two bottles, labeled bottle A and bottle B at their liberty. Over a period of three weeks, with the initial week of water only, ethanol concentrations in bottle B will be increased from two percent to eight percent (2%, 4%, and 8%) volume/volume in tap water. Over this period of time, water consumption versus the initial preference for ethanol will be measured, total fluid intake independent of ethanol, and total ethanol intake will be measured daily. The bottles will be weighed every morning, and the concentrations will be increased on sixth day of the week. Animals will be videotaped so that home cage behavior can be accounted for during this period. A habituation period of eight weeks to the 8% ethanol solution in the presence of the water

²These studies have been preformed for the CRH receptor 1 mutants in already published literature; this portion of the study serves as a method to compare the CRH receptor 1-/- and double null mutant model^{32, 5}. This study will be the platform for the withdrawal experiments to follow.

only bottle will be allowed before the stress induction studies began.

Forced Alcohol Drinking Paradigm

The forced drinking paradigm will be followed as described by Timpl⁴¹.

Stress Induction

Repeated Exposure to Stressor Conditions

After the habituation period has been established, in which the genotypes will not express any change in ethanol intake, the animals will be exposed to each of the stressor conditions for consecutive days. Restraint, social defeat, which will serve as a psychological stressor, and Porsolt swim test which is noted as a severe stressor, this test is known to have an emotional component, in which behavioral despair can be analyzed. Crawley et al. found that time spent floating instead of swimming measure depression-like behavior in mice. In this instance the animal is displaying a behavior of giving up and is not seeking escape for survival. Voluntary daily ethanol intake will be monitored post stress conditions.

Restraint Stress

A restraint experiment will be conducted, in which the animal will be placed in a ventilated 50- ml polypropylene tube for thirty minutes. The tubes are fitted so that the animal can breathe with ease, but mobility is limited. After the thirty minutes of restraint stress, the animal will be returned back to his cage for recovery with free access to water and ethanol.

Social Defeat

In a (20x12x20 cm) cage, the test animals (WT, CRHR1, or the double null mutant) will be introduced into a cage in which he is the intruder. The resident mouse will have been housed in this cage approximately one month. Upon the initial attack of the resident mouse on the intruder, animals will be separated after two minutes by a wire mesh partition for 15 minutes, and the intruder will placed in a small section of the cage (7x12x20 cm). During the two minute attack, the latency of the resident to attack, number of bites should be scored. This serves as strong component of aggressive and passive behavior in comparison to the intruder. After the 15 minute wait following defeat, the intruder should be returned to his home cage to recover with access to water or ethanol.

Porsolt Swim Test

Animals will be placed in a cylinder filled with water ~21°C. The depth of the water will be 12 inches, and mice will be monitored for time spent swimming versus time spent floating. The total time allowed to swim will be ten minutes, after which the animal will be dried and returned to his home cage for recovery with access to water of ethanol. In the case of the double mutant model, voluntary intake of ethanol will be measured in a separate set of experiments where the mutants will not be exposed to stress, in order to rule out the possibility that the mutation causes increased intake instead of stress.

For statistical significance, ANOVA with Newman-Keuls multiple comparison *post hoc* test will be used (Sigma Plot and Sigma Stat Software). Sample sizes, (for studies associated with knock-out animals, n-values of ten or more are suggested¹⁰), weight and body temperature will be as equal as possible among subjects.

Food Deprivation

Food Deprivation studies will be carried out as described by Bale et al.⁴

Pharmacokinetic Alcohol Assessment

Because the CRH receptor 1 and the wild type animal display normal profiles for the metabolism of alcohol; the double null mutant will be subjected to blood alcohol concentrations in comparison to the wild type and the CRH receptor 1 deficient animal. The animal will receive an intraperitoneal (i.p.) injection with 3.5 g/kg ethanol (20% ethanol solution, v/v). Blood samples will be collected (25-30µl) to measure blood alcohol levels at time points from (30 to 340mins). Blood alcohol levels will be assessed via NADH enzyme spectrophotometric method (Sigma).

Preparation of Brain Tissue NMDA Receptor

After establishment of the stress induced alcoholism, mice will be decapitated and brain tissue will be dissected into hippocampus, amygdala, and nucleus accumbens. Tissue may be frozen and stored at -80°C until ready to use if necessary.

Western Blot Analysis for NR2B Subunit

The above tissue samples will be homogenized in HEPES buffer 1% NP40 and proteinase inhibitors²⁶. The sample will be removed from cellular debris via centrifugation and the supernatant will be used as the total protein. Quantitation of the protein will be access by (BioRad DC Protein Kit). Samples will be loaded on 9% SDS-PAGE and transferred to nitrocellulose using a mini transfer cell apparatus (BioRad). Membranes will be blocked with 5% BSA in TBS buffer and incubated with the appropriate primary antibodies overnight^{5, 26}. Following overnight incubation, samples will be incubated in secondary antibodies for 2 hours. All antibody incubations, washes and dilutions will be performed with TBS-T buffer. Antibody detection will be analyzed using the Amersham Life Science ECL protocols. All chemicals will be purchased from (Sigma Inc). Expression will be observed by optical densitometry (Eastman Kodak).

In Situ Hybridization for Ucn, Ucn II and III mRNA Expression

Mice will be decapitated at various times following stress, and brains sites will be frozen in isopentane, and stored at ~-80°C until they are ready to be sectioned. Tissues will be sliced 12µm thick (Leica Cryostat) and collected on SuperFrost plus slides (Fisher). The slides will be dried at room temperature and stored at -20°C with desiccant. Tissue samples from the EWN for Ucn, from the paraventricular, supraoptic, and the arcuate nuclei of the hypothalamus for Ucn II, and from the hypothalamus, brainstem, and lateral septum and the bed nucleus of stria terminalis for Ucn III will be probed. Slides will be pretreated as described⁴⁴ by the protocol. Riboprobes will be generated for each of the peptides. For urocortin, briefly cRNA probes will be made complimentary to nucleotides 660-882 of the coding region of the 3' coding and untranslated region of Ucn mRNA⁴⁶. Ucn II, mouse cDNA will be sub-cloned into TOP-II plasmid, cRNA probes will be made by linearizing the plasmid²⁷. Ucn III, cRNA probes will be made from Ucn cDNA. The probes will be radio-labeled by using T3 enzymes and [α -³³P] UTP as described^{33, 48}. The control probes will be made by using brain paste instead of commercialized standards, because they provide better standards. Following the generation of the probes, they will be stored at -20°C in hybridization solution. (50% deionized formamide/10mM Tris, pH8/1mMEDTA/500µg/ml yeast tRNA/10mMDTT) until ready to use. Upon hybridization, the cRNA probes will be heated to 65°C for approximately 3 minutes. 100 µl of hybridization solution with the probe will be applied to each slide, air bubbles must be avoided. The slides will be saturated with 50% formamide/0.5 M sodium chloride and hybridized for 16 hours at 60°C. Post hybridization washes will be followed as described⁴⁸. Probes will be made as described⁴⁹ by CRH and CRH receptor 2 riboprobe will be constructed. For CRH probe, a 1.2 kb EcorR1 fragment that is the length mouse cDNA can be subcloned into a pBluescript II-Sk+ plasmid. For the CRH receptor 2 probe will be constructed from the 275 base pair fragment that is encoded for the N-terminal of the receptor, the construct will then be ligated into a pBluescript II-Sk+ plasmid. The antisense cRNA will be incorporate [α -³³P] UTP into the CRH receptor probe. All transcription reactions will follow (Ambion Transcript kits) as described⁵. For optimal anatomical localization, slides will be dipped in (Kodak) NBT-2 liquid nuclear emulsion diluted 1:1 in distilled water, followed by exposure at 4°C (x-ray film; Kodak) for 20-28 days, followed by photographic processing. Analysis of slides will be examined for detection above background.

CRH receptor 2 Autoradiography

Protocol can be followed as described by Skelton³³.

Both *in situ* hybridization and receptor autoradiography images will be digitized via Kodak Image Station 2000R (Eastman Kodak Co. 2003).

Withdrawal from Chronic Ethanol

After repeated periods of stress, mice will be allowed to drink ethanol as there only drinking fluid (8 %v/v)³⁶. Two groups based on periodicity will be established: a) three month period b) six month period. During these periods of chronic ethanol exposure, animals will be monitored for behavioral changes by using the open field behavior test and through continued observations of home cage behavior. Animals will be withdrawn from ethanol at the respective periods of time, in which the ethanol solution will be replaced with pure water, after twelve hours, the animals will be tested on the open field and light-dark test. Blood

alcohol levels will be monitored as described³². In parallel, a control group of animals will be exposed to chronic ethanol exposure under the forced ethanol drinking paradigm as described^{37, 40}, with some modifications. This will provide evidence of the wild type under withdrawal from alcohol, the forced paradigm is used because mice and rats do not drink ethanol voluntarily unless genetically modified.¹² Statistical Analysis can be measured by ANOVA followed by *post-hoc* Scheffé's test using Sigma Plot and Sigma Stat software.

Hippocampal Slice for Neurodegeneration

Tissues from the withdrawn subjects were prepared as described^{30, 39}, but slightly modified. Briefly, the mice will be properly anesthetized with urethane or halothane and perfused via the ascending aorta with Streck tissue fixative. The tissue will be post fixed overnight in Streck fixative, if not used immediately after the overnight storage; the tissue will be stored in a 20% sucrose solution and refrigerated. Samples will be sectioned at a thickness of 12 μ m. The remainder of the protocol will be followed as described³⁰. Appropriate stock solutions will be made for staining. Slides will be stained with Fluoro-Jade B (Histo-Chem Inc., Jefferson AR) for 20 minutes and rinsed (3X) with distilled water, properly drained of excess water, and placed on the slide warmer until completely dried. Slides will be immersed in xylene to remove residues for 1 minute, and cover slipped with a non-aqueous, non-fluorescent plastic mounting media (Sigma Chemicals). The tissues will be analyzed by using Epi-fluorescent microscopy with blue (450-490nm) or blue-violet (420-490nm) excitation light³⁰. Filters that visualize FITC may be used as an alternative, however the emission color will be green (515-565nm). Results will be measured as percentage (mean) of condensed or fragmented cells $\pm$ SEM; n values of 5 or more from individual experiments will be needed for statistical significance.

Western Blotting for GABA Receptor Expression

Animals will be decapitated and brains will be homogenized as stated above. Protein preparation, blotting, and ECL detection will be followed as stated above. GABA receptor antibodies will be purchased from (Sigma and Alomone Labs).

It is possible that the mutant animals are functioning under allostatic parameters. The term allostasis was coined by McEwen, which is defined as "the concept of physiology in which all of the parameters of the internal milieu are varied to match perceived and anticipated environmental demands in an attempt to maintain stability"¹⁶. Therefore, I expect that withdrawal from alcohol may cause the mutant animals to experience allostatic loading, in which a small challenge will cause a spiraling cycle of breakdown in systems. If this should occur, relapse to alcohol may occur. I would expect the CRH receptor 1-/- to have a greater chance of recovering and coping with stress. Because the double null mutant displays a more blunted (decreased) response to stress in basal conditions, the systems maybe working to in an allostatic manner, which could eventually lead to pathology or possible neuronal cell death. This model should be able to handle acute stress; however, the animals' responses to chronic stress may be problematic. I would expect the double-null mutant animal to exhibit greater allostatic load than the CRHr1 animal.

In summary, using genetically altered animal models may serve as a tool to unravel the web of stress related alcohol dependence. However, a complete characterization of the involved primary receptors and ligands of the corticotropin releasing hormone system, along with extrahypothalamic regions of the brain must be studied to gain a comprehensive understanding of the effects of stress induced alcohol drinking and other patho-physiological disorders. Although the experimental methods used may not be novel; the results may provide a basic road map to understanding the phases of stress and alcohol drinking. Furthermore, targeting influential components of the HPA stress axis may advance our knowledge to efficient methods of treatment and possible cures.

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