THE RELATIONSHIP BETWEEN STRESS AND ETHANOL SELF-ADMINISTRATION: CIRCULATING STRESS HORMONES AND GLUTAMATE SIGNALING IN THE HYPOTHALAMIC PARAVENTRICULAR NUCLEUS

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CERTIFICATE OF APPROVAL

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LIST OF ABBREVIATIONS

3V: Third Ventricle

A1: First abstinence phase

A2: Second abstinence phase

A3: Third abstinence phase

AC: Anterior Commissure

aCSF: Artificial Cerebral Spinal Fluid

ACTH: Adrenocorticotropin Hormone

ALLO: Allopregnenolone

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AVP: Arginine-vasopressin

AUD: Alcohol Use Disorder

BEC: Blood Ethanol Concentration

BDA: Biotinylated Dextran Amine

CAR: Cortisol Awakening Response

Cd: Caudate

Cm: Membrane Capacitance

CRH: Corticotropin Releasing Hormone

CSF: Cerebral Spinal Fluid

Cyno: Cynomolgus

CYP: Cytochrome P450

DAB: Diaminobenzadine

DAPI: 4',6-diamidino-2-phenylindole

DHEAS: Dehydroepiandrosterone sulfate

DOC: Deoxycorticosterone

eCB: Endocannabinoid

EM: Electron Microscopy

EPSC: Excitatory Postsynaptic Current

EPSP: Excitatory postsynaptic Potential

FR: Fixed-ratio

FT: Fixed-time

GABA: Gamma-aminobutyric acid

GR: Glucocorticoid Receptor

HPA axis: Hypothalamic Pituitary Adrenal axis

HSD: Hydroxysteroid

IPSC: Inhibitory Postsynaptic Current

IPSP: Inhibitory Postsynaptic Potential

LTS: Low Threshold Spike

mGR: Membrane bound Glucocorticoid Receptor

MR: Mineralocorticoid Receptor

MSN: Medium Spiny Neuron

NESARC: National Epidemiologic Survey on Alcohol and Related Conditions

NHP: Nonhuman Primate

NMDA: N-methyl-D-aspartate

NO: Nitric Oxide

OC: Optic Chiasm

o-CRH: Ovine Corticotropin Releasing Hormone

OD: Optical Density

Open-access: daily 22-hr sessions

PB: Phosphate Buffer

PBS: Phosphate Buffered Saline

Pu: Putamen

PVN: Paraventricular Nucleus of the Hypothalamus

Rm: Membrane Resistance

ROI: Region of Interest

SIP: Schedule-Induced Polydipsia

StAR: Steroidogenic Acute Regulatory protein (StAR)

THDOC: Allotetrahydrodeoxycorticosterone

VGCC: Voltage-Gated Calcium Channel

Vm: Resting Membrane Voltage

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ABSTRACT

Alcohol use disorders (AUDs) are a significant global burden. In 2014, 5.1% of the global burden of disease and injury was attributed to alcohol use (World Health Organization, 2014). Stress is an important etiological factor in the initiation and continuation of alcohol consumption and stress increases the risk for relapse (Blaine et al., 2015; Sinha et al., 2011). Stress is defined as any physiological or psychological stimulus that challenges homeostasis and is quantified by its activation of the hypothalamic-pituitary-adrenal (HPA) axis (Smith & Vale, 2006). The relationship between stress and ethanol consumption is complex and bidirectional. On the one hand, stress influences consumption in both humans and animals (Becker et al., 2012; Blaine et al., 2015; Sinha et al., 2011), while on the other, acute and repeated ethanol influences the HPA axis (Richardson et al., 2008; Adinoff et al., 2005a and 2005b).

This dissertation is a study of the peripheral and central components of the HPA axis using a macaque model of ethanol self-administration. Chapter 3 focuses on the primary stress hormones, adrenocorticotropic hormone (ACTH) and cortisol, under two distinct conditions, to test two hypotheses. The first is that long-term ethanol self-administration selectively dampens stress hormones under low, but not mild, stress conditions. The second is that repeated forced abstinence elevates the HPA axis response under low stress conditions and leads to a dampened response to mild stress. Compared to pre-ethanol, there was a decrease in ACTH, independent of ethanol. The concentration of ACTH during open-access was negatively correlated with average daily intake, while low stress cortisol and the response to mild stress (both ACTH and cortisol) is generally

preserved. Repeated abstinence revealed elevated cortisol under low stress and dampened ACTH under the mild stress condition. Chapter four focuses on parvocellular neurons in the hypothalamic paraventricular nucleus (PVN), the apex of the HPA axis. In females, a unique relationship between the relative glutamate immunogold density in putative recurrent terminals and average daily ethanol intake was found. When compared to ethanol-naïve controls, no differences were found in frequency of glutamatergic events in males with approximately 7.5-mo of open-access conditions while male macaques with a history of open-access and repeated abstinence had a higher frequency than ethanol-naïve controls. A binge concentration of ethanol (20mM) decreased the frequency of excitatory events in males with a history of ethanol access. Interestingly, ethanol-naïve males with no history of repeated abstinence did not respond to this concentration of ethanol while the ethanol-naïve subjects with repeated abstinence did, suggesting that the stress of repeated forced abstinence (indicated by elevated cortisol even in the ethanol-naïve controls) may sensitize the parvocellular PVN neurons to an intoxicating concentration of ethanol, perhaps contributing to the comorbidity of stress and ethanol consumption. These data suggest that glutamate signaling in the PVN is related to ethanol self-administration, and altered by repeated periods of abstinence, although these relationships and alterations may differ between males and females.

CHAPTER 1: General Introduction

1.1 The Physiological Stress Response

Stress is a reality for all living creatures. Survival is dependent on the ability to generate and terminate an appropriate stress response, often in the face of simultaneous challenges to an individual's internal and external environment. A healthy stress response mobilizes energy stores for immediate use, increases heart rate and cardiovascular tone to bring oxygen to the muscles and sharpens concentration. However, a stress response that is activated too often or for too long has negative consequences including sleep disturbances, hypertension, impaired reproductive function and a compromised immune response.

Because stress is a universal experience, use and understanding of the word can carry many different interpretations. The diverse nature of stressors was pivotal in discovering the biologic basis of the body's response to it. In the early 1930's a physician named Hans Selye observed that across a wide range of physiological insults there was a consistent bodily response (Selye, 1936). Selye observed and characterized the temporal dynamics of this response, calling the early (6-48 hours) phase the "general alarm reaction" and later phase (after 48 hours) the "general adaptation syndrome", and in the case of prolonged stressors, a third phase "exhaustion". Around the same time, a physiologist named Walter B. Cannon was describing how the stability of the internal environment (or the milieu intérieur as Claude Bernard described it) was maintained by a series of feedback loops and physiological set-points, describing the internal state of the body as a "condition in which may vary, but which is relatively constant", which he

termed homeostasis (Cannon, 1932). This was followed shortly thereafter with the description and definition of allostasis, or the ability to maintain stability through change (Silver and Eyer, 1988). The homeostatic framework arguably applies to relatively few physiological processes such as maintaining blood pH, body temperature, blood oxygen tension, etc. Allostasis is applicable to a wider range of physiological processes and incorporates the demands of the individual's environment to the physiological response. In terms of stress, regulation of the primary glucocorticoid (cortisol) provides a good example. Glucocorticoids fluctuate in a diurnal pattern within a range that is relatively similar across healthy individuals, and in fact glucocorticoids themselves are a negative feedback signal for their own synthesis and secretion. In response to an acute environmental stimulus, for example the presence a predator, glucocorticoid concentrations will quickly rise and eventually return to their homeostatic levels when the threat has gone. If the threat remains for long periods of time, glucocorticoid concentration will remain high. This prolonged activation will negatively impact glucose metabolism, immune system response, sleep and cognitive function, known as allostatic load (McEwen, 2000). Stress is operationally defined as any physical or psychological stimulus that challenges homeostasis (Smith & Vale, 2006), and it is often measured by activation of the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1, page 3). In the years since Walter Cannon and Hans Selye, the study of stress has flourished. Understanding the fundamental mechanisms of central and peripheral responses to stress, as well as their interactions with physiological and psychological disease states is a major focus for the scientific community.

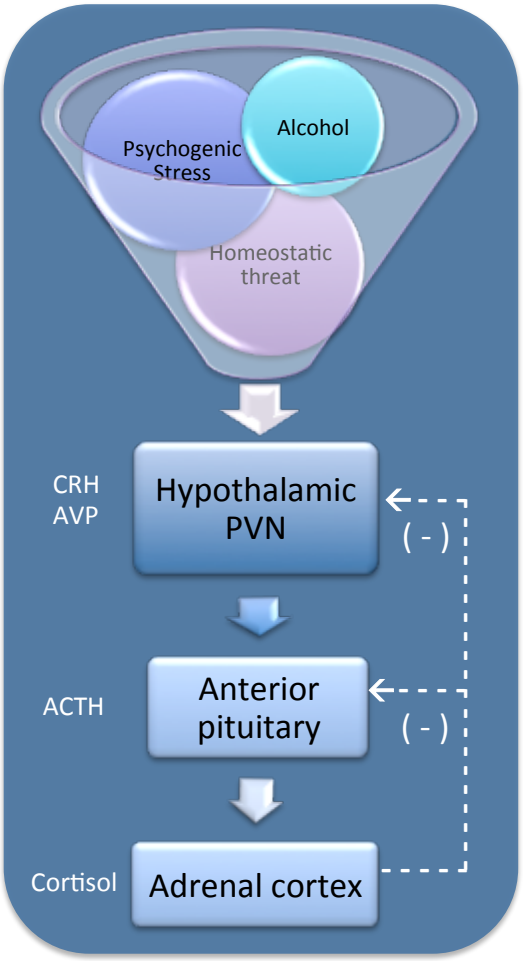

Figure 1 | The hypothalamic-pituitary-adrenal (HPA) axis. Information regarding a variety of stressors converge onto parvocellular neurons of the hypothalamic PVN. These neurons synthesize and release corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) into the median eminence where they reach the corticotropes in the anterior pituitary and stimulate adrenocorticotropic hormone (ACTH). ACTH is secreted into circulation where it acts on the adrenal cortex to stimulate steroidogenesis. Cortisol, the primary glucocorticoid in humans and nonhuman primates, acts as a negative feedback signal at the hypothalamus and pituitary to inhibit further activation of the HPA axis and restore homeostasis.

The fundamental components of the stress response include the hypothalamus, pituitary and adrenal, together referred to as the HPA axis (Figure 1, page 3). At the apex of the HPA axis is the hypothalamic paraventricular nucleus (PVN), a bilateral nucleus bordering the walls of the third ventricle with a diverse neuronal population. There are two major cell populations within the PVN, the magnocellular and parvocellular neurons. Magnocellular neurons secrete vasopressin (AVP) and oxytocin (OXY). Their axons project to the posterior pituitary where their contents are released into general circulation during lactation, parturition and osmotic challenge. Parvocellular neurons are generally smaller in size and, depending on the species, located in identifiable regions within the PVN. Parvocellular neurons are further divided into two populations, preautonomic and neuroendocrine parvocellular neurons. Preautonomic parvocellular neurons project to the brainstem and spinal cord where they participate in the regulation of sympathetic activity (Pyner and Coote, 2000; Stocker et al., 2004; Son et al., 2013), including activation of the sympathetic stress response (see Nunn et al., 2011 for review). Neuroendocrine parvocellular neurons project to the external zone of the median eminence and are the apex of the HPA axis. Information about the internal and external environments converge onto neuroendocrine parvocellular neurons from across the brain in a hierarchical manner (Herman et al., 2003) to initiate a cascade of stress hormones resulting in physiological and behavioral changes. The majority of synaptic contacts are GABAergic or glutamatergic. Parvocellular neurons express multiple neuropeptides (including corticotropin-releasing hormone (CRH), AVP, vasoactive intestinal peptide, cholecystokinin, angiotensin II, neurotensin and enkephalin) (Sawchenko et al., 1992). In addition, these peptides can be coexpressed with CRH under a variety of physiological

(restraint stress, neuroimmune challenge or tail shock, for example) and pharmacological (colchicine, an axonal transport inhibitor) challenges (for review, see Watts, 1996; Carrasco and Van de Kar, 2002). It is important to note that the expression of neuropeptides is more complex than described above. Neuropeptides and neurotransmitters are expressed and coexpressed across multiple populations of neurons, subject to differential regulation and they stimulate ACTH to varying degrees alone or in combination with other neuropeptides. This dissertation will focus on CRH and AVP, which serve a lead role in ACTH stimulation in response to a variety of stressors, including ethanol, which is detailed below.

When stimulated, neuroendocrine parvocellular neurons release CRH and AVP from their axons in the external zone of the median eminence. These neuropeptides travel via portal vasculature to the anterior pituitary where they stimulate their respective g-protein coupled receptors (CRH_{R1} and AVP_{1b}) on pituitary corticotroph cells. Activation of CRH_{R1} increases adenylyl cyclase, cAMP and protein kinase A via the G_s pathway (Aguilera & Liu, 2012) while activation of AVP_{1b} receptor increases phospholipase C via the G_q pathway (Koshimizu et al., 2012), ultimately stimulating the synthesis and secretion of adrenocorticotropin hormone (ACTH) into circulation (Figure 1, page 3). Radiolabeled binding studies approximate that 50-80% of corticotropes bind AVP while at least 80% bind CRH, demonstrating that there is a sizeable proportion of corticotropes that express both receptors (Childs and Unabia, 1990). CRH has the greatest stimulatory effect on pituitary corticotrophs (Rivier & Vale, 1983). AVP is capable of stimulating a mild response on its own, however when co-released with CRH the magnitude of ACTH secretion from the anterior pituitary is greatly potentiated (Rivier and Vale, 1983; Antoni,

1993). A recent report demonstrates that activation of both CRH_{R1} and AVP_{1b} receptors inhibit TREK-1 channels, a potassium channel responsible for setting the resting membrane potential of corticotropes, resulting in an additive and sustained depolarization compared to activation of either receptor alone (Lee et al., 2015). While the interaction between TREK-1 and each receptor likely accounts for the differences in ACTH-secretion between the two receptors, the individual signaling pathways also contribute. As described above, CRH_{R1} activates the Gs signaling pathway, which leads to activation of voltage-gated calcium channels (VGCCs) while AVP_{1b} activates the Gq signaling pathway, resulting in a release of intracellular calcium stores. The increase in calcium via these two pathways may have an additive effect on exocytosis leading to increased vesicle fusion and ACTH release. Evidence for CRH_{R1} and AVP_{1b} receptors forming homo- and hetero-dimers has been found (Young et al., 2007); however the functional implications of this require further investigation.

ACTH increases steroidogenesis in the adrenal cortex by activation of the melanocortin type 2 (MC_{R2}, G_s signaling pathway) receptor (Dores et al., 2016). An increase in ACTH causes the rapid synthesis of steroids from the adrenal gland, a transcription-independent event where cholesterol is mobilized from lipid stores and moved from the outer to inner mitochondrial membrane by phosphorylation of the steroidogenic acute regulatory protein (StAR). Cholesterol is then metabolized by P450scc into pregnenolone, the rate-limiting step in steroidogenesis. The remaining steps (see Figure 2, page 8) occur within the mitochondria and endoplasmic reticulum within the specific layers of the adrenal cortex: the zona glomerulosa, zona fasciculata and zona

reticularis. The principal adrenal steroids secreted from each layer (aldosterone, cortisol (or corticosterone in rodents) and DHEAS, respectively) are increased following an increase in circulating ACTH (Gallo-Payet, 2016).

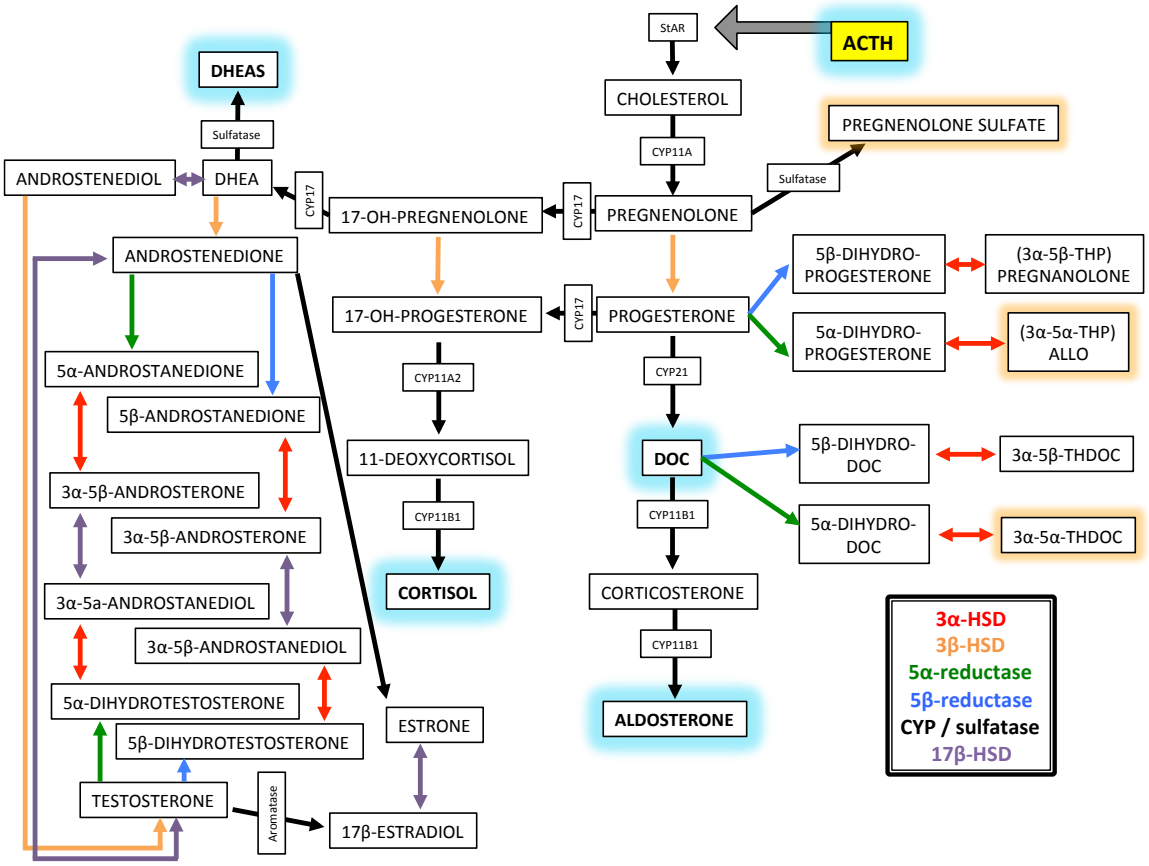

Figure 2 | Simplified steroidogenic pathway. ACTH stimulates phosphorylation of StAR, resulting in cholesterol mobilization into the inner mitochondrial membrane and metabolism into pregnenolone by P450scc. Synthesis of aldosterone, cortisol and DHEAS are produced primarily in the zona glomerulosa, zona fasciculata and zona reticularis, respectively. Each enzyme is represented with a colored arrow. The adrenal steroids presented in this dissertation are indicated with a blue halo and neuroactive metabolites with positive/negative allosteric modulatory roles at GABAa and/or NMDA receptors are indicated by orange halos. This figure was modified from: Porcu et al., 2009; Helms et al., 2012; Snelling et al., 2014; Gallo-Payet, 2016.

Just as appropriate activation of the stress response is crucial for survival, so too is appropriate termination. This is primarily accomplished by the ACTH-dependent increase in cortisol which acts as a negative feedback signal. Cortisol binds to two receptors, the mineralocorticoid (MR) and glucocorticoid (GR) receptors. Cortisol has a 6- to 10-fold lower affinity for GR than MR (Reul and de Kloet, 1985). During the diurnal peak and activation of the HPA axis, cortisol saturates the MRs and spills over to GRs, which inhibit further activation of the HPA axis on two timescales: rapid (seconds to minutes) and slow (hours to days) (de Kloet et al., 2008). The rapid effects are mediated by non-genomic retrograde signaling. Although not yet fully understood, this likely involves membrane bound GRs (mGR) and the release of retrograde signaling molecules, including endocannabinoids and nitric oxide (Di et al., 2003; Di et al., 2005). The slower actions of GRs follow dimerization of bound GRs translocating to the nucleus where they inhibit CRH and AVP gene transcription (Erkut et al., 1998; Watts, 2005). Prolonged or over-activation of the HPA axis has been implicated in mood disorders (McEwen, 2003), dysregulation of the immune system (Sternberg, 2001) and neuronal remodeling (McEwen, 2016).

1.2 Complex Regulation of Parvocellular Neurons

The majority of synaptic contacts onto parvocellular neurons of the PVN are GABAergic and glutamatergic (Van den Pol et al., 1990; Miklós and Kováks, 2002), although monoamines represent a sizable contribution as well (see Bains et al., 2015 for review). Understanding the unique contributions of specific neurotransmitters on

regulating the activity of parvocellular neurons has proven to be complex, involving neuromodulation by monoamines, intra-PVN signaling, neuronal crosstalk and regulation by adrenal steroids.

An acute microinjection of glutamate into the PVN leads to the expected activation of the HPA axis, and this effect is blocked by GR activation (Feldman & Weidenfeld, 1997). However, the HPA axis response to glutamate was blunted when either the ventral noradrenergic bundle or dorsal raphe nucleus were chemically lesioned (Feldman & Weidenfeld, 1997), suggesting that these two monoamine pathways serve a regulatory role in glutamatergic activation of the HPA axis. Consistent with these findings, Tasker and colleagues reported that approximately 50% of parvocellular neurons responded to bath application of norepinephrine (Daftary et al., 2000). Of those that responded, the majority (36%) had an increase in the frequency of excitatory postsynaptic potentials (EPSP), consistent with noradrenergic regulation of glutamate activity, while the remainder (14%) demonstrated a sustained hyperpolarization (Daftary et al., 2000). More recent evidence suggests that the role of noradrenergic regulation of parvocellular neurons is even more complex. The frequency of inhibitory postsynaptic potentials (IPSP) onto parvocellular neurons were altered by bath application of norepinephrine (Yang et al., 2008). Consistent with the subpopulations in excitatory responses reported previously (Daftary et al., 2000), the effect of noradrenaline on inhibitory activity was not consistent across all neurons; approximately equal proportions responding with either an increase or decrease in IPSP frequency. More interesting though, is that adrenalectomy increased the proportion of neurons that responded with a decrease in IPSP frequency to nearly 90% and low levels of glucocorticoid replacement

(subcutaneous cortisol pellet) prevented this response. These data provide evidence that monoamines, particularly noradrenaline, have a significant role in modulating both excitation and inhibition of parvocellular neurons in the PVN. Furthermore, this modulation is sensitive to adrenal steroid concentration.

In addition, hypotheses regarding the ability of PVN neurons to communicate within and between the different sub-populations of the PVN have been made. One possibility for intra-PVN signaling are axon collaterals, or recurrent terminals. Recurrent terminals form synapses onto other cells in the region of origin or onto itself, in the case of autapse synapses. Recurrent axon collaterals have been found in the retina of rodents and primates and may serve in amplifying the signal to noise ratio for optimized visual signal across varying degrees of light (Hannah et al., 2016). The formation of recurrent axon collaterals does not appear to be random, as in the striatum the D1-expressing medium spiny neurons (MSNs) preferentially form recurrent synapses with other D1-expressing MSNs while D2-expressing MSNs do not seem to have a preference (Traverna et al., 2008). Furthermore, the strength of the recurrent connections made by these two cell populations differed (Traverna et al., 2008). Recurrent axon collaterals have been reported from parvocellular neurons in the PVN (Liposits et al., 1985; Rafols et al., 1987; Ray and Choudhury, 1990). However, as illustrated Figure 3 (page 14), the specific populations involved have not been characterized, especially the population on the receiving end of the recurrent terminals. Consistent with these structural findings, parvocellular neurons are responsive to both CRH and AVP (Inenaga and Jamashita, 1986; Qiu et al., 2005). In vitro, parvocellular excitability increased following bath application of CRH via an interaction between CRH_{R1} and hyperpolarization-activated

nucleotide-gated (HCN) I_H current (Qiu et al., 2005). In addition to recurrent terminals, there is evidence of dendritic release of vasopressin as a means of cellular signaling between populations of neurons in the PVN (Son et al., 2013).

The role of glucocorticoids in negative feedback and inhibition of HPA axis activation is well documented. Glucocorticoids as well as other adrenal steroids and their neuroactive metabolites also regulate activity of parvocellular neurons. The genomic mechanism of glucocorticoid regulation is well documented in CRH and AVP gene transcription (Erkut et al., 1998). Tasker and colleagues have shown that glucocorticoids also modulate synaptic activity on a faster timescale (seconds to minutes) via interactions with membrane bound glucocorticoid receptors. These receptors were shown to be linked to G_s signaling pathways whose alpha and beta-gamma subunits were found to increase the release of retrograde signaling molecules that decrease the frequency of miniature EPSPs and increase the frequency of miniature IPSPs onto magnocellular neurons in the PVN and supraoptic nucleus (Di et al., 2003; Di et al., 2005; Di et al., 2009). In addition to glucocorticoids, neuroactive metabolites of adrenal steroids have been shown to influence the activity of parvocellular neurons. In cultured cells with human CRH gene transfection allopregnenolone (ALLO) and allotetrahydrodeoxycorticosterone (THDOC) dose-dependently decreased activity of a reporter gene (Budziszewska et al., 2010). Consistent with these findings, THDOC applied to preautonomic parvocellular PVN neurons in vitro inhibited action potential frequency (Womack et al., 2006). The effect of THDOC on activity of parvocellular PVN neurons is particularly important as regulation of the precursor, deoxycorticosterone (DOC), is influenced by activation of the HPA axis (Porcu et al., 2006) and the combination of HPA axis activation and alcohol self-

administration altered adrenal and hypothalamic mechanisms regulating DOC secretion in monkeys (Jimenez et al., 2017a) and humans (Porcu et al., 2008).

The magnitude and duration of the stress response is a highly coordinated and tightly regulated physiological response that is essential for survival. During prolonged challenge, a new physiological set point, or an allostatic state, can be established (McEwen, 1998; McEwen et al., 1993). Importantly, dysregulation of the stress response has been implicated in risk for developing psychiatric disorders (Naughton et al., 2014), including addiction (Blaine and Sinha, 2017). Additionally, long-term ethanol consumption results in dysregulation of the HPA axis (Adinoff et al., 1991) and contributes to risk for relapse (Breese et al., 2011).

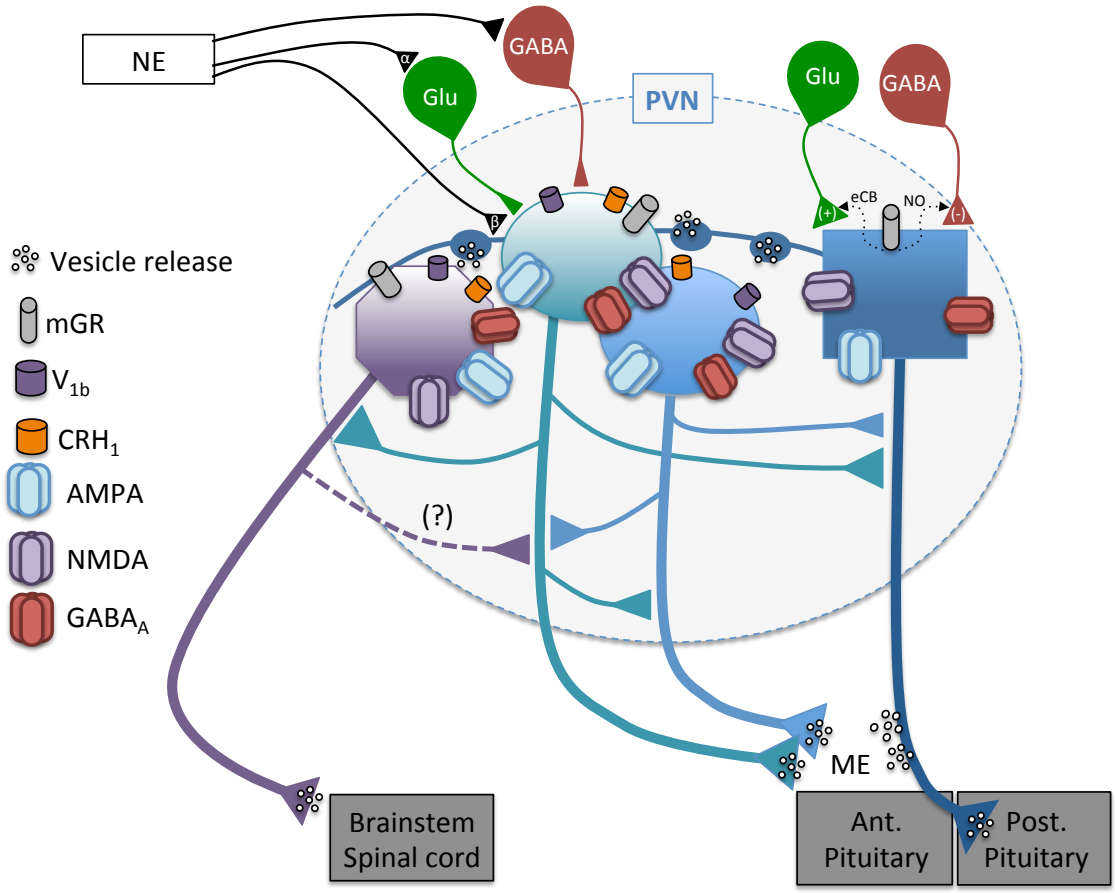

Figure 3 | Microcircuitry of the hypothalamic PVN. Parvocellular neuroendocrine cells are indicated with circular cell bodies, parvocellular preautonomic cells are indicated with octagon cell bodies and magnocellular with a square cell bodies. The three primary outputs of the PVN are the anterior pituitary, brainstem or spinal cord and posterior pituitary, respectively. Although GABA and glutamate represent the majority of synaptic contacts in the PVN, regulation of neuronal activity is complex. Noradrenergic (NE) inputs have direct and indirect effects on parvocellular neurons (Daftary et al., 2000; Yang et al., 2007). Activation of a G-protein coupled membrane glucocorticoid receptor (mGR) results in synthesis of nitric oxide (NO) and endocannabinoids (eCB) that act as retrograde signals to increase or decrease presynaptic GABA or glutamate release,

respectively (Di et al., 2003; Di et al., 2005; Di et al., 2009). Vasopressin, released from magnocellular dendrites, interacts with AVP_{1b} receptors on preautonomic parvocellular neurons to regulate sympathetic nerve activity (Son et al., 2013). Recurrent axon collaterals have been found in rodents (Liposits et al., 1985; Ray and Choudhury, 1990) and monkeys (Rafols et al., 1987) and participate in intra-PVN communication across the different neuronal populations. The specific populations for projecting and receiving recurrent axon collaterals remains unknown, as indicated by the dashed line.

1.3 Alcohol Use Disorders

Alcohol use disorders (AUDs) are a major public health concern. In 2014, 5.1% of the global burden of disease and injury was attributed to alcohol use (World Health Organization (WHO), 2014). The impact and cost on personal health combined with legal fees and lost productivity stemming from harmful alcohol use represents a huge economic burden, estimated at more then $235 billion in the United States (Whiteford et al., 2013). Importantly, AUDs are not distributed equally among all individuals who consume alcohol. According to analyses from the National Epidemiological Survey on Alcohol and Related Conditions (NESARC), a majority of adults in the United States have consumed alcohol while only a subset of these, approximately 30%, will develop an AUD in their lifetime. After controlling for sociodemographic variables, a strong association is found between AUD and mood and anxiety disorders (Hasin et al., 2007). Post-traumatic stress disorder, a psychiatric disorder characterized by an aberrant stress response, affects nearly twice as many military personnel than the general public (Gates et al., 2012) and is associated with comorbid AUD (Gilpin and Weiner, 2017). Consistent with this, enlisted military service members returning from combat were found to have increased risk for harmful heavy drinking and alcohol related problems (Jacobson et al., 2008).

While the need to identify risk factors for harmful drinking is apparent, the NESARC data also indicates that treatment strategies are stagnant, with similar rates of treatment seeking throughout recent decades (Hasin et al., 2007). A possible explanation for this could be the stigma of alcohol use disorders negatively impacting individuals from seeking treatment (Keyes et al., 2010). Understanding the neural consequences of

harmful alcohol use and the substrates of addiction can provide the potential for the development of novel treatment strategies. Furthermore, with a greater public understanding that addiction is a treatable disease, the stigma around it decreases (McGinty et al., 2015), which has significant implications for future treatment seeking individuals.

1.4 Benefits of the Nonhuman Primate Model of Ethanol Self-Administration in Studying the Relationship Between Stress and Ethanol

In order to dissect the interactions of stress and excessive drinking, animal models play a key role. Nonhuman primate (NHP) models of ethanol self-administration, as with other animal models, are able to reduce the impact of several key factors (e.g., nutritional status, housing environment, age at first intoxication, exposure to stressors, etc.) and isolate critical variables related to excessive alcohol consumption (Grant and Bennett, 2003). NHPs have a prolonged adolescence and young adulthood phase, close genetic similarities, similar expansion of the cerebral cortex, and in the case of old world monkeys, neuroendocrine similarities to humans. These similarities are especially advantageous when studying endocrine physiology, where there are notable differences between primates (human and NHP) and rodents. For example, the relative distribution of opioid receptors in the frontal cortex differs substantially between rodents and humans (Mansour et al., 1988), corticotropin releasing factor binding protein (CRF-BP) is found both centrally and peripherally in primates while only in the brain and pituitary of rodents

(Bowman et al., 2001; Seasholtz et al., 2002), and important morphological differences in the pituitary gland exist between rodents and humans (Kelberman et al., 2009). At the level of the adrenal gland, the primary glucocorticoid, providing negative feedback to the brain and pituitary to restore homeostasis following HPA axis activation, is corticosterone in rodents and cortisol in primates. The zona reticulata, where the primary adrenal androgen dehydroepiandrosterone (DHEA) is synthesized, is absent in rodents (Conley et al., 2004). And finally, rodents have low levels of 5β-reduced neuroactive steroids indicating species differences in neuroactive metabolites of adrenal steroids that may contribute to the subjective effects of alcohol (Helms et al., 2012c; Morrow et al., 2006; Porcu et al., 2009). In comparisons to humans, macaque monkeys have similar alcohol absorption and metabolism rates and can self-administer large quantities of alcohol over months and years (Green et al., 1999; Vivian et al., 2001; Baker et al., 2014). As discussed above, the adaptive response to chronic stress and chronic ethanol self-administration can result in a pathological state that involves the physiological integration of multiple organs impacting the primate HPA axis and brain. Thus, studies that address allostatic mechanisms involving longitudinal adaptations in brain circuitry are uniquely possible in NHPs (Grayson et al., 2014; Miranda-Dominguez et al., 2014). Importantly, like humans, NHPs show wide individual differences in their daily ethanol intake, with a large proportion voluntarily drinking amounts similar to human alcoholic chronic drinking levels (Mello and Mendelson, 1972; Baker et al., 2014; Baker et al., 2017).

1.5 Evidence of Centrally-Mediated Allostasis in the NHP Model

Across species, structural imaging techniques have revealed decreases in brain volume as a result of alcohol-related damage to both grey and white matter (Human: Kril and Halliday, 1999; Rodent: Pfefferbaum et al., 2008; Monkey: Kroenke et al., 2013; Kroenke et al., 2014). In the NHP model of ethanol self-administration, we have reported blunted diurnal rhythms (Helms et al., 2013). Consistent with these findings, oscillatory brain regions have been altered with long-term ethanol self-administration. Welsh and colleagues (2011) found hyperexcitability related to T-type calcium current activation in neurons within the inferior olive in cynomolgus monkeys following chronic ethanol self-administration. Interestingly, slices from monkeys in extended abstinence (35 days) had below-normal function, suggesting overcompensation, or an allostatic state in movement related circuits. The lateral geniculate nucleus of the thalamus has also been studied in this model (Breckinridge Carden et al., 2006). This area of the thalamus regulates sleep/wake cycles, arguably one of the most critical daily rhythms that is disrupted in subjects with AUDs (see Chakravorty et al., 2016 for review). Burst firing within the lateral geniculate was significantly dampened following long-term ethanol self-administration in cynomolgus males (Breckinridge Carden et al., 2006), providing a possible cellular basis for understanding, and treating, ethanol-induced sleep disruption. Although currently unexplored, long-term ethanol self-administration may disrupt the master oscillator, the suprachiasmatic nucleus (Chung et al., 2011; Nicolaides et al., 2015). Disruptions in central control of oscillatory rhythms and their downstream physiological (ie homeostatic) effects are, by definition, stressors.

Central mechanisms of allostasis following ethanol self-administration have been

demonstrated in brain regions that contribute to activation of the HPA axis. The amygdala and bed nucleus of the stria terminalis (BNST) are critical regions for integration of emotionally relevant, or psychogenic, stressors (Sawchenko et al., 1999; Jankord and Herman, 2008; Crestani et al., 2013). Ethanol interactions in the BNST have been found in humans (O'Daly et al., 2012) and rodents (Kash et al., 2009; Kash, 2012; Olive et al., 2002). Recently, Pleil and colleagues (2015) reported an increase in the frequency of spontaneous inhibitory postsynaptic currents (sIPSC) in the BNST of male rhesus macaques following 12-months of ethanol self-administration. Using cluster analysis, 40% of the variance in sIPSC frequency was accounted for by DOC (negative association), lifetime ethanol consumption and neuronal capacitance (positive associations). Because the BNST has a heterogeneous neuronal population, these data suggest that inhibitory signaling onto a particular neuronal phenotype (indicated by capacitance) is altered by chronic ethanol self-administration, albeit possibly through ethanol's effects on circulating adrenal steroids. Inhibitory signaling to the BNST, in part, comes from the central and medial nuclei of the amygdala (Lebow and Chan, 2016; Prewitt and Herman, 1998). The amygdala receives information from the prefrontal cortex and can indirectly influence PVN activity via projections to the peri-PVN or via the posterior BNST. In a cohort of male and female cynomolgus monkeys Floyd and colleagues (2004) found a decrease in GABA receptor potency within the basolateral amygdala following long-term ethanol self-administration. Although this relationship with anxiety and stress response is currently unexplored in the NHP ethanol self-administration model, there is evidence that the amygdala and BNST are a central hub for alcohol use disorders (Gilpin et al., 2015).

1.6 Dissertation Studies

There is strong evidence that the mammalian response to stress is an orchestration of endocrine, neural and behavioral processes that, in the face of chronic ethanol, can become maladaptive and propagate further escalations in ethanol intake (Becker, 2012; Blaine et al., 2015; Sinha et al., 2011). This underscores the phenomenon that the relationship between stress and ethanol is bidirectional. On the one hand, stress is an etiological factor in the development of alcohol use disorders (Keyes et al., 2012) while on the other hand, alterations (i.e., allostatic) or adaptations in the stress response occur due to continued ethanol consumption (Richardson et al., 2008; Sinha, 2012).

The studies presented here were designed to test the hypothesis that long-term ethanol self-administration dampens circulating stress hormones (ACTH and cortisol) under a low stress condition, while the response to a mild stressor is maintained. Furthermore, the hypothesis that adaptation occurs at the level of the hypothalamic paraventricular nucleus via an increase in presynaptic GABAergic signaling was tested. Chapter three demonstrates that ACTH measured under the low stress condition is decreased in males during open-access. This decrease was independent of group (alcohol or control), however low stress concentration of ACTH was negatively correlated with average daily intake for all animals with ethanol access. Conversely, ACTH and cortisol in response to mild stress is preserved with long-term ethanol self-administration. No differences were seen in DOC, DHEAS or aldosterone under low stress, but the mild stress condition showed sex- and species-specific changes between baseline and open-access that were independent of ethanol access. Rhesus males, but not females, had a decrease in DOC under mild stress during open access and cynomolgus males had a

significant increase in aldosterone. Similarly, suppression of ACTH and adrenocortical steroids showed sex- and species-specific differences between baseline and open-access. Repeated forced abstinence revealed disruptions to both low and mild stress response, with cortisol being responsive to forced abstinence – an effect that was initially present in ethanol-naïve controls but not during the third abstinence. Repeated forced abstinence also revealed significant disruptions in dexamethasone-suppression of DOC, much more than the other adrenocortical steroids. Chapter four focuses on the apex of the HPA axis, the parvocellular neurons of the hypothalamic PVN. These data are the first to show a unique ultrastructural relationship between putative recurrent glutamatergic parvocellular collaterals and ethanol consumption. This relationship was only found in female rhesus macaques, indicating important sex-, species- or duration-specific effects on glutamate signaling within the PVN. Whole-cell patch-clamp electrophysiology revealed that a history of ethanol self-administration or stress (as indicated by elevated cortisol) sensitizes parvocellular neurons to a physiologically relevant ethanol dose. These data support the development of an allostatic state, particularly with glutamatergic regulation of parvocellular neurons in the PVN and suggest that the different layers of the adrenal cortex are uniquely sensitive to stress and ethanol.

CHAPTER 2: Materials and Methods

2.1 Animals

The subjects of the experiments described here are detailed in Table 1 (page 25). All animals participated in an ethanol self-administration protocol as shown in the experimental timeline (Figure 4, page 24). The duration of the self-administration protocol varied by cohort and the experimental end-points are indicated by black arrows. Importantly, analysis of the low and mild stress response and dexamethasone suppression occurred at the same experimental phases, allowing for multi-cohort analyses, as indicated by the red arrows.

All animals were housed in quadrant cages (0.8 x 0.8 x 0.9 m) with constant temperature (20-22^{0}C), humidity (65%) and an 11-hour light cycle. Animals had visual, auditory and olfactory contact with other conspecifics. On weekdays, females were pair-housed for two hours between sessions. All animals were maintained on a positive caloric and fluid balance throughout the experiment and body weights were measured weekly. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Oregon National Primate Research Center IACUC and the NIH guidelines for the care and use of laboratory animal resources.

These studies include both rhesus (Macaca *mulatta*) and cynomolgus (Macaca *fascicularis*) macaques. The use of two species adds to the genetic diversity (Yan et al., 2011). Genetics have proven to be a critical component in immune function (Flynn et al., 2009), depression (Mullins and Lewis, 2017) and addiction (Reilly et al., 2017), including AUDs (Tawa et al., 2016). There is also genetic variation in stress-related

circuitry that may contribute to the relationship between stress and harmful ethanol drinking (Rogers et al., 2013).

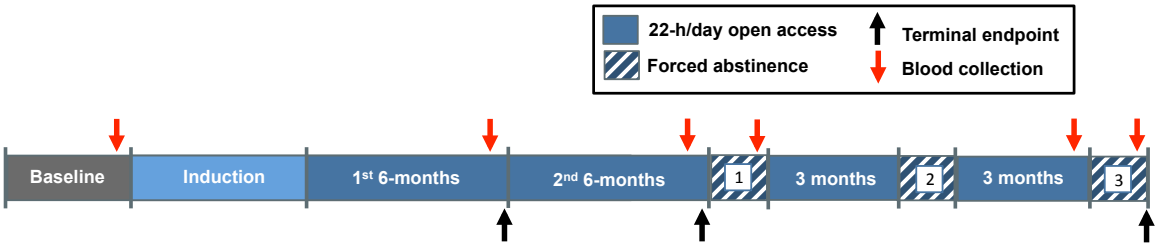

Figure 4 | Experimental timeline. All animals were trained to obtain all food and fluids from an operant panel in their housing cage and participate in blood collection procedures during the baseline phase. The induction procedure began with a water equivalent of 1.5 g/kg/day for approximately 30 days. A 4% w/v ethanol solution was introduced and progressed every 30 days from 0.5 g/kg/day, to 1.0 g/kg/day to 1.5g/kg/day ethanol. The terminal endpoint differed for each cohort where animals were sent to necropsy after approximately 6-months of open-access (cohorts 9 and 13: cynomolgus males), approximately 12-months of open-access (cohorts 5, 6a, 6b, 7a and 7b: rhesus males and females) or following three forced abstinence periods (cohort 10: rhesus males). Analysis of the endocrine response are shown with red arrows and were consistent across the in-life phase for all cohorts.

Cohort	Sex / Species	Number of animals (Ethanol / Controls)	Age (years) at onset	Duration of open-access
5	Male Rhesus	12 (12 / 0)	6.9 – 9.7 Middle-aged	12-mo
6a	Female Rhesus	9 (6 / 3)	4.2 – 4.6 Late adolescents	12-mo
6b	Female Rhesus	8 (5 / 3)	5.5 – 6.0 Young adults	12-mo
7a	Male Rhesus	12 (8 / 4)	4.0 – 4.6 Late adolescents	12-mo
7b	Male Rhesus	5 (5 / 0)	5.0 – 5.6 Young adults	12-mo
9	Male Cynomolgus	11 (8 / 3)	5.9 – 6.9 Middle-aged	6-mo
10	Male Rhesus	12 (8 / 4)	5.1 – 6.2 Young adults	18-mo + repeated abstinence
13	Male cynomolgus	12 (9 / 3)	6.8 – 7.1 Middle-aged	6-mo

Table 1 | Cohort composition and experimental details. Analyses of specific cohorts or combination of cohorts are specified in the text.

2.2 Water and Ethanol Access

2.2.1 Operant Panel

Each cage had an operant panel on one wall that dispensed food and fluids, as previously described (Vivian et al., 2001; Grant et al., 2008). Briefly, the panels had two spouts, each below a set of three stimulus lights (white, red, and green) that indicated an active session, food or fluid availability, respectively. A centrally located recessed dowel activated the fluid spouts and an infrared finger-poke activated the pellet dispenser. Each spout was connected via Nalgene tubing to a 1-L fluid reservoir set on a digital scale (Ohaus Navigator Balances N1B110, Ohaus Corporation, Pine Brook, NJ). Dowel pulls, finger pokes and fluid consumption were recorded in real time (inputs polled approximately every 500-ms) via a computerized system (Macintosh G4, Apple Computer, Cupertino, CA) using custom hardware and programing using National Instruments interface and Labview Software (Austin TX). Data were downloaded, husbandry tasks were performed, food and fluids were replenished and fresh fruit was provided each day by technicians during the 2-hr session break.

2.2.2 Schedule-Induced Polydipsia

Schedule-induced polydipsia (SIP) was used to induce ethanol self-administration in daily 16-h sessions, as has been previously described (Vivian et al., 2001; Grant et al., 2008). Briefly, a 1-g banana food pellet (Research Diets Incorporated, New Brunswick, NJ) was dispensed every 300-s (fixed time 300 seconds) until a water volume equivalent

of 1.5 g/kg of 4% (w/v) ethanol was consumed. Following water induction, 4% ethanol replaced water. In 30-day increments, each animal consumed increasing doses of 4% ethanol; 0.5 g/kg/day, 1.0 g/kg/day then 1.5 g/kg/day (1.5 g/kg/day induction remained in effect for 44-60 sessions). Following consumption of the programmed volume, water was immediately available and any remaining pellets were available on a fixed-ratio 1 (FR-1) schedule after two hours. Daily sessions started at 0800, 0900 or 1000 each day and was consistent within specific cohorts.

2.2.3 Open-Access Self-Administration

Daily 22-h sessions during which water and ethanol were concurrently available began following 1.5 g/kg/day induction. The operation of the panel did not change, except that fluid was available at any time on either spout following a dowel pull. Food pellets were available on a FR-1 schedule in at least three daily meals in 2-h intervals starting at the session onset. According to Figure 4 (page 24), animals were sent to necropsy during either an active drinking phase (following approximately 7.5- or 13-months open-access conditions) or in abstinence (28-35 days after the last ethanol session).

2.2.4 Ethanol-Naïve Control Subjects

Ethanol-naïve controls were also housed with their respective cohorts (see Table

1, page 25). All controls were housed with an operant panel and participated in experimental manipulations (endocrine profiles, blood collections, etc). The conditions SIP and self-administration were identical, with the exception that both spouts dispensed water.

A maltose-dextran solution (10% in water) was given to ethanol-naive subjects to calorically match the drinkers and controls. Each control subject was yoked to an ethanol drinker within the same cohort of similar body weight. Each week the average daily ethanol intake was used to calculate the average number of calories consumed from ethanol. This value was used to make an isocaloric maltose-dextran solution for the yoked ethanol-naïve animal. Maltose-dextran was given at the beginning of each daily session by attaching a bottle to the front of the cage (i.e. not available through the operant panel) beginning in 0.5 g/kg/day induction and continuing until the final open-access session prior to necropsy. During forced abstinence (cohort 10) the maltose-dextran was not available.

2.2.5 Forced Abstinence

AUDs are a chronically relapsing condition. To understand the physiology of the stress response under conditions relevant to the affected population, an extended self-administration protocol was used. Cohort 10 had approximately 14-months of open-access before three repeated forced abstinence conditions for 28 days, with intervening ethanol open-access conditions (see Figure 4, page 24). During this time the ethanol

reservoir was replaced with water while all other conditions were identical. Ethanol was available between the forced abstinence phases for three months. This cohort was sent to necropsy in abstinence (28-35 days after their last ethanol session).

2.3 Blood Collection and Mild Stress-Induced Activation of the HPA Axis

After acclimating to the laboratory and staff, monkeys were trained to comply with awake venipuncture. Training was performed up to twice daily and advanced for each animal individually as they readily performed each step with minimal signs of observable distress. Fresh fruit, vegetables or nuts were offered when the animal would sit at the front of the cage and present its leg through an opening (10 x 10 cm). Once the animal would reliably respond to this request, a dental pick was used to simulate a needle stick. Finally, a 3-cc blood sample was collected through a 22-gauge needle into an EDTA Vacutainer tube (BD, Becton Dickinson) from the femoral vein.

The animals were next trained to sit in a primate chair for sample collection. Briefly, each monkey wore a size-appropriate nylon collar that was designed to attach to a pole. The pole was inserted into the cage and clipped onto the collar and this was used to guide the animal out of the housing cage and into the chair. Once seated, the collar was secured into place and the pole removed. A chest plate was used to restrict the animals' ability to reach towards the femoral region. This condition was a mild stressor as indicated by elevated cortisol, but behavioral signs of distress (resistance to being moved

and secured into the chair, vocalizations etc) diminish rapidly (Ruys et al., 2004; Jimenez et al., 2015; Jimenez et al., 2017b).

These two locations (housing-cage or chair) served as distinct stressor conditions (low and mild stress, respectively) in which hormones associated with HPA axis activation can be measured and compared within animal and between groups prior to and following ethanol self-administration. The concentration of cortisol reported under these conditions is slightly elevated when compared to blood samples collected from indwelling catheters in male and female rhesus macaques (Williams et al., 2003; Pascoe et al., 2008), indicating a low level of HPA axis activation during the blood collection procedure. All training occurred during the 'baseline' phase of the experimental timeline (Figure 4, page 24). Blood collection from the home-cage occurred at least once per week for a minimum of two months prior to the samples assayed for the current project. Hormonal response to these two conditions was measured after the animals had been trained to obtain all food and fluids from the operant panel, but prior to the start of the induction procedure (baseline) and again following ethanol exposure (approximately 6- and/or 12-months) and during repeated abstinence and open-access conditions (red arrows, Figure 4, page 24). All blood samples under low stress condition were collected between 0800-0900, within the first two hours of the onset of the light cycle. For the mild-stress condition, animals were seated in a primate chair between approximately 0800 or 1130 and a blood sample was collected within 35-60 minutes. Figure 5 (page 31) demonstrates the ACTH response to low and mild stress conditions, as well as the response to a moderately high dose of naloxone, a μ-opioid receptor antagonist that activates the HPA axis. These data, collected from a group of ethanol-naïve adult

cynomolgus males, shows that both hormones are significantly elevated in the mild stress condition compared to the low stress. Additionally, this shows that the mild stress condition is not at a ceiling, as disinhibition of the HPA axis results in significantly higher ACTH and cortisol.

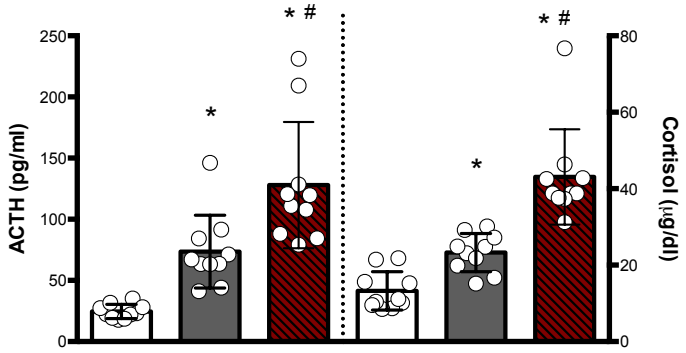

Figure 5 | ACTH (left) and cortisol (right) response to the low stress condition (white bars), the mild stress condition (grey bars) and 375 µg/kg naloxone (red hatched bars). Mean ± SD. * $p < 0.05$ compared to low stress, # $p < 0.05$ compared to mild stress.

2.3.1 Plasma Assays

Samples for hormone assay were stored on ice for up to 60 minutes until centrifuged (3000 rotations per minute) for 15 minutes at 4^{o}C (Beckman Coulter, Model Allegra 21R). Plasma was aliquoted and stored at -80^{o}C until assayed for cortisol, adrenocorticotropin hormone (ACTH), aldosterone and sulfated dehydroepiandrosterone (DHEAS). Assays were conducted by the Oregon National Primate Research Center

Endocrine Technology Services Laboratory. A Roche Cobas e411 automatic clinical platform was used to assay ACTH (1-2,000 pg/ml sensitivity, 0.8% inter-assay variation), cortisol (0.036-63.4 μg/dl sensitivity, 1.1% inter-assay variation) and DHEAS (sensitivity, 0.001–10 μg/ml; inter-assay variation, 4.4 %). A commercially available enzyme-linked immunosorbent (ELISA) assay was used for aldosterone (15-1,000 pg/ml sensitivity and 7.8% inter-assay variation). Deoxycorticosterone (DOC) was assayed using radioimmunoassay (RIA) as previously described (Porcu et al., 2006). The sensitivity of the assay is 10 pg/ml and the inter-assay variation was less than 5%.

Blood (20 μl) was collected from the saphenous vein approximately every fifth day for analysis of blood ethanol concentration (BEC) using gas head-space chromatography (Hewlett-Packard 5890 Series II, Avondale, PA, equipped with a headspace auto-sampler, flame ionization detector, and a Hewlett Packard 3392A integrator). Blood was added to 500-μl of sterile water and frozen in a sealed glass vial until assayed. Duplicate standards of known concentration ranging from 25 to 400 mg/dl were used to generate a standard curve. Standards and samples were run on the same day.

2.4 Necropsy and Tissue Collection

At the end of the experimental timeline (black arrows, Figure 4, page 24), each animal was sedated with 10 mg/kg ketamine, intubated, and maintained with isoflurane and pentobarbital. A craniotomy was performed followed by perfusion with chilled artificial cerebral spinal fluid (aCSF). Once the perfusion was complete (within 4 minutes) the brain was immediately removed, placed into a brain block (TedPella, Inc,

Redding, CA) and dissected into 4-mm coronal sections, as described previously (Daunais et al., 2010). The anterior commissure and hippocampus served as anterior/posterior landmarks and the fornix and third ventricle served as lateral/medial landmarks that helped identify the location of the PVN. The PVN was removed from both hemispheres of a single 4-mm block. The approximate area dissected is shown as the boxed area in Figure 6, page 34. The left hemisphere was placed into electron microscopy (EM) fixative (2.5% gluteraldehyde, 0.1% picric acid and 0.5% paraformaldehyde in 0.1M phosphate buffer) for 24-hours then 4% paraformaldehyde for 24 hours before being stored in 0.1M phosphate buffer until sectioned. The right hemisphere was handled differently between males and females. For the females, the right hemisphere was preserved for light microscopy by fixing in 4% paraformaldehyde solution for 48 hours then 30% sucrose until sectioned (see Light microscopy processing below). For males, the right hemisphere was placed into ice-cold oxygenated aCSF and quickly sectioned for electrophysiology (see Tissue Preparation for Electrophysiology below).

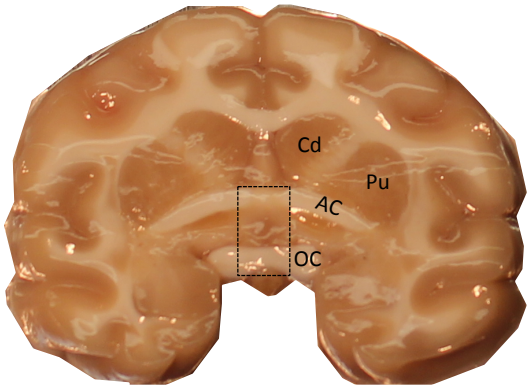

Figure 6 | Representative brain slice. Rostral face of a 4mm coronal brain block containing the caudate (Cd), putamen (Pd), anterior commissure (AC) and optic chiasm (OC). The hypothalamic paraventricular nucleus is outlined within this block and was dissected as shown by the dotted line.

2.4.1 Electron Microscopy Processing

The EM-fixed block of tissue was sectioned at 60-µm using a vibratome (Leica Microsystems, Buffalo Grove, IL). Five sections containing the PVN were selected and processed for EM using pre-embed diaminobenzidine (DAB) immunohistochemical labeling for localization of AVP (Millipore, 1:4000, rabbit) or CRH (Santa Cruz, 1:500, rabbit) using a modified microwave procedure. Tissue was incubated in the microwave (Pelco BioWave, TedPella, Inc., Redding, CA) for 5 min, 550 W, at 35°C with the vacuum off (all the remaining steps occurred at this temperature) with the vacuum cycling down to 20 Hg, then back to atmosphere repeatedly in 10 mM sodium citrate, pH 6.0 (antigen retrieval), rinsed in 0.1 M phosphate buffer saline (PBS) for 1 min at 150 W

with the vacuum off, exposed to 3% hydrogen peroxide at 150 W for 1 min with the vacuum off, rinsed in PBS at 150 W for 2 x 1 min with the vacuum off, exposed to 0.5% Triton X-100 for 5 min, 550 W with the vacuum cycling, then exposed to one of the primary antibodies for 48-h at 4-5^{o}C. The tissue was then rinsed in PBS 1 min x 2 each at 150 W with the vacuum off, then exposed to the secondary antibody (bioatinylated goat anti-rabbit, 1:100; Vector Laboratories, Burlingame, CA) for 16 min at 200 W for 2 cycles of the following: 4 min on, 3 min off, 4 min on, 5 min off, all on a continuous vacuum (20 Hg). The tissue was then rinsed in PBS for 1 min, followed by a rinse in working imidazole buffer (0.01M imidazole, 0.016M Na Acetate aqueous) at 150 W with the vacuum off and then exposed to ABC (Vector Elite Kit, 1% solution A and B in working imidazole buffer) for 16 min at 200 W under constant vacuum using the following cycle: 4 min on, 3 min off, 4 min on, 5 min off. The tissue was then rinsed in working imidazole buffer twice at 1 min each, at 150 W with the vacuum off and then exposed to DAB (0.5 mg/ml + 1.5% hydrogen peroxide) for 10 min under constant vacuum at 200-W. The tissue was embedded in Epon-Spurs at 60^{o}C for approximately 16-h. The region of interest (2-mm dorsal-ventral between the ventricular edge and the fornix) was removed and attached to a resin block for thin sectioning. Sections were cut (60-nm) on an ultra-microtome (EM UC7; Leica Microsystems, Buffalo Grove, IL) using a diamond knife (Diatome, Hartford, CT) and collected onto 100-mesh formvar covered grids (Electron Microscopy Sciences, Hatfield, PA). Post-embed immunogold electron microscopy was performed using glutamate (non-affinity-purified, rabbit polyclonal; G-6642 purified glutamate conjugated to KLH as the immunogen, Sigma-Aldrich, St. Louis, MO) and GABA antibodies (A2052 antibody is isolated from antiserum by

immunospecific methods of purification, Sigma-Aldrich, St. Louis, MO) on adjacent thin sections that had been previously DAB stained for CRH or AVP. The primary glutamate antibody, as described previously (Phend et al., 1992), was diluted 1:250 in TBST 7.6 and aspartate (1 mM) was added to the glutamate antibody mixture 24-h before incubation with the thin-sectioned tissue to prevent any cross- reactivity with aspartate within the tissue. The GABA antibody was diluted in TBST pH 8.2 immediately prior to use. The secondary antibody for both GABA and glutamate was goat anti- rabbit IgG conjugated with 12-nm gold particles (diluted 1:50 in TBST, pH 8.2; Jackson ImmunoResearch, West Grove, PA). Meshul et al. (1994) previously demonstrated the specificity of the glutamate and GABA antibodies using a competition assay in which incubation of the antibody with 3 mM of glutamate or GABA, respectively, resulted in no immunogold labeling.

2.4.2 Electron Microscopy Analysis

Photomicrographs (50-60/animal) were taken on a JEOL 1400 transmission electron microscope of presynaptic terminals contacting DAB-labeled postsynaptic structure at a final magnification of 8,000x using a digital camera (Advanced Microscopy Techniques, Woburn, MA). For quantification of immunogold labeling, the number of gold particles within a terminal are counted. Gold particles located within the mitochondria (i.e. the metabolic pool) were subtracted from the total. The density of gold particles per square micrometer of nerve terminal area was measured using Image-Pro version 6.3 software (Media Cybernetics, Inc., Rockville, MD, USA). A mean density

was calculated for each animal from a minimum of 12 terminals and the mean density for each group was calculated. Non-specific binding has been reported previously (approximately 10 μm^2; Meshul et al., 1994), a minimum density threshold of 30 μm^2 was used in these analyses to capture immuno-labeled terminals. Only synaptic terminals contacting an immuno-labeled (CRH- or AVP-positive) postsynaptic structure were included in these analyses.

2.4.3 Light Microscopy Processing

Both primary antibodies were produced in rabbit; in order to perform double-label immunohistochemistry the CRH antibody was conjugated to biotin using the following protocol: 500-µl of CRH anti-rabbit primary antibody was concentrated using a VivaSpin500 centrifuge tube (Satorius, Bohemia, New York) for 10 minutes at 15,000g. The concentration of antibody was measured using NanoDrop spectrophotometer and diluted in phosphate buffer (PB) to ~1 mg/ml. A Biotin-XX kit (Molecular Probes, Grand Island, NY) was used to covalently link the concentrated CRH antibody to biotin. Once sectioned, six 30-µm serial free-floating sections (180-µm distance apart) from each animal were incubated in the microwave (Pelco BioWave, TedPella, Inc, Redding, CA) for 5 min, 550 W, at 35°C with the vacuum off (all the remaining steps occurred at this temperature) in 10 mM sodium citrate, pH 6.0 (antigen retrieval), rinsed in 0.1 M PB for 2 x 1 min at 150 W with the vacuum off, exposed to 3% hydrogen peroxide at 150 W for 1 min with the vacuum on, rinsed in PB at 150 W for 2 x 1 min with the vacuum off, exposed to 0.5% Triton X- 100 for 5 min, 550 W with the vacuum on, washed in PB for 2

x 1 min at 200 W with the vacuum off, then exposed to the AVP primary antibody (1:4,000), as described above, for 48 hours at 4-5^{o}C. All remaining steps were done on the bench-top. Tissue was rinsed in PB (4 x 5- min, 3 x 2-min, 2 x 20-s rinses). Secondary (AlexaFluor 488 donkey anti-rabbit, 1:1,000) was applied at room temperature for one hour on a rotating plate while protected from light. The tissue was rinsed in PB, then incubated in biotinylated-CRH primary antibody (1:500) for 72-h in the refrigerator on a rotating plate while protected from light. The tissue was rinsed in PB prior to use of a Tyramide Signal Amplification kit (Molecular Probes, T20936) using a detection using a streptavidin-linked AlexaFluor 647 secondary antibody. All sections were counterstained using Hoeschst (Life Technologies, Grand Island, NY, 1:15,000 in PB) for one minute before the final set of rinses. The tissue sections were mounted onto slides and cover-slipped with 200-µl ProLong Gold anti-fade mounting reagent (Life Technologies, Grand Island, NY). Additional tissue sections were processed in the absence of primary antibody to confirm secondary antibody specificity.

2.4.4 Light Microscopy Analysis

Images were acquired using a Marianas imaging workstation (Intelligent Imaging Innovations, Denver, CO), using Slidebook 5.5. Excitation light was provided by a DG-4 fluorescence illumination system (Visitron Systems GmbH) and filtered through a Sedat Quad set (Chroma Technology) and detected by a CoolSNAP HQ CCD camera (Photometrics). A 10x NA 0.45 Plan-Apochromat objective was used to acquire and construct a large montage of 3 x 5 fields of view. All image acquisition parameters for

each channel, including the exposure times and the histogram domain selected for display and tiff export were kept constant throughout the experiment: 500ms in FITC channel captured AVP signal, 20ms exposure in DAPI (4',6-diamidino-2-phenylindole) captured the nucleic marker, 500ms in the Cy3 channel captured CRH signal and 900ms in the Cy5 channel to capture non-specific background autoflorescence that was subtracted from the AVP and CRH images during analysis. Images were analyzed in ImageJ (NIH) using the following procedure: Images were imported (as .tiffs) and displayed as individual channels. For each section, the region of interest was defined as the area of densely packed fluorescently labeled cells, outlined and saved to the region of interest (ROI) manager to apply the same ROI to each channel. The mean intensity for each channel was measured in six serial sections and used to calculate a mean intensity for each animal which was then used to calculate a group mean.

2.4.5 Tissue Preparation for Electrophysiology

The isolated tissue section was blocked to ~10mm wide (approximately 5mm on either side of the third ventricle) x ~8mm high and 4mm thick to contain the hypothalamic paraventricular nucleus. The tissue block was placed in a 15ml conical tube of ice-cold oxygenated perfusion solution and transported on ice for slicing. Tissue was then transferred to ice-cold cutting solution containing in mM: sucrose, 194; NaCl, 30; KCl, 4.5; $MgCl_2$, 1; $NaHCO_3$, 26; NaH_2PO_4, 1.2; and glucose, 10, aerated with a mixture of 95% O_2/5% CO_2 gas. Coronal slices at a thickness of 250mm were obtained using a ceramic blade (Camden Instruments Limited, Lafayette, IN) attached to a VTI200S

vibrotome (Leica, Buffalo Grove, IL). Slices were equilibrated for 1 h in aCSF containing in mM: NaCl, 124; KCl, 4.5; $MgCl_2$, 1; $NaHCO_3$, 26; NaH_2PO_4, 1.2; glucose, 10; and $CaCl_2$, 2; continuously aerated with a mixture of 95% O_2/5% CO_2 gas at a temperature of 33^0C. Slices were transferred to room temperature until experimental use. Cells remained viable for up to 10 hours after cutting.

2.4.6 Electrophysiology

PVN slices were transferred to a recording chamber on the stage of an upright microscope (SliceScope, Scientifica, Clarksburg, NJ) and stabilized with an overlying platinum ring. The tissue was continuously perfused with aCSF maintained at 28–32^0C (Automatic Temperature Controller, Warner Instruments, Hamden, CT). A 40x water immersion objective and infrared optics were used to identify neurons and guide the placement of the recording pipette. Recording pipettes were pulled from borosilicate glass capillaries (1.5mm outer diameter, 1.12mm inner diameter, World Precision Instruments, Sarasota, FL) and filled with potassium gluconate based internal solution (in mM: K-gluconate, 126; KCl, 4; HEPES, 10; Mg-ATP, 4; Na-GTP, 0.3; Phosphocreatine, 10; and 1mg/kg Dextran Cascade Blue (Molecular Probes, Grand Island, NY). When filled with internal solution, the patch pipettes had a resistance or 3-5 MΩ. Recordings were made using a MultiClamp 700B amplifier (Molecular Devices, Foster City, CA). Whole-cell membrane currents were digitized at 1 kHz using Clampex v10.5 and DigiData 1440A (Molecular Devices, Foster City, CA).

Two salient electrophysiological features were used to distinguish magnocellular

from parvocellular neurons. Using a current clamp protocol, neurons were clamped at 0pA and a 500ms current step was applied each second, beginning near -100mV and becoming progressively more depolarizing with each step. Putative magnocellular neurons display a transient outward rectification following the hyperpolarizing current steps resulting in a delay to action potential onset while putative parvocellular neurons generate non-bursting low threshold spikes (see Figure 7, page 42; Hoffman and Tasker, 1991; Tasker and Dudek, 1991; Luther et al., 2000; Stern, 2001). Only the putative parvocellular neurons (those displaying low threshold spikes and the absence of the transient outward rectification) were included in spontaneous EPSC analysis.

Putative parvocellular neurons were voltage clamped at -55mV (10 min duration) in the presence of picrotoxin (100 μM in aCSF; Sigma-Aldrich, St. Louis, MO) to isolate spontaneous EPSCs. A test pulse (-65 mV, 10ms duration) was automatically applied each minute to monitor the series resistance. Cells were excluded from analysis if no action potentials were generated in response to a current clamp protocol or if the series resistance increased to over 30 MΩ during the course of the experiment. A subset of putative parvocellular neurons were included in an acute ethanol challenge where 20mM ethanol was added to the picrotoxin bath solution following the 10-min “basal” recording.

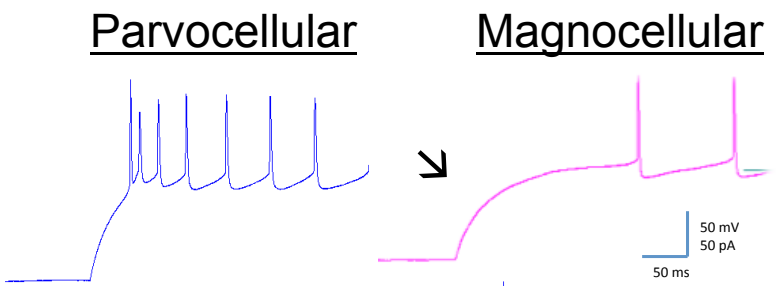

Figure 7 | Electrophysiological identification of PVN neurons. After a hyperpolarizing current step, putative parvocellular (left) and magnocellular (right) neurons elicit characteristic responses. Putative parvocellular neurons display a low threshold spike (LTS) dependent on T-type currents and putative magnocellular neurons display a transient outward rectification driven by A-type currents (arrow) that result in a delay to action potential onset.

2.4.7 Electrophysiology Analysis

Because of their principal role in activation of the HPA axis, spontaneous EPSCs were recorded only from parvocellular neurons, as previously mentioned. The frequency, amplitude and event kinetics of spontaneous EPSCs were analyzed over three minutes using MiniAnalysis (Synaptosoft v6.0.7, Decatur, GA), a semi-automated threshold-based software, and were visually confirmed. Because the average daily intake of ethanol varied by subject, the average frequency, amplitude and event kinetics were calculated per animal from a minimum of two parvocellular neurons.

CHAPTER 3: The Relationship Between Ethanol Self-Administration and HPA Axis During Low and Mild Stress Conditions in Macaque Monkeys

3.1 Introduction

In humans, the state of the HPA axis following long-term ethanol consumption depends on whether the individual is intoxicated, sober but an active drinker, or if they are in acute or protracted withdrawal. In non-alcoholic men, a moderate (0.75 g/kg) dose of ethanol blunted the ACTH response to ovine-CRH (oCRH; Waltman et al., 1993). Actively drinking alcoholics did not have significantly different basal ACTH or cortisol when compared to age-matched controls, however their ACTH response to insulin-induced hypoglycemia was blunted relative to age-matched non-alcoholic controls despite no differences in blood glucose concentration (Berman et al., 1990). Similar results were found in 4-6 week abstinent alcohol-dependent subjects where the cortisol response to exogenous ACTH and o-CRH challenge was dampened relative to age-matched controls (Adinoff et al., 2005a; Adinoff et al., 2005b). Mid-day, but not morning, ACTH was higher in actively drinking alcoholics than non-alcoholic age-matched controls and again, a dampened ACTH, but not cortisol, response to oCRH was found (Wand & Dobs, 1991). In treatment-seeking alcoholics with three weeks of abstinence, no differences were found in basal ACTH or cortisol concentration or oCRH-stimulated ACTH and cortisol, suggesting that the HPA axis recovers following a

relatively short period of abstinence (Adinoff et al., 1991, but see Adinoff et al., 2005a and 2005b). Similarly, incarcerated alcoholics with at least 60 days of abstinence did not differ from age-matched controls with respect to basal cortisol concentration (Mendelson et al., 1971).

These studies provide evidence that HPA axis function is disrupted with chronic alcohol consumption and withdrawal, but the conclusions are complicated by several factors. First, the existence of behavioral and environmental factors such as a family history of alcohol use disorders (Capone and Wood, 2008), the age when individuals started drinking alcohol (Prescott and Kendler, 1999; Ehlers et al., 2006) and socioeconomic status (Keyes & Hasin, 2008) all have strong associations with the development of harmful patterns of ethanol consumption. Comorbid psychiatric disorders are also associated with aberrant HPA axis response (Depression: Lopez-Duran et al., 2009; Belvederi et al., 2014; Anxiety: Faravelli et al., 2012) and contribute to risk for harmful drinking (Boden and Fergusson, 2011; Brady and Lydiard, 1993). And finally, self-selection in the human population is a confound that cannot be ignored. The consequences of long-term heavy ethanol consumption likely interact with the physiology that contributed to that behavioral pattern. Similarly, obtaining samples from treatment seeking versus subjects in forced abstinence (incarceration) likely impacts the state of the individual and their physiological response to stress and ethanol.

Animal models have shown that acute ethanol stimulates the HPA axis in rodents. In the two-hours following administration there is a rapid increase in ACTH and corticosterone (Rivier and Lee, 1996; Ogilvie and Rivier, 1997; Lee and Rivier, 1997). It has been demonstrated that corticosterone elevation mirrors the timecourse of blood

ethanol concentration (Ellis, 1966). The dose of ethanol used in these studies (> 3.0 g/kg) is quite high, resulting in blood ethanol concentrations (BECs) well over 100 mg/dl (Ogilvie et al., 1997). In humans, an acute dose of ethanol only resulted in an increase in circulating stress hormones when the BEC exceeded 100 mg/dl (ACTH: Waltman et al., 1993; cortisol: Mendelson et al., 1971; Jenkins and Connolly, 1968), particularly if accompanied by gastrointestinal distress. However, these studies were not done using ethanol-naïve subjects and tolerance in the HPA axis response to ethanol has been shown in rodents (Rivier et al., 1997). As mentioned previously, rodents and primates have significant differences in adrenal physiology that may lead to unique responses to ethanol. In cynomolgus macaques, an acute nasogastric dose of 1.0 and 1.5 g/kg ethanol (20 % w/v resulting in peak BEC of 88.8 ± 10.1 mg/dl and 146.9 ±15.7 mg/dl, respectively) did not increase ACTH or cortisol (unpublished observation). In fact, ACTH at 90- and 120-minutes post-ethanol were significantly lower than 15-minutes after ethanol administration, suggesting suppression of ACTH. These results are in agreement with a suppression of circulating deoxycorticosterone (DOC) from the adrenal zona glomerulosa following acute ethanol challenge (Jimenez et al., 2017a).

In terms of animal models, the monkey model of ethanol self-administration is uniquely situated to study the interaction between stress physiology and long-term voluntary ethanol consumption. Using a within-subject analysis, two hypotheses were tested. The first is that long-term ethanol self-administration selectively dampens stress hormones under low, but not mild, stress conditions. The second hypothesis is that repeated forced abstinence further disrupts the HPA axis response, resulting in elevated stress hormones under low stress conditions and a dampened response to mild stress.

3.2 Statistical Analysis

All statistical analyses were conducted as mixed multi-level analyses with repeated measures, using monkey as the subject (random) variable. Fixed effects were experimental phase (two levels: baseline and 6- or 12-months for cynomolgus and rhesus, respectively), and group (five levels: controls and low, binge, heavy or very heavy drinkers). Drinking categories are described on www.MATRR.com and in Baker et al., 2014. Briefly, very heavy drinkers had >3.0 g/kg/day average ethanol self-administration with >10% of sessions exceeding 4.0 g/kg. Heavy drinkers consumed >3.0g/kg/day at least 20% of days. Binge drinkers had at least 55% of days >2.0 g/kg and at least one BEC exceeding 80 mg/dl. Light drinkers included individuals not captured in the other categories. When evaluating the effect of repeated forced abstinence experimental phase had six levels (baseline, 6-month, 12-month, abstinence 1, 18-month and abstinence 3; see the experimental timeline in Figure 4, page 24). All factors were checked for normal distribution using Shapiro-Wilks normality test and log-transformed when necessary. The dependent variables were ACTH and cortisol concentration under low and mild stress conditions (assayed from a single timepoint under each stress condition) and percent suppression by dexamethasone (calculated as a percentage of pre-dexamethasone hormone concentration). A reverse stepwise model selection was performed where all interaction terms were initially included then dropped from the model if non-significant. Significant results are reported based on Tukey post hoc analysis. Statistical analyses were conducted using the NLME (Pinheiro et al., 2016) and multcomp (Hothorn et al., 2008) packages in R Statistical Computing software (version 3.1.2: R Core Team, 2015). Figures and correlations were generated using Prism (GraphPad, version 6). Data are

presented as mean ± standard deviation unless otherwise stated. Alpha < 0.05 was considered significant.

3.3 Results

3.3.1 Ethanol Self-Administration and Blood Ethanol Concentration During Open-Access

In this population of macaques, average daily intake showed wide individual differences (Figure 8, page 49). Rhesus macaques included in this analysis had approximately 12-months of open-access conditions (see Table 1, page 25). The number of daily open-access sessions included in the calculation of average daily intake excludes those where sessions were abnormal (for example, the animal had been sedated for a procedure, a technical error or days of endocrine profiles). The number of sessions included in the average daily intake are: cohort 5: 355 ± 1 day, cohort 6a: 330 ± 1 days, cohort 6b: 341 ± 1 days, cohort 7a: 350 ± 1 days, cohort 7b: 341 ± 0.4 days and cohort 10: 350 ± 2 days, respectively. The male cynomolgus monkeys had approximately 6-months of open-access conditions, each cohort (9 and 13) had on average 188 ± 1 days of open-access in the average daily intake.

Blood samples collected 7-hours into the session were collected approximately every 5 days and the averages included 57 – 65 samples/animal for rhesus macaques and 34 – 43 samples/animal for the cynomolgus macaques (Figure 8, page 49). Intake at the time of sample collection is highly correlated with blood ethanol concentration across cohorts (Grant et al., 2008; Siciliano et al., 2016).

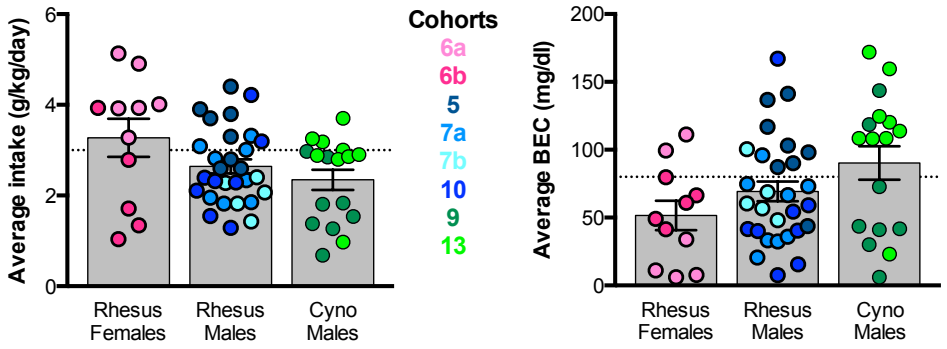

Figure 8 | Average Intake and Blood Ethanol Concentrations. Average intake (g/kg/day) (left) and average blood ethanol concentration (BEC, mg/dl) (right) over the duration of open-access conditions. Rhesus cohorts had approximately 12-months of open-access while the cynomolgus males had approximately 6-months of open-access (see text for number of open-access session per cohort). The dashed lines represent 3.0 g/kg/day and 80 mg/dl, respectively, indicating heavy drinking and the legal limit of intoxication in the United States. Mean ± SEM.

3.3.2 Effect of Ethanol Self-Administration on ACTH and Cortisol under Low and Mild Stress Conditions

Low stress: Blood samples obtained while the animals are in their housing environments reliably show low levels of circulating stress hormones (Helms et al., 2012; Helms et al., 2014; Jimenez et al., 2015; Jimenez et al., 2017b). Figures 9A and B (page 51) show ACTH and cortisol from rhesus (cohorts 5, 6a, 6b, 7a, 7b and 10: n = 17 females, n = 37 males) at baseline and following approximately 12-months of open-

access. A linear mixed model with repeated measures revealed a significant main effect of experimental phase ($F_{(1,52)} = 98.53$, $p < 0.0001$) where ACTH was lower following long-term open-access when compared to the baseline phase. There was a trend towards an effect of group, but this did not reach significance ($p = 0.07$) and no effect of sex ($p > 0.05$). However, a significant interaction between experimental phase and sex was found ($F_{(1,52)} = 16.11$, $p = 0.002$). Tukey's post-hoc analysis revealed that at baseline rhesus males had significantly higher ACTH than rhesus females (male average: 79.46 ± 44.35 pg/ml, female average: 50.50 ± 17.78 pg/ml; $p = 0.014$). ACTH declined significantly during open-access in rhesus males (average open-access: 36.53 ± 19.44 pg/ml, $p < 0.001$), but not females (average open-access: 40.26 ± 17.87 pg/ml; $p > 0.05$). Low stress ACTH following approximately 12-months of open-access was negatively correlated with average daily intake ($r^2 = 0.15$, $p = 0.015$; Figure 9C, page 51). For cortisol, an interaction between experimental phase and sex was found in the rhesus macaques ($F_{(1,52)} = 6.18$, $p = 0.016$; Figure 9B, page 51) where cortisol significantly declined between baseline and open-access for males (Baseline: 26.06 ± 6.84 μg/dl, open-access: 18.49 ± 3.93 μg/dl; $p < 0.0001$), but not females (baseline: 29.06 ± 7.30 μg/dl, open-access: 27.14 ± 6.64 μg/dl) and a significant sex difference was found during open-access where rhesus males had significantly lower cortisol when compared to rhesus females ($p < 0.0001$).

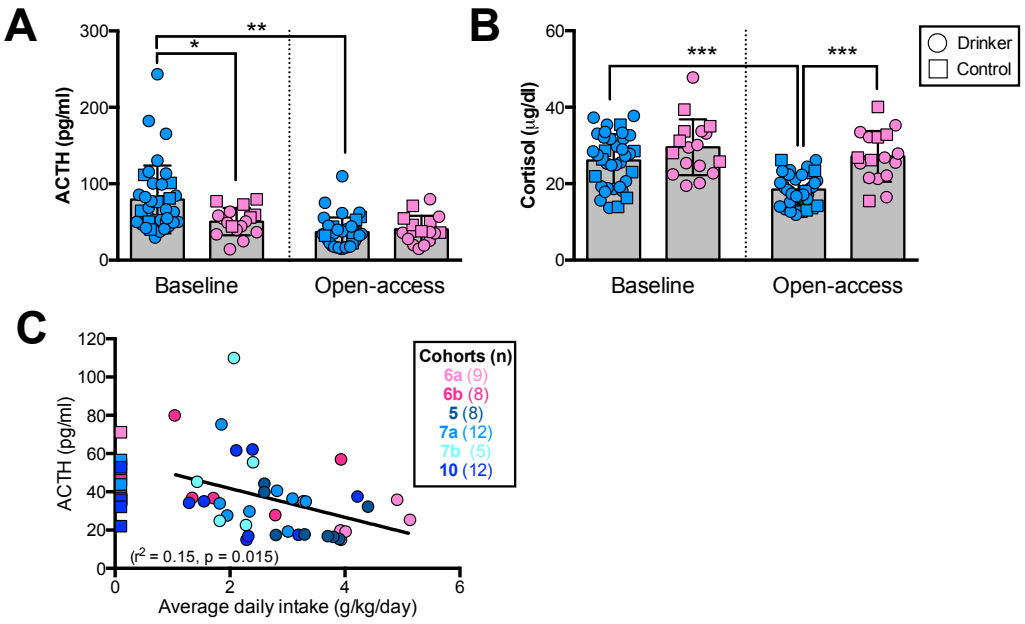

Figure 9 | Low Stress ACTH and Cortisol (Rhesus). Concentration of ACTH (A) and cortisol (B) under the low stress condition in male and female rhesus macaques prior to and following approximately 13-months of daily ethanol self-administration. Males are represented with blue symbols, females with pink. Low stress ACTH following approximately 13-months of open-access is negatively correlated with average daily intake (C). Data are presented as mean ± SD. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

Similar to the rhesus males, cynomolgus males (n = 23) with open-access to ethanol self-administration for approximately 7.5-months had a significant decrease in ACTH when compared to baseline ($F_{(1,19)} = 35.87$ $p < 0.0001$; baseline: 42.27 ± 17.59 pg/ml, open-access: 25.27 ± 16.96 pg/ml; Figure 10A, page 52) and the concentration of ACTH under the low stress condition during open-access was also negatively correlated with average daily ethanol intake ($r^2 = 0.40$, $p = 0.006$; Figure 10C, page 52). The effect

of group was not significant, however there was a trend towards an interaction between group and experimental phase ($F_{(3,19)} = 2.93$, $p = 0.060$). Cortisol under the low stress condition was not significantly different between groups or experimental phase (baseline: 23.88 ± 8.82 µg/dl, open-access: 22.79 ± 5.86 µg/dl; $p > 0.05$; Figure 10B).

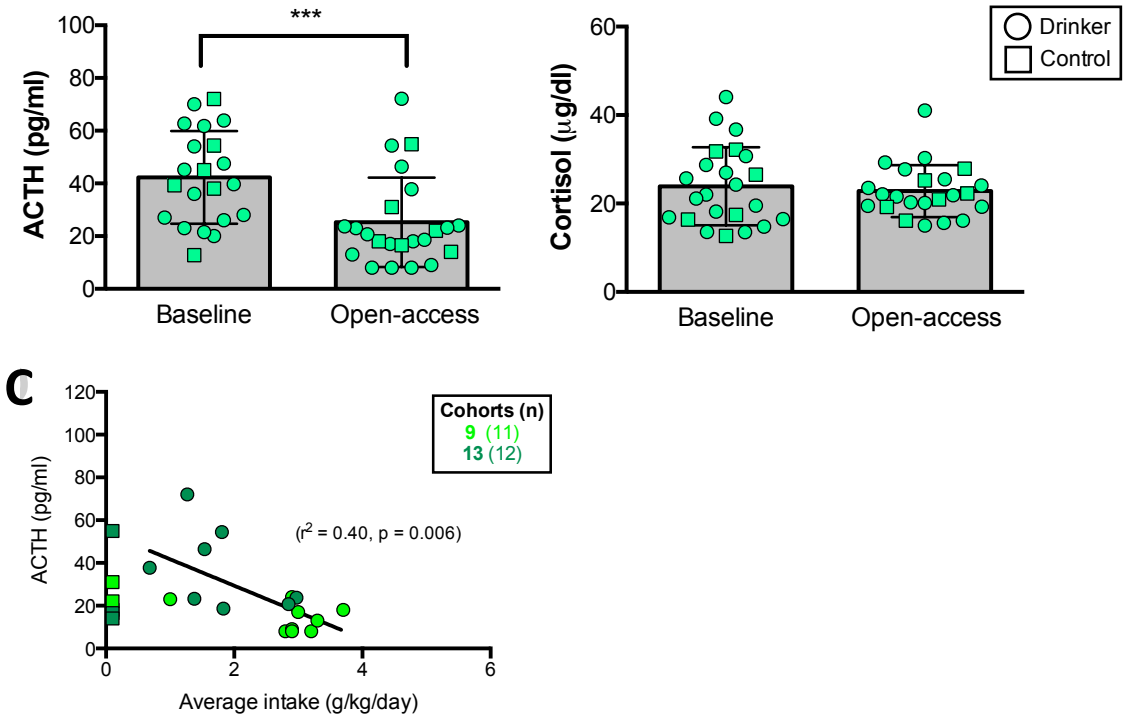

Figure 10 | Low Stress ACTH and Cortisol (Cyno). Concentration of ACTH (A) and cortisol (B) under the low stress condition in male cynomolgus macaques prior to and following approximately 7.5-months of daily ethanol self-administration. Basal ACTH following open-access is decreased, although this was not significantly different between drinkers and controls. ACTH following approximately 7.5 months of open-access negatively correlated with average daily ethanol intake (C). Data are presented as mean ± SD. *** $p < 0.0001$.

Mild stress: Both ACTH and cortisol are reliably elevated when animals are removed from their home cage and seated in a primate restraint chair (Ruys et al., 2004; Jimenez et al., 2015; Jimenez et al., 2017b). For the analysis of the response to mild-stress, a subset of the previously analyzed cohorts were used. These included rhesus cohorts 6a, 6b, 10 (n = 17 females, n = 12 males) and cynomolgus cohort 13 (n = 12 males).

In rhesus macaques, a significant interaction between sex and experimental phase was found for ACTH concentration ($F_{(1,27)} = 4.62$, $p < 0.041$, Figure 11, left, page 54). When comparisons between baseline and open-access were made rhesus males showed a 30% decrease while females showed a 25% increase, although these were not detected with post-hoc analysis. These sex-specific directional changes in the ACTH response to the mild stress condition contributed to a potentiation of the relative low to mild stress response. At baseline, rhesus males and females showed a 2.8- and 3.0-fold increase in ACTH under mild stress compared to low stress. During open-access this increased to 4.2- and 4.7-fold increase for males and females, respectively. These data point towards a potentiation of HPA axis activation to a novel stressor during chronic ethanol self-administration, and further suggests that the physiology underlying this response may be sex-dependent. For cortisol, a main effect of experimental phase was found ($F_{(1,27)} = 33.64$, $p < 00001$, Figure 11, right, page 54) where compared to baseline (43.12 ± 5.52 µg/dl), cortisol was lower during open-access conditions (33.33 ± 6.41 µg/dl).

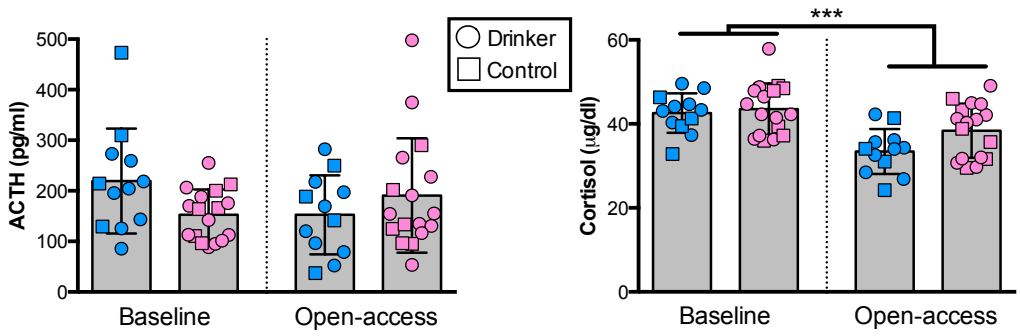

Figure 11 | Mild Stress ACTH and Cortisol (Rhesus). ACTH (left) and cortisol (right) in rhesus males (blue) and females (pink) under the mild stress condition. No differences were found between sex or experimental phase for ACTH. Cortisol concentration did not differ by sex, but was lower following approximately 12-months of open-access when compared to baseline. Mean ± SD. *** $p < 0.0001$.

In cynomolgus males, neither experimental phase nor group were significant factors in ACTH (Baseline: 90.36 ± 34.95 pg/ml, open-access: 90.00 ± 62.13 pg/ml; $p > 0.05$; Figure 12, left, page 55) or cortisol (Baseline: 38.21 ± 7.45 pg/ml, open-access: 33.77 ± 10.00 pg/ml; $p > 0.05$; Figure 12, right, page 55) concentration under mild stress conditions.

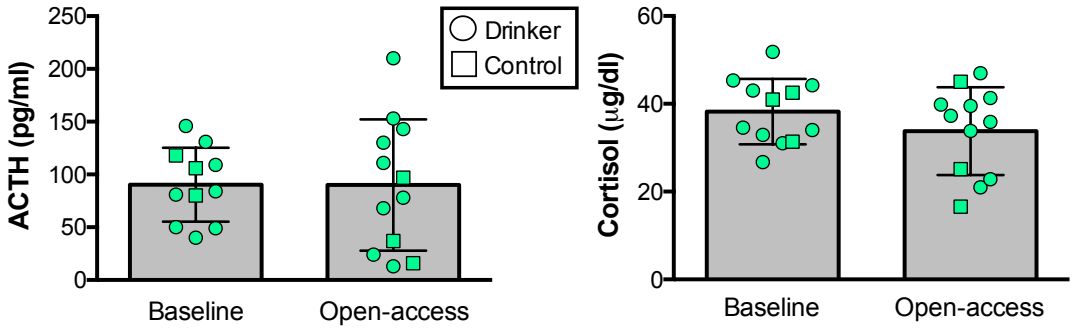

Figure 12: Mild Stress ACTH and Cortisol (Cyno). ACTH (left) and cortisol (right) in rhesus males (blue) and females (pink) under the mild stress condition. No differences were found between sex or experimental phase for ACTH. Cortisol concentration did not differ by sex, but was lower following approximately 12-months of open-access when compared to baseline. Mean ± SD. *** $p < 0.0001$.

3.3.3 Effect of Ethanol Self-Administration on Adrenocortical Steroids under Low and Mild Stress Conditions

ACTH also stimulates the secretion of DOC (Porcu et al., 2006; Jimenez et al., 2017a), DHEAS (Izawa et al., 2008; Maninger et al., 2010) and aldosterone (Jimenez et al., 2015; Gallo-Payet, 2016), see Figure 2 page 8. The following analyses were aimed at understanding whether these hormones are altered in monkeys during ethanol self-administration under low or mild stress conditions. These analyses included 17 female (cohorts 6a and 6b) and 29 male (cohorts 7a, 7b and 10) rhesus macaques and 23 male (cohorts 9 and 13) cynomolgus macaques, except when noted otherwise.

Low stress: A main effect of sex was found in rhesus macaques ($F_{(1,48)} = 16.30$, $p = 0.0002$; Figure 13A, page 57) where female rhesus had significantly higher circulating DOC than males prior to ethanol (females: 0.435 ± 0.242 ng/ml; males: 0.256 ± 0.106 ng/ml) and following approximately 12-months of open-access (females: 0.501 ± 0.301 ng/ml; males: 0.256 ± 0.106 ng/ml). Experimental phase and group were not significant factors in DOC concentration under low stress.

Neither long-term ethanol self-administration nor sex were significant factors in DHEAS concentration under low stress conditions ($p > 0.05$; Figure 13B, page 57). However, there was a significant main effect of group ($F_{(1,40)} = 3.35$, $p = 0.019$) where posthoc analysis revealed that animals categorized as binge or heavy and light or very heavy drinkers were significantly different ($p = 0.036$ and $p = 0.063$, respectively). However, because no main effect or interaction with experimental phase was found, these differences are not a consequence of long-term ethanol self-administration.

Aldosterone secretion under low stress conditions was not influenced by experimental phase or sex ($p > 0.05$), but a main effect of group was found ($F_{(4,36)} = 1.50$, $p = 0.025$; Figure 13C, page 57). Posthoc analysis revealed a significant difference between light and very heavy drinkers ($p = 0.019$). However, similar to DHEAS, this effect was not related to experimental phase and is not a consequence of long-term ethanol self-administration. The aldosterone analysis excludes cohort 10, bringing the number of male rhesus down to 17 (cohorts 7a and 7b). This was a result of a change in the manufacturer-supplied assay kit that occurred after the baseline samples had been assayed. The newer version of the assay kit, while intra-assay reliability is tolerable, does not yield results that are comparable with the older assay kit.

Under low stress, no significant main effects or interactions were found among male cynomolgus monkeys between group and experimental phase for DOC (Figure 13D, page 57), DHEAS (Figure 13E, page 57) or aldosterone (Figure 13F, page 57).

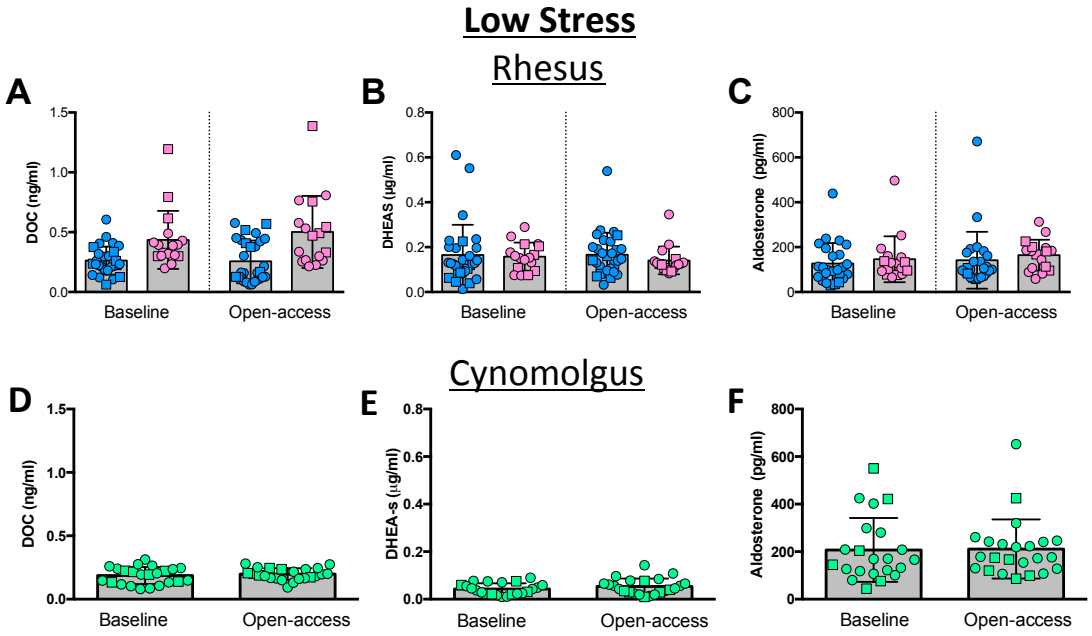

Figure 13 | Low Stress DOC, DHEAS and Aldosterone (Rhesus & Cyno). Concentration of DOC (A, D), DHEAS (B, E) and aldosterone (C, F) under low stress condition. Rhesus males (blue) and females (pink) are shown in the upper row (A, B, C) and cynomolgus males are shown below (D, E, F). Under low stress, DOC differed significantly between males and females rhesus macaques, independent of experimental phase and group. No main effects of experimental phase, group or sex were found for DHEAS and aldosterone under low stress conditions for rhesus macaques. No significant differences were found in cynomolgus males between baseline and open-access among cynomolgus macaques. Drinkers: circles, controls: squares. Mean ± SD.

Mild stress: These analyses included 17 female (cohorts 6a and 6b) and 12 male (cohort 10) rhesus macaques and 12 male (cohort 13) cynomolgus macaques. Due to the above-mentioned assay change, aldosterone measurements were not available for the mild stress condition from male rhesus. The cohorts excluded from these analyses were not trained to sit in a primate chair, thus no samples were available.

Under the mild stress condition, a significant interaction between sex and experimental phase was found in DOC concentration among rhesus macaques ($F_{(1,27)}$ = 8.19, p = 0.008; Figure 14A, page 60). At baseline, no differences were found between males (0.522 ± 0.173 ng/ml) and females (0.539 ± 0.210 ng/ml) while following open-access male rhesus had a significant decrease in DOC (0.370 ± 0.137 ng/ml; p = 0.035) while females had a non-significant increase (0.648 ± 0.297 ng/ml), leading to a significant sex difference during open-access (p = 0.002).

For DHEAS, similar to the low stress condition, rhesus macaques had a significant main effect of group ($F_{(4,23)}$ = 3.01, p = 0.039; Figure 14B, page 60). However, posthoc analysis revealed that this difference was between binge and light drinkers (p = 0.025), which were not significantly different in the low stress condition. However, because this difference was not related to experimental phase it is not a consequence of ethanol self-administration. No other main effects were found.

Aldosterone was found to be stable across the experimental phases in rhesus females (baseline: 235.07 ± 128.07 pg/ml; open-access: 219.56 ± 79.09; Figure 14C, page 60). There was no main effect of experimental phase (p = 0.93) and group revealed a trend-level of significance (p = 0.071).

Because only one cohort was included in the analysis of mild stress among cynomolgus males (cohort 13, n = 12) and eight of nine drinkers were categorized as heavy or very heavy drinkers, the group comparison in this analysis only included two levels (control and drinker). Under mild stress, there was a trend for drinkers to have higher DOC than controls, but this was not significant ($p = 0.054$; Figure 14D, page 60). Furthermore, because this was not related to experimental phase this is not believed to be a consequence of ethanol self-administration). No main effects or interactions were found for DHEAS in cynomolgus males under the mild stress condition (Figure 14E, page 60). Aldosterone increased significantly during open-access in cynomolgus males under mild stress ($F(1,10) = 5.56$, $p = 0.04$; Figure 14F, page 60). However, this effect was independent of group.

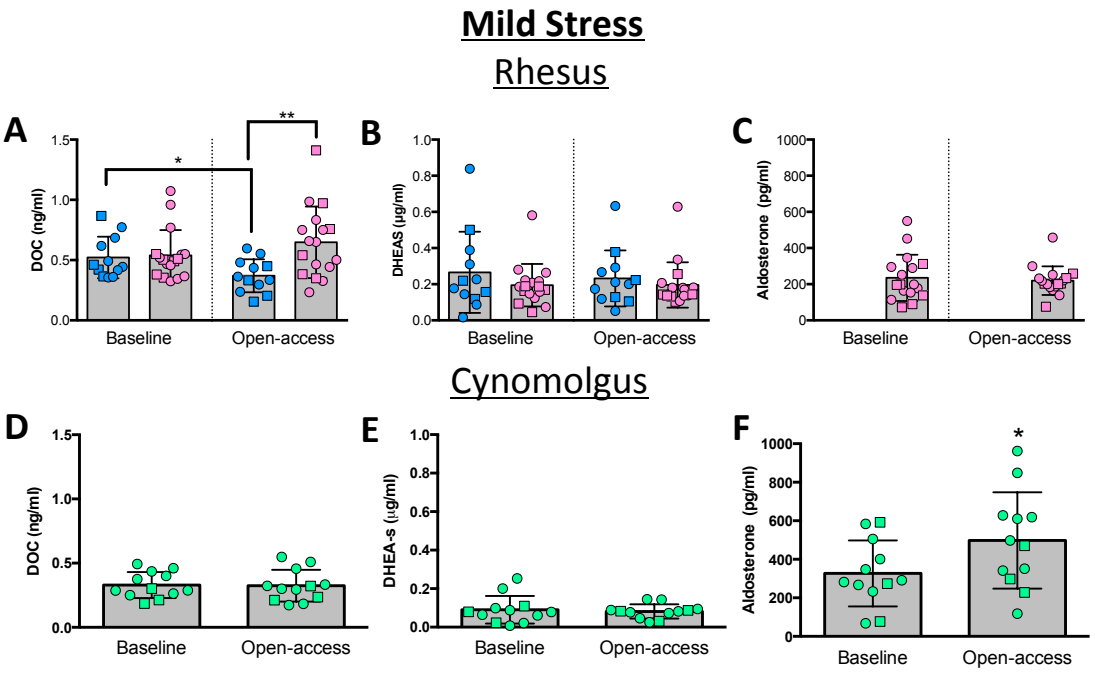

Figure 14 | Mild Stress DOC, DHEAS and Aldosterone (Rhesus & Cyno). Concentration of DOC (A, D), DHEAS (B, E) and aldosterone (C, F) under mild stress condition. Rhesus males (blue) and females (pink) are shown in the upper row (A, B, C) and cynomolgus males are shown below (D, E, F). Under mild stress, an interaction between sex and experimental phase was found for DOC among rhesus macaques, posthoc analysis revealed significant decrease in DOC across experimental phases for males and a significant difference between males and females during open-access (A). No main effects of experimental phase, group or sex were found for DHEAS and aldosterone in rhesus macaques, or in DOC, DHEAS or aldosterone in the cynomolgus males. Drinkers: circles, controls: squares. Data are presented as mean ± SD. * $p < 0.05$, ** $p < 0.01$

3.3.4 Effect of Ethanol Self-Administration on Glucocorticoid Feedback

Dexamethasone, a synthetic glucocorticoid with high affinity for the glucocorticoid receptor, is used to measure sensitivity to negative feedback. Across the experimental timeline dexamethasone remained a potent inhibitor of both ACTH, cortisol and DHEAS (Figure 15, page 63). ACTH suppression was greater in males (-93 ± 19 percent suppression) than females (-90 ± 10 percent suppression; main effect of sex: $F_{(1,48)} = 5.03$, $p = 0.030$). No differences were found in dexamethasone-induced suppression of ACTH by experimental phase ($p = 0.38$) or group ($p = 0.68$).

A significant main effect of experimental phase was found for dexamethasone-induced suppression of cortisol ($F_{(1,52)} = 11.47$, $p = 0.00014$; Figure 15B, page 63). A trend towards a main effect of sex was found ($p = 0.08$) and no effect of group ($p = 0.81$). There was a significant interaction between experimental phase and sex ($F_{(1,52)} = 19.78$, $p < 0.0001$). Post-hoc analysis revealed that cortisol among rhesus males were less sensitive to dexamethasone suppression during open-access (-86 ± 16%) compared to pre-ethanol baseline (-92 ± 3%; $p < 0.001$) and that during open access females had greater suppression than males ($p < 0.001$).

Experimental phase was a significant factor in dexamethasone suppression of DOC ($F_{(1,50)} = 37.91$, $p < 0.0001$), and a significant interaction between sex and experimental phase was found ($F_{(1,50)} = 10.57$, $p = 0.0002$; Figure 15C, page 63). Posthoc analysis revealed that prior to ethanol, male and female rhesus macaques differed significantly in dexamethasone-suppression of DOC (males: -58 ± 19% suppression; females: -38 ± 20% suppression; $p = 0.004$) and males had a significant reduction in

suppression during open-access (-13 ± 37% suppression; $p < 0.001$). Group and sex were not significant factors in DOC suppression following dexamethasone ($p = 0.42$ and $p = 0.28$, respectively).

An interaction between experimental phase and sex ($F_{(1,44)} = 7.51$, $p = 0.0009$; Figure 15D, page 63) was found for dexamethasone suppression of DHEAS in rhesus macaques. Posthoc analysis revealed a significant reduction in dexamethasone suppression of DHEAS among male rhesus between baseline (-92 ± 3% suppression) and open access (-72 ± 23% suppression; $p < 0.001$). Group was not a significant factor ($p = 0.57$).

As noted above, cohort 10 was excluded from aldosterone analyses, so the dexamethasone suppression of aldosterone included 25 male rhesus macaques (cohorts 7a, 7b and 5). In general, dexamethasone suppression of aldosterone was weak, but stable across the experiment (males: baseline -32 ± 31%, open-access: -37 ± 36%; females: baseline -37 ± 28%, open-access: -35 ± 36%). Experimental phase, group and sex were not significant factors in dexamethasone suppression of aldosterone ($p = 0.76$, $p = 0.76$ and $p = 0.88$, respectively; Figure 15E, page 63).

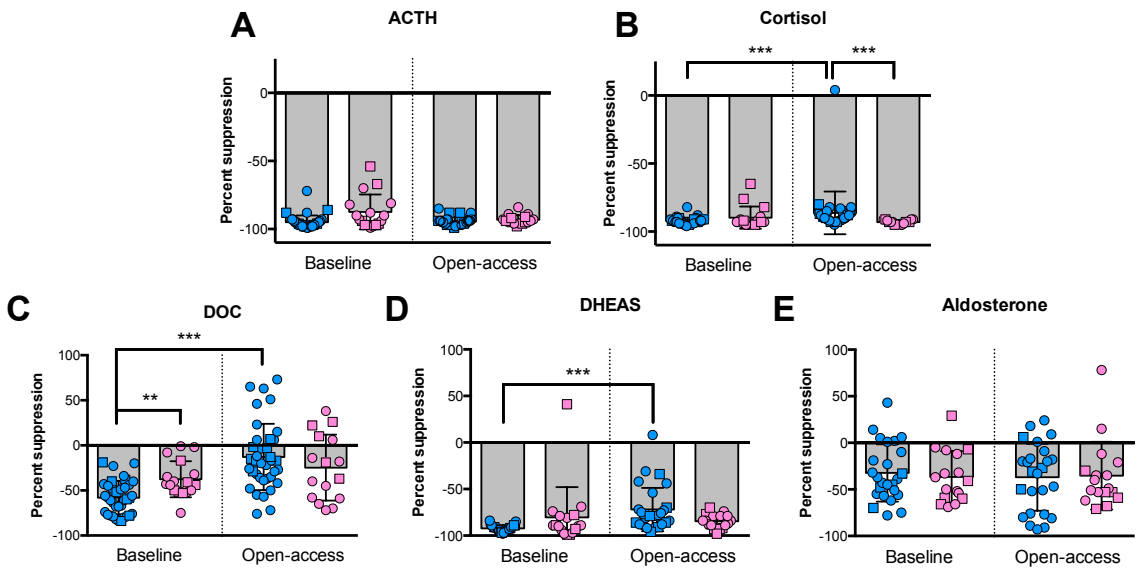

Figure 15 | Dexamethasone Suppression of ACTH, Cortisol, DOC, DHEAS and Aldosterone (Rhesus). Dexamethasone suppression of ACTH (A), cortisol (B), DOC (C), DHEAS (D) and aldosterone (E) from rhesus males (blue) and females (pink). No differences between sex or experimental phases were found for ACTH, however cortisol suppression was reduced in males following open-access leading to a significant difference when compared to baseline and females in open-access. Males and females differed significantly in dexamethasone-suppression of DOC at baseline and dexamethasone suppression of DOC (C) and DHEAS (D) decreased significantly in males following open-access. No differences between sex or experimental phase were found in dexamethasone suppression of aldosterone. Drinkers: circles, squares: controls. Females: pink, males: blue. Mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In cynomolgus males with approximately 7.5-months of open-access conditions, ACTH suppression by dexamethasone was significantly blunted (baseline: -92 ± 7%, open-access: -88 ± 7%; $F_{(1,19)} = 9.37$, $p < 0.0001$), independent of group ($F_{(3,19)} = 0.07$, $p = 0.97$; Figure 16A, page 65).

There was a weak trend towards a dampened suppression of cortisol ($F_{(1,19)} = 3.56$, $p = 0.075$) and no effect of group ($F_{(3,19)} = 0.03$, $p = 0.99$; Figure 16B, page 65).

Dexamethasone suppression of DOC increased in cynomolgus males between baseline (-46 ± 25% suppression) and open-access (-61 ± 21% suppression; $F_{(1,18)} = 12.31$, $p = 0.0025$; Figure 16C, page 65), but the change was independent of group ($p = 0.40$).

DHEAS suppression following dexamethasone decreased significantly between baseline (-85 ± 9% suppression) and open-access (-69 ± 15% suppression; $F_{(1,19)} = 31.37$, $p < 0.0001$; Figure 16D, page 65). Similar to DOC, this was unrelated to group ($p = 0.16$).

Similar to rhesus, dexamethasone suppression of aldosterone in cynomolgus males was weak relative to the other adrenal steroids. Across experimental phases, dexamethasone suppression of aldosterone did not significantly differ (baseline: -24 ± 29% suppression; open-access: -36 ± 35% suppression; $p = 0.21$; Figure 16E, page 65) and there was no effect of group ($p = 0.34$).

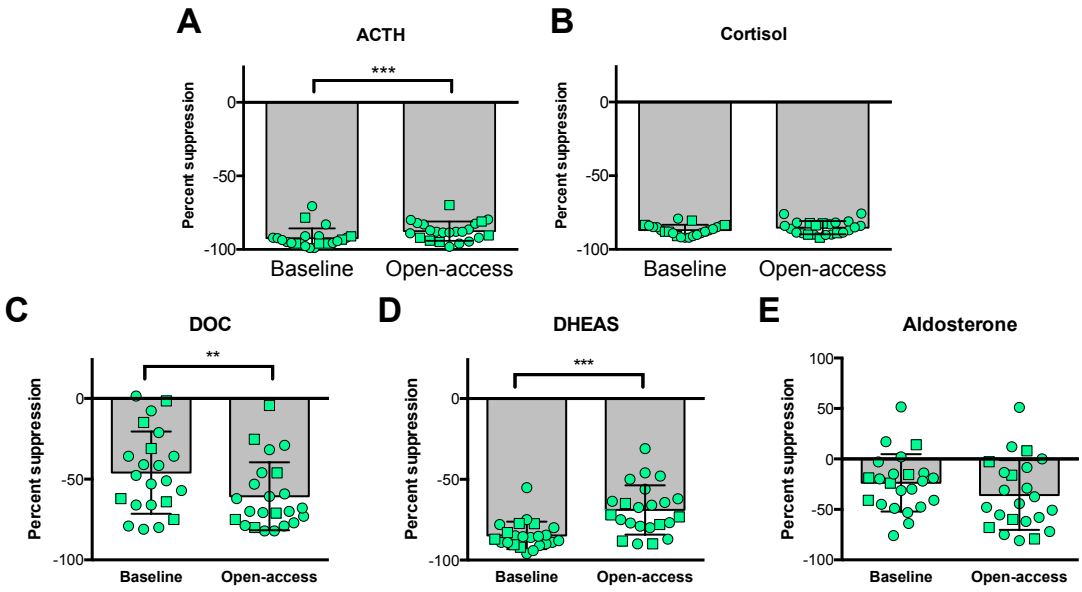

Figure 16 | Dexamethasone Suppression of ACTH, Cortisol, DOC, DHEAS and Aldosterone (Cyno). Dexamethasone suppression of ACTH (A), cortisol (B), DOC (C), DHEAS (D) and aldosterone (E) from male cynomolgus macaques. Dexamethasone-suppression of ACTH (A) and DHEAS (D) following open-access were reduced compared to baseline, while DOC suppression (C) was enhanced cortisol (B) or aldosterone (E) suppression did not differ between experimental phases. Drinkers: circles, squares: controls. Females: pink, males: blue. Data are presented as mean ± SD. ** $p < 0.001$, *** $p < 0.0001$

3.3.5 Effect of Repeated Abstinence on Low and Mild Stress Response and Glucocorticoid Feedback

Twelve rhesus males (cohort 10) were subjects in an extended protocol where following approximately 12-months of open-access ethanol was replaced with water during three 28-day forced abstinence periods, with three months of open-access conditions in between (see experimental timeline, Figure 4, page 24). Blood samples under low and mild stress conditions were during the first abstinence (A1), after approximately 18 months of open-access (prior to the third abstinence phase), and again during the third abstinence phase (A3).

Under low stress conditions, there was a main effect of experimental phase ($F_{(5,50)} = 10.12$, $p < 0.0001$), but not group ($p > 0.05$) on ACTH concentration (Figure 17A, page 68). Post-hoc analysis revealed that ACTH was significantly lower than baseline at each experimental phase ($p < 0.05$), with the exception of the first abstinence, where the difference did not reach significance ($p = 0.06$).

A main effect of experimental phase ($F_{(5,50)} = 7.97$, $p < 0.0001$) was found for cortisol concentration with a trend towards a main effect of group ($F_{(1,10)} = 3.90$, $p = 0.08$; Figure 17B, page 68) Posthoc analysis for experimental phase revealed that cortisol was significantly elevated during the first and third abstinence phases when compared to all open-access phases. All open-access phases (6-mo, 12-mo and 18-mo) showed a trend level decrease when compared to baseline ($p = 0.09$, $p = 0.07$ and $p = 0.06$, respectively).

For DOC, experimental phase was a significant main effect ($F_{(5,50)} = 13.48$, $p <$

0.0001; Figure 17C, page 68). Posthoc analysis revealed that compared to baseline, DOC was significantly lower after approximately 12- ($p < 0.001$) and 18-months ($p = 0.010$) of open-access with a trend-level difference between baseline and the first abstinence phase ($p = 0.053$). Compared to 12-months of open-access, DOC had significantly increased during the first and third abstinence ($p = 0.0014$ and $p < 0.001$, respectively). Group was not a significant factor ($p = 0.23$).

DHEAS under the low stress condition was not related to experimental phase ($p = 0.60$) or group ($p = 0.91$; Figure 17D, page 68).

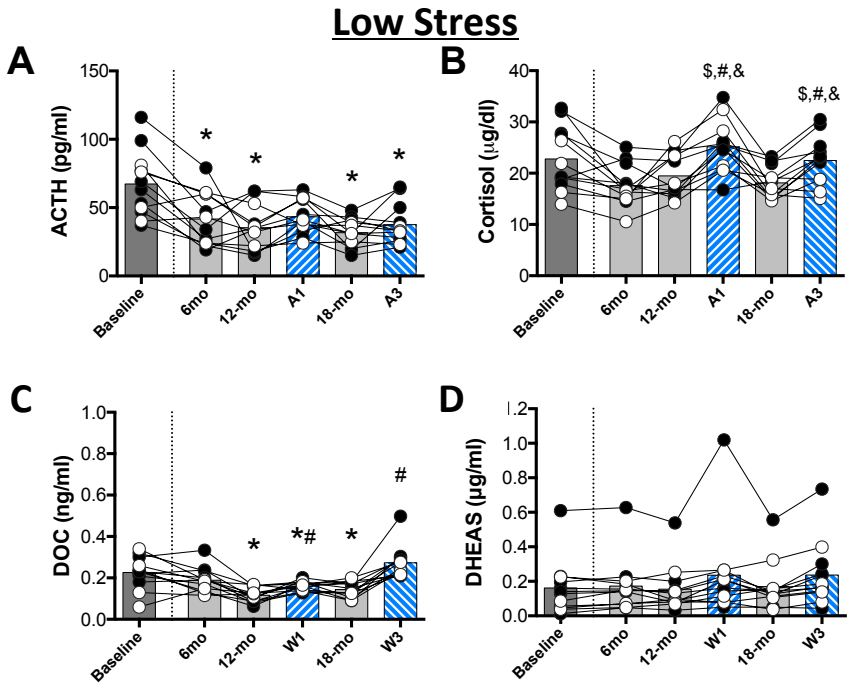

Figure 17 | ACTH, Cortisol, DOC and DHEAS under Low Stress Conditions in Open-Access and Repeated Forced Abstinence . ACTH (A), cortisol (B), DOC (C) and DHEAS (D) under the low stress condition across open-access and repeated abstinence. Forced abstinence phases are indicated by blue hatched bars. The ethanol drinkers and ethanol-naive controls are indicated by black and white, respectivelyBars represent mean, individual animals represented. * $p < 0.05$, compared to baseline, # $p < 0.05$ compared to 6-mo, $ $p < 0.05$ compared to 12-mo, & $p < 0.05$ compared to 18-mo.

Under mild stress, a main effect of experimental phase, but not group, was found for ACTH concentration ($F_{(5,49)} = 7.11$, $p < 0.0001$; Figure 18A, page 70). Interestingly, where under low stress nearly every experimental phase was decreased relative to baseline, under mild stress ACTH was significantly dampened relative to baseline

(219.34 ± 103.72 pg/ml) at each experimental phase following the first forced abstinence (A1: 104.69 ± 47.47 pg/ml, p = 0.033; 18-mo: 96.31 ± 51.45 pg/ml, p = 0.020; A3: 92.76 ± 59.37 pg/ml, p = 0.012).

Cortisol, under mild stress conditions (Figure 18B, page 70), was significantly lower at all experimental phases relative to baseline ($F_{(5,50)} = 12.46$, $p < 0.0001$), independent of group (p = 0.14).

A main effect of experimental phase was found for DOC concentration ($F_{(5,50)} = 3.78$, p = 0.006; Figure 18C, page 70). Posthoc analysis revealed that there was a significant decrease in DOC during the third abstinence (0.321 ± 0.128 ng/ml) compared to baseline (0.522 ± 0.173 ng/ml; p = 0.003), but this was independent of group (p = 0.25).

Experimental phase and group were unrelated to DHEAS concentration across this repeated forced abstinence protocol (p = 0.56 and p = 0.90, respectively; Figure 18D, page 70).

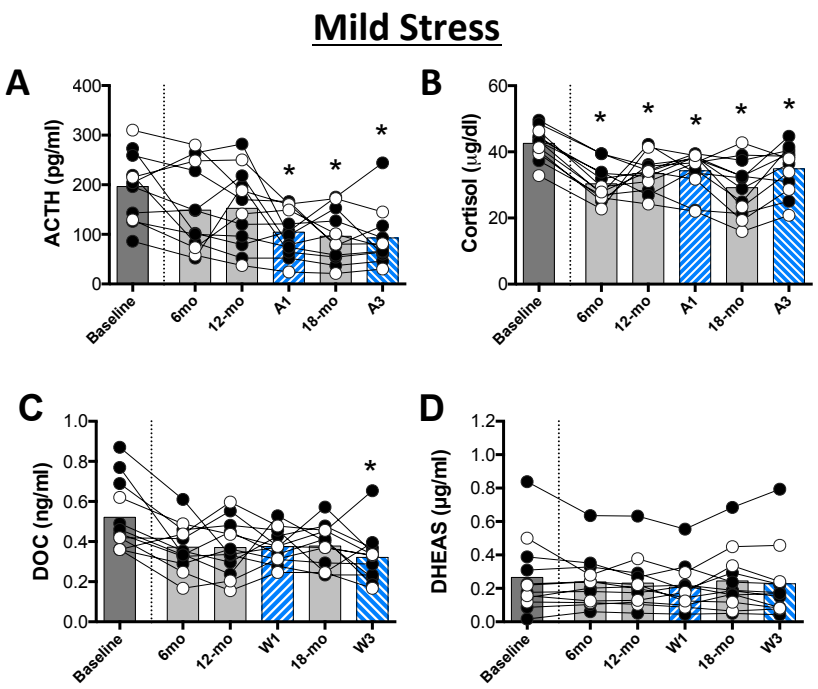

Figure 18 | ACTH, Cortisol, DOC and DHEAS under Mild Stress Conditions in Open-Access and Repeated Forced Abstinence. ACTH (A), cortisol (B), DOC (C) and DHEAS (D) under mild stress conditions across open-access and repeated abstinence. Forced abstinence phases are indicated by hatched bars. Bars represent mean, individual animals represented. The ethanol drinkers and ethanol-naive controls are indicated by black and white, respectively. * $p < 0.05$, compared to baseline.

Dexamethasone suppression of ACTH and cortisol were also influenced by experimental phase (ACTH: $F_{(5,55)} = 2.16$, $p = 0.034$, Figure 19A, page 72; cortisol: $F_{(5,55)} = 6.81$, $p < 0.0001$, Figure 19B, page 72), but not group ($p = 0.95$ and $p = 0.32$, respectively). Post hoc analysis showed that compared to baseline (-96 ± 2%) ACTH suppression was dampened following 12-months of open-access (-93 ± 3%, $p = 0.010$)

and a trend-level decrease following 18-months of open access (-94 ± 3%, $p = 0.09$). Cortisol suppression followed a similar, but more robust pattern with significantly less suppression when baseline (-92 ± 2%) was compared to 12-months (-87 ± 5%, $p < 0.001$), the first abstinence (-88 ± 3%, $p = 0.0023$) and 18-months (-87 ± 4%, $p = 0.016$).

Experimental phase was found to be a significant main effect in dexamethasone suppression of DOC ($F_{(5,50)} = 27.54$, $p < 0.0001$), but this was unrelated to group ($p = 0.98$; Figure 19C, page 72). Posthoc analysis revealed that compared to baseline (-70 ± 18%), percent suppression of DOC was significantly different at 6-months (-37 ± 20%, $p < 0.001$), 12-months (19 ± 33%, $p < 0.001$), first abstinence (-27 ± 20%, $p < 0.001$), 18-months (12 ± 33%, $p < 0.001$) and the third abstinence (-53 ± 22%, $p = 0.01$). Additionally, dexamethasone suppression during the third abstinence differed significantly from both 12- and 18-months of open-access ($p < 0.001$ for both experimental phases).

Dexamethasone suppression of DHEAS was unrelated to experimental phase ($p = 0.062$) or group ($p = 0.21$; Figure 19D, page 72).

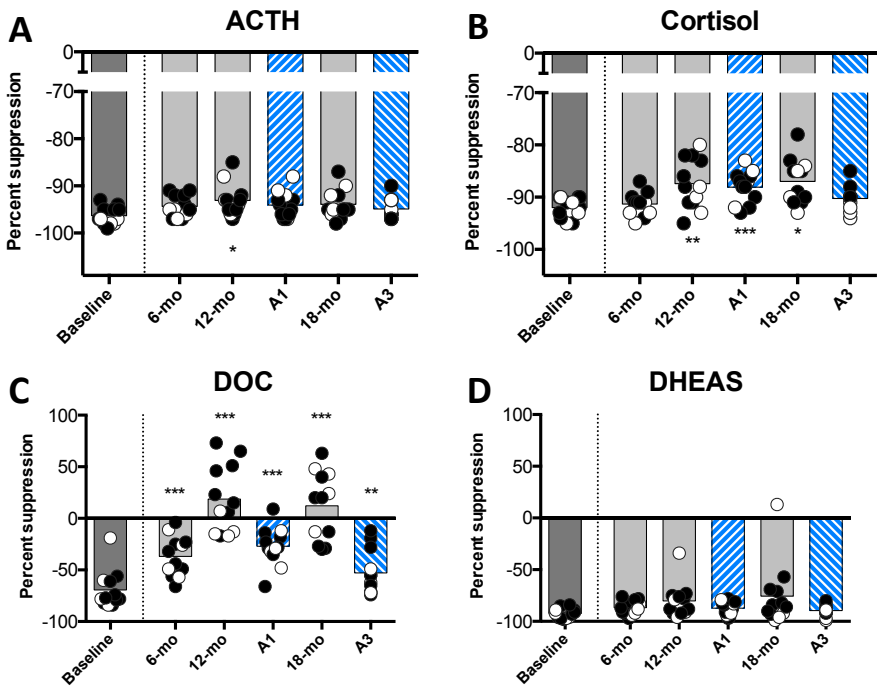

Figure 19 | Dexamethasone Suppression of ACTH, Cortisol, DOC and DHEAS in Open-Access and Repeated Forced Abstinence. Dexamethasone suppression of ACTH (A), cortisol (B), DOC (C) and DHEAS (D) across an extended repeated abstinence procedure. The first and third forced abstinence phases are indicated by blue hatched bars. The ethanol drinkers and ethanol-naive controls are indicated by black and white, respectively. Bars represent mean, individual animals represented. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to pre-ethanol baseline.

3.4 Discussion

This chapter was an analysis of pituitary and adrenocortical hormones under low and mild stress conditions aimed at evaluating the effect of ethanol self-administration on the HPA axis. The first hypothesis was that long-term ethanol self-administration would selectively dampen stress hormones under low, but not mild, stress conditions. These analyses revealed several significant changes between baseline and open-access, however contrary to the hypothesis, these were independent of ethanol access. Among males (both rhesus and cynomolgus), there was a significant decrease in ACTH under the low stress condition and although no effect of group was found, the concentration of ACTH measured during the open-access phase was negatively correlated with average daily ethanol intake. Cortisol also decreased in male rhesus, but not female rhesus or cynomolgus males, under the low stress condition. Under mild stress, cortisol decreased in rhesus, but not cynomolgus macaques and no changes in ACTH were found. Thus, the hormonal response to mild stress was generally unaltered. The second hypothesis was that repeated forced abstinence would result in elevated HPA axis activity under low stress conditions and a dampened response to mild stress. ACTH declined during open-access, again independent of group, and remained low during repeated abstinence. Cortisol was elevated during forced abstinence. Although effect was initially seen in both drinkers and controls, a trend-level interaction suggests that the ethanol-naïve controls did not respond to the final abstinence phase. Under mild stress, ACTH was dampened following the first forced abstinence phase, but this was not specific to animals with a history of ethanol access. Cortisol under the mild stress condition was not responsive to forced abstinence.

The most important aspect of these studies is the voluntary consumption of ethanol during the open-access phase, resulting in individual differences in the preferred pattern of intake ("sipping" and maintaining low BEC or "gulping" to obtain higher BEC). For example, the average daily intakes shown in Figure 8 (page 49) do not always reflect the average BEC, particularly with the female rhesus. In general, BEC is highly correlated with ethanol intake at the time of the sample (7-h into the 22-h session; Jimenez et al., 2015; www.MATRR.com). However, the pattern of intake is variable between animals. Some animals drink the majority of their daily ethanol by the time BECs are collected, while others will continue to drink throughout the night and again in the morning, consuming up to an additional 1.5 g/kg than what had been consumed when the BEC was collected. In particular, the female rhesus (cohort 6a) consumed ethanol overnight and in the morning (the last two hours of the 22-h session; see www.MATRR.com), which accounts for the high average daily intake but comparatively low BECs shown in Figure 8 (page 49). While BEC is an important measure of intoxication, this demonstrates that because the ethanol consumption in this model is voluntary, a multivariate approach to the data is best. Perhaps similar to alcoholic liver disease (O'Shea et al., 2010), breast cancer (Smith-Warner et al., 1998) and coronary heart disease (Tolstrup et al., 2006), ethanol dose represents a risk for harmful consequences (or benefit, in the case of coronary heart disease) but the relationship is not necessarily linear and influences, generally, only a portion of those at risk. Other variables may include family history, lifestyle (diet, activity level, etc), drinking typography (low levels daily or binge-like behavior), among others. In the monkey model described here, a multivariate approach has been used to categorize drinking

severity where average daily intake, percentage of days over a given dose, and BEC were found to reliably cluster animals into stable drinking categories (see Baker et al., 2014; Baker et al., 2017).

The studies described here follow a standard experimental timeline (Figure 4, page 24). The monkeys were experienced in weekly blood collection procedures from the home cage environment prior to the collection of samples analyzed here. As such, stress hormones assayed under these conditions reliably reflect a low stress condition (Jimenez et al., 2015; Jimenez et al., 2017a) similar to blood samples collected with an indwelling catheter (Williams et al., 2003; Pascoe et al., 2008). This allows a within-subject analysis of stress hormones across the ethanol self-administration protocol to evaluate the effect of daily ethanol self-administration on the HPA axis.

Prior to ethanol access, evidence for a sex difference was apparent among rhesus macaques. At baseline, rhesus males and females differed significantly in the concentration of ACTH (Figure 9A, page 51) and dexamethasone suppression of DOC (Figure 15C, page 63). Generally, the pituitary-adrenal response to mild stress did not differ between males and females, with the exception of DOC, which was higher in rhesus females following open-access. The sex comparison under mild stress should be interpreted with caution, however, as the sample size was much smaller than the low-stress condition. In addition to sex differences, these data indicate that species may be an important consideration. For example, prior to ethanol rhesus macaques had much higher concentrations of ACTH, DOC and DHEAS compared to cynomolgus macaques under the low stress condition. These differences were not due to rhesus macaques having higher concentrations in pituitary and adrenocortical hormones in general, as aldosterone

was found to be higher in cynomolgus macaques and no differences were found in the concentration of cortisol. After long-term open-access conditions adrenocortical suppression by dexamethasone showed species-specific directional shifts. Dexamethasone suppression of cortisol was reduced during open-access in male rhesus macaques (Figure 15B, page 63), while cynomolgus males showed no change (Figure 16B, page 65). Furthermore, dexamethasone suppression of DOC decreased in male rhesus (Figure 15C, page 63), but increased in cynomolgus males (Figure 16C, page 65), while dexamethasone suppression of DHEAS decreased in males from both species (Figure 15D and 16D, pages 63 and 65). Because these adrenal steroids are produced in different layers of the adrenal cortex, these data suggest these layers may have unique sensitivity to ethanol that are species-specific. Future analyses should include cynomolgus females to further explore the relationship between sex and species on the adrenocortical steroids. At the level of the anterior pituitary, dexamethasone suppression of ACTH was dampened following medium-term (ie approximately 6-months) open-access conditions in the cynomolgus, but not rhesus, males (Figures 15A and 16A, pages 63 and 65). Due to the differences in the duration of open-access between the rhesus and cynomolgus cohorts (14-months and 7.5-months, respectively), additional studies are needed to better characterize these preliminary observations.

Basal (low stress) ACTH after approximately 6- or 12-months of ethanol self-administration was negatively correlated with the average daily ethanol intake for rhesus (males and females) and cynomolgus macaques (Figure 9C and 10C, pages 51 and 52, respectively). Importantly, a significant decrease in the ACTH response under the low stress condition in males, but not females, was found between baseline and open-access

(Figure 9A, page 51). Because this effect was found in both drinkers and controls, one possible explanation is that male rhesus continued to habituate to the blood collection procedure after the baseline sample had been collected. Another possibility is that the induction procedure alters the HPA axis and contributes to the decrease in circulating ACTH, independent of ethanol. Previous work in rodents and monkeys has shown that SIP of water leads to activation of the HPA axis (Brett and Levine, 1981; Helms et al., 2013). Using the paradigm presented here, HPA axis regulation of the mineralocorticoid DOC was decreased in response to pharmacological stimulation of the adrenal cortex (exogenous ACTH; 10 ng/kg) and anterior pituitary (ovine-CRH; 1 μg/kg), but increased in response to hypothalamic disinhibition (naloxone; 375 μg/kg) during schedule-induced polydipsia (Jimenez et al., 2017a). The response to these pharmacological challenges normalized following 6-months of open-access conditions, suggesting ethanol-induced allostasis of adrenal steroid secretion involving central mechanisms (Jimenez et al., 2017a). Although there were no ethanol-naïve controls in this analysis, previous work has shown that the introduction of ethanol following water SIP, despite a 2/3 reduction in fluid requirement, further increased ACTH and flattened the ACTH diurnal rhythm (Helms et al., 2013), demonstrating that the combination of the SIP procedure and ethanol has an additive effect on HPA axis activation. The hypothesis that HPA axis sensitivity to the SIP procedure is related to future ethanol drinking has not been analyzed directly, but excitatory and inhibitory synapses in the hypothalamic PVN have been shown to be altered by adrenocortical steroids and stress. Specifically, adrenalectomy resulted in a robust increase in the number of GABAergic synaptic terminals contacting CRH-immunopositive parvocellular neurons (Miklós and Kovács, 2002) and chronic

variable stress resulted in an increase in glutamatergic and noradrenergic appositions onto CRH-immunopositive neurons (Flak et al., 2009).

The relative change between low (Figure 9A, page 51) and mild (Figure 11, left, page 54) stress conditions was potentiated during open-access, with rhesus males and females showing a 4.2- and 4.7-fold increase during open access compared to 2.8- and 3.0-fold at baseline, respectively. The explanation of this potentiation was sex-dependent. For males, ACTH decreased approximately 54% under the low stress condition and approximately 30% in response to the mild stress condition between baseline and open-access. The ACTH response to low stress did not change for females between baseline and open-access, but the there was approximately a 25% increase in response to mild stress during open-access compared to baseline.

As mentioned previously, 1.0 and 1.5 g/kg ethanol challenge to ethanol-naïve cynomolgus macaques resulted in significantly lower ACTH concentration at 90- and 120-minutes following the challenge (unpublished observation). In healthy (nonalcoholic) men, a moderate dose of ethanol (0.75 g/kg) blunted the ACTH response to o-CRH (Waltman et al., 1993). These data, together with the results here, indicate that both acute and chronic ethanol lead to dampened pituitary secretion of ACTH. An important consideration of these results is the BEC at the time of the stress challenges. Blood samples for BEC are not routinely collected in the morning from these macaques, but measurable BECs have been found on occasion and acute intoxication may further suppress circulating stress hormones. These data suggest that, in addition to adaptations at the level of the adrenal cortex, consequences of long-term ethanol occur at the pituitary or upstream in the hypothalamus.

The hypothesis that ethanol results in an allostatic state is supported by the stress hormone response to repeated forced abstinence in a subset of rhesus males. In this cohort, ACTH under low and mild stress were consistent with the multi-cohort analysis up to the first abstinence: decreased ACTH at 6- and 12-months of open-access under low stress and no change in ACTH during mild stress (group analyses shown in figures 9 and 11, pages 51 and 54). However, in response to mild stress, ACTH was significantly blunted relative to baseline following the first abstinence phase (Figure 18A, page 70). Cortisol under low stress conditions, on the other hand, was uniquely responsive to forced abstinence phases where concentrations were significantly higher than each open-access phase while cortisol concentrations under the mild stress condition remained consistently lower than baseline across the experimental timeline, similar to previous findings from cynomolgus macaques (Cuzon Carlson et al., 2011).

The effect of abstinence on the HPA axis in humans has been, at times, inconsistent. One-month abstinent alcoholics have been reported to have a dampened basal cortisol, but not ACTH, concentration (Adinoff et al., 2005), consistent with the results presented here. Although three-week abstinent alcoholics did not differ from healthy non-alcoholic subjects in basal ACTH or cortisol or in their response to o-CRH (Adinoff et al., 1991). In both rhesus (Allen et al., under review) and cynomolgus macaques (Cuzon Carlson et al., 2011), repeated abstinence markedly changed patterns of ethanol consumption. In the cynomolgus macaques, the variability surrounding the average daily dose reduced with each abstinence phase resulting in higher BECs (Cuzon Carlson et al., 2011). In the current cohort, all subjects had an increase in their average daily intake. This was a transient effect in light and binge drinkers, but sustained with the

heavy and very heavy drinkers (Allen et al., under review). This is an important consideration in evaluating the seemingly discrepant findings with the human literature as human subjects enrolled into these studies are typically treatment-seeking individuals and are likely to have experienced abstinence (forced or voluntary) previously.

A dissociation between ACTH and cortisol secretion has been a consistent finding in the non-human primate model of ethanol self-administration (Helms et al., 2014; Helms et al., 2012). This was also found with long-term social housing manipulations in ethanol-naïve cynomolgus males (Jimenez et al., 2017a), suggesting this is a response to chronic stress, rather than ethanol-specific. A potential mechanism for this is an upregulation of catecholamine (epinephrine and norepinephrine) secretion that has been shown to potentiate adrenocortical responsiveness to ACTH (Edwards and Jones, 1987). Support for this has been reported in cynomolgus macaques (Cohen et al., 1997) and tree shrews (Fuchs et al., 1993), where changes in social settings were associated with higher concentrations of circulating norepinephrine. Furthermore, acute ethanol has been shown to increase plasma epinephrine and norepinephrine in light to moderate drinking young adult men (Ireland et al., 1984) and norepinephrine was elevated in alcoholics (Patkar et al., 2004). Interestingly, human alcoholics with a minimum of three weeks of abstinence did not differ from healthy controls in their plasma norepinephrine (Patkar et al., 2004), suggesting the effect is reversible. The increase in adrenomedullary release could be mediated by centrally projecting autonomic parvocellular neurons in the hypothalamic PVN that incorporate the sympathetic nervous system and increase epinephrine and norepinephrine, although this is outside the scope of this dissertation.

It is important to exercise caution when interpreting data from a single timepoint.

Although these samples were obtained at approximately the same time of day across the experimental timeline, previous data using this model has shown that the ACTH diurnal rhythm is flattened and peak cortisol is shifted with ethanol induction and self-administration (Helms et al., 2012; Helms et al., 2013). Similarly, mid-day but not morning ACTH was found to be higher in alcoholics (Wand and Dobs, 1991), an effect that is unlikely to be captured using a single timepoint. In addition to analyzing diurnal fluctuations, pharmacological assessment of the HPA axis is a useful tool that can also provide clues as to the location and system involved in aberrant responding. In this regard, ovine-CRH and naloxone have been used in humans and monkeys (Hernandez-Avila et al., 2002; Adinoff et al., 2005; Jimenez et al., 2017a) and can provide information about the location and mechanism of HPA axis function. In addition, samples collected several hours into the morning (low stress condition) or late morning / early afternoon (mild stress condition) miss the cortisol awakening response (CAR) that is an important indicator in psychiatric disease (see Fries et al., 2009 for review), including alcohol dependent patients (Junghanns et al., 2007).

In summary, this chapter aimed to identify consequences of long-term daily ethanol self-administration on stress hormones under low and mild stress conditions. The hypothesis that ACTH and cortisol under a low stress condition would be dampened following long-term ethanol self-administration while the response to mild stress would be preserved is not supported by the data presented here. These data show that under the low stress condition ACTH is negatively correlated with average daily ethanol self-administration (Figures 9C and 10C, pages 51 and 52, respectively). The hypothesis that repeated forced abstinence would result in elevated stress hormones under low stress and

a dampened response under mild stress was partially supported. These data demonstrate that long-term open-access and forced abstinence have unique effects on HPA axis response to low and mild stress. These findings are particularly important given that AUDs are characterized as a chronically relapsing condition. The similar endocrine physiology between monkeys and humans, combined with volitional ethanol self-administration, provides a powerful opportunity to understand the physiological consequences of ethanol self-administration and identify novel targets for treatment.

CHAPTER 4: An Ultrastructural and Functional Analysis of Long-term Ethanol Self-Administration on the Parvocellular Neurons of the Hypothalamic Paraventricular Nucleus

4.1 Introduction

Several studies have provided evidence that ethanol influences parvocellular neurons in the PVN. Elevated CRH mRNA expression in parvocellular neurons and secretion of stress hormones (ACTH and corticosterone) have been reported after a single administration of 1.5 g/kg of ethanol to rats (Richardson et al., 2008) and neuronal activation, measured by c-Fos, is found in parvocellular neurons of the PVN after an acute ethanol administration (Ogilvie et al., 1998). Continued ethanol exposure, however, leads to a dampened neuroendocrine response to ethanol (Richardson et al., 2008; Lee et al., 2000; Mohn et al., 2011). Lee and colleagues (1997) demonstrated that the effect of acute ethanol on ACTH and corticosterone is dependent on CRH and AVP signaling from the PVN (Lee et al., 1997). This was the first study to demonstrate that the PVN is required for ethanol's effects on the HPA axis. The effect of repeated ethanol exposure on heterotypic stressors is more variable. Some groups have reported that animals with a history of ethanol exposure have a dampened HPA axis activation to heterotypic stressors (immune challenge, shock: Mohn et al., 2011; Lee et al., 2000; Richardson et al., 2008) while others have reported a selective tolerance to ethanol while the response to

heterotypic stressors is preserved (Lee and Rivier, 1997; Silva and Madeira, 2012). These different results may be duration- or stressor-specific. Nonetheless, the PVN shows consistent and robust changes to repeated ethanol exposure. In adult male rats with a brief history of ethanol exposure (4.5 g/kg/day for three consecutive days) no differences were found in CRH or AVP mRNA concentration under basal conditions, but following an ethanol challenge the concentration of CRH and CRH_{R1} mRNA were selectively decreased, while AVP mRNA did not change (Lee et al., 2001). Chronic ethanol exposure (6-month liquid diet) revealed differential changes in parvocellular and magnocellular PVN neurons. The number of CRH- and AVP-immunoreactive neurons was decreased in animals with chronic ethanol exposure, but no differences were found in the total number of neurons (Silva et al., 2002a). The number of immunoreactive neurons recovered slightly following two-months of abstinence, suggesting temporary loss of activity without neuronal-loss of putative parvocellular neurons. Using the same paradigm, the magnocellular neurons suffered an irreversible loss of OXY- and AVP-immunoreactive neurons (Silva et al., 2002b). The loss of neurons is supported by an overall decrease in the neuronal cell counts, however it is notable that the optical density (an arbitrary unit for protein immunoreactivity) for both neuropeptides recovered suggesting a compensatory response by the surviving neurons. It is important to note that the PVN response to ethanol appears sex-specific. Silva and Madeira (2012) have shown that long-term alcohol consumption and withdrawal result in sex-dependent corticosterone response with males showing a recovery of basal (low stress) corticosterone and females having a further attenuation. In the same study, Silva and Madeira (2012) report a similar pattern with CRH mRNA in the PVN where males had a

significant decrease during ethanol consumption that recovered following prolonged withdrawal while females did not show a significant difference during alcohol consumption but had a significant decline in CRH mRNA following prolonged abstinence. The sex-dependent effects of drugs of abuse, including ethanol, across the addiction cycle was recently reviewed (Becker and Koob, 2016), highlighting the importance for future research.

One question that remains unanswered is whether the effects of long-term ethanol on the PVN are a result of direct or indirect interaction with parvocellular neurons. As described above, the effects of ethanol have been reported in numerous brain areas, including stress-sensitive regions known to influence the parvocellular neurons of the PVN (see Figure 20, page 87). In general, physiological stressors have direct connections to parvocellular neurons while psychological stressors are relayed through a network of nuclei (Herman et al., 2003). Additionally, the PVN is surrounded by a net of primarily GABAergic neurons that are thought to provide a hierarchal filter where incoming information can be gated or modified by simultaneous information (Herman et al., 2002). This is supported by data showing that the degree of c-Fos activation in parvocellular neurons depends on both the type and magnitude of the stressor (Pacák and Palkovits, 2001), suggesting varied sources of synaptic input that code for stress intensity. The majority of synaptic inputs onto parvocellular neurons of the PVN are glutamatergic and GABAergic (gamma-aminobutyric acid; Decavel and van den Pol, 1992; Miklos and Kovacs, 2002); although other neuropeptides (see Carrasco and Van de Kar, 2003; Watts, 1996) and neurotransmitters are also present such as norepinephrine (Liposits et al., 1986; Daftary et al., 2000) and serotonin (Qi et al., 2009) (see Figure 20, page 87).

Consistent with the wide range of inputs, there is evidence for an equally wide range of receptors in the PVN. Of importance for this dissertation are the ionotropic and metabotropic glutamate receptors (Herman et al., 2000; Ziegler et al., 2005) and $GABA_A$ receptors (Cullinan, 2000), which are important in ethanol pharmacology (Grant and Lovinger, 1995; Roberto and Varodayan, 2017). These receptors are known to be sensitive to pharmacologically relevant concentrations of acute ethanol, and demonstrate compensatory changes with chronic ethanol exposure. Furthermore, the effects of ethanol on presynaptic GABA and glutamate signaling are gaining appreciation (Siggins et al., 2005; Roberto et al., 2006; Weiner et al., 2006).

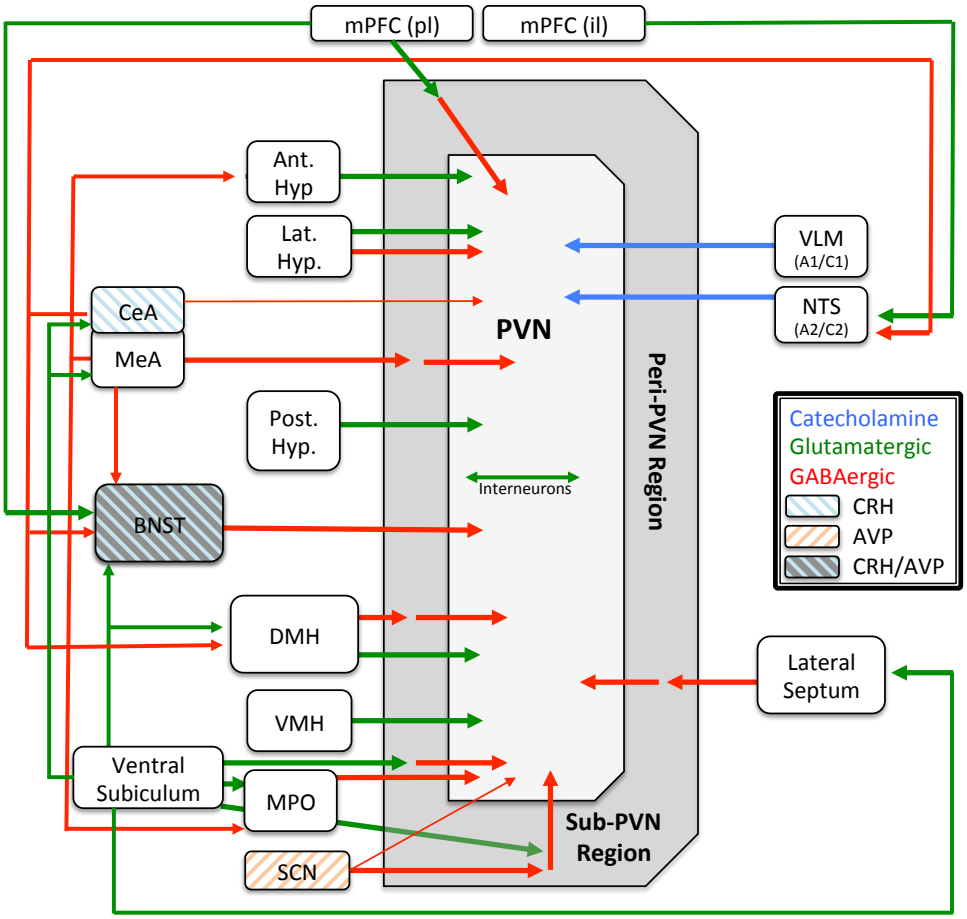

Figure 20 | Projections to the PVN and peri-PVN. Simplified circuitry of major inputs to the hypothalamic PVN. Abbreviations: (Ant. Hyp) anterior hypothalamus, (BNST) bed nucleus of the stria terminalis, (CeA) central nucleus of the amygdala, (DMH) dorsal medial hypothalamus, (Lat. Hyp) lateral hypothalamus, (MeA) medial nucleus of the amygdala, (mPFCpl) prelimbic medial prefrontal cortex, (mPFCil) infralimbic medial prefrontal cortex, (MPO) medial preoptic nucleus, (NTS) nucleus of the tractus solitarius, (SCN) suprachiasmatic nucleus, (VLM) ventrolateral medulla, (VMH) ventromedial hypothalamus. This schematic is adapted from: Prewitt and Herman, 1998; Herman et al., 2004; Herman et al., 2005; Dong and Swanson, 2006; Herman and Meuller, 2006; Ulrich-Lai et al., 2011; Crestani et al., 2013.

This chapter will focus on the structure and function of parvocellular neurons of the hypothalamic paraventricular nucleus. GABA and glutamate are well known targets for ethanol pharmacology (Grant and Lovinger, 1995), as well as principal signaling components in the PVN (Miklós and Kovács, 2002; Van Den Pol et al., 1990; Van Den Pol, 1991; Van Den Pol and Trombley, 1993; Evanson and Herman, 2015; Figure 20, page 87). Long-term ethanol exposure has been found to decrease $GABA_A$ receptor expression. While this has not been investigated in the PVN, blocking $GABA_A$ receptors by administering an antagonist to the PVN, but not the lateral ventricles, decreased voluntary consumption in male rats (Li et al., 2011). These data suggest that in addition to regulation of the HPA axis, GABAergic signaling in the PVN contributes to self-administration behavior. The objective of this study was to test the hypothesis that presynaptic GABA density is positively correlated with ethanol intake. These data are the first to describe the unique relationship between chronic ethanol self-administration, ultrastructural measures of GABA and glutamate immunogold density and whole-cell patch clamp electrophysiological glutamate activity in parvocellular neurons in a primate brain.

4.2 Statistical Analysis

The subjects in these analyses include a subset of those previously described in the endocrine response to chronic ethanol self-administration (Chapter 3). They included a cohort of medium length self-administration (6-months open-access, cohort 13), long-term self-administration (12-months open-access, cohort 6b) and long-term self-administration with repeated forced abstinence (18-months total open-access, cohort 10). Analyses were run independently for each cohort. Due to the limited number of animals in each cohort 'Group' was analyzed with two levels (control vs drinker) instead of five to preserve statistical power. Independent Student's t-tests were used to compare immunogold density parameters, immunofluorescence and electrophysiological measures between experimental groups. The effect of acute ethanol was calculated as a percent of basal spontaneous EPSC frequency (bath application of aCSF + 100μM picrotoxin). The effect of the ethanol challenge was analyzed using a paired Student's t-test and groups (ethanol vs control) were compared using an independent t-test. To evaluate the effect of ethanol self-administration, Pearson correlations were used to assess the relationship between immunogold density, optical density, hormone concentrations, spontaneous EPSC frequency and average daily ethanol (g/kg/day). All values are reported as mean ± standard deviation (SD), unless otherwise stated. Analyses were performed in R Statistical Computing software (version 3.1.2: R Core Team, 2015), $\alpha < 0.05$ was considered significant.

4.3 Results

4.3.1 Long-term Ethanol Self-Administration:

The subjects of these experiments were rhesus females (cohort 6b; 3 controls and 5 drinkers) with approximately 13-months open-access conditions (341 ± 1 consecutive days). These animals were sent to necropsy during the open-access phase of the protocol.

Effect of Ethanol on Relative GABA Immunogold Density

The relative presynaptic density of GABA and glutamate was measured in terminals contacting immunohistochemically-identified CRH or AVP dendrites. This analysis revealed two populations of presynaptic terminals: those immunoreactive for a neuropeptide (CRH or AVP) and those that were not (Figure 21, page 92).

Table 2 (page 91) lists the mean GABA and glutamate immunogold density within these immunopositive and immunonegative terminals. Presynaptic GABA density did not differ by group, nor did it correlate with average daily ethanol intake (g/kg/day) over 12 months (CRH: $r = 0.45$, $p = 0.26$; AVP: $r = 0.47$, $p = 0.29$). Analysis of both GABA and glutamate density related to AVP was limited to two ethanol-naïve controls due to inadequate resin infiltration during tissue processing.

Neurotransmitter	Postsynaptic Immunoreactivity	Presynaptic Immunoreactivity	Mean density (particles/μm^2) controls	Mean density (particles/μm^2) drinkers	p-value
GABA	AVP $^{\Phi}$	AVP	99.9 (2.9)	113.7 (4.0)	0.19
		Non-AVP	130.3 (7.0)	102.5 (11.3)	0.32
	CRH	CRH	118.7 (9.8)	164.3 (14.5)	0.15
		Non-CRH	107.0 (5.7)	143.8 (14.2)	0.31
Glutamate	AVP $^{\Phi}$	AVP	48.8 (0.7)	71.6 (8.3)	0.10
		Non-AVP	77.4 (4.2)	87.9 (8.6)	0.60
	CRH	CRH	86.2 (7.2)	87.1 (6.4)	0.97
		Non-CRH	79.5 (7.9)	80.1 (5.9)	0.95

Table 2 | Mean (± SEM) GABA and glutamate immunogold density in control (n = 3) and ethanol-drinking monkeys (n = 5). $^{\Phi}$ only two ethanol-naïve animals were available for these data.

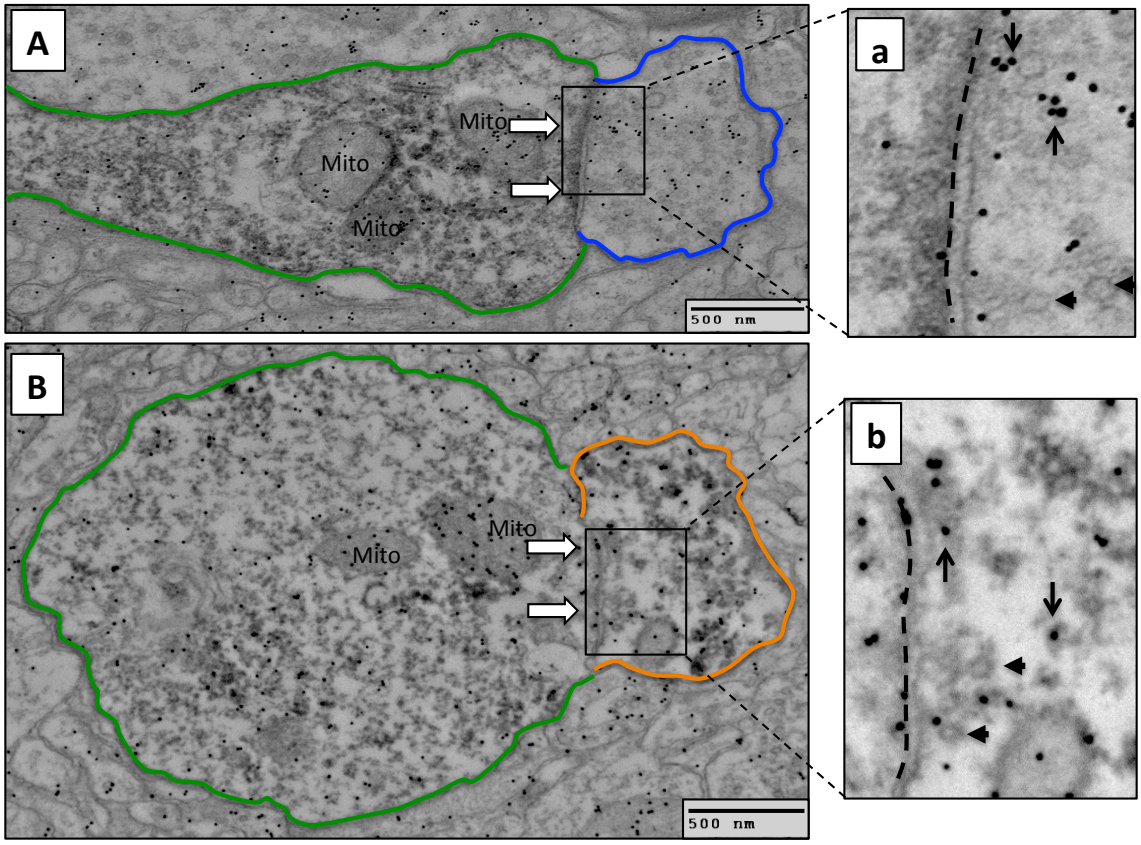

Figure 21 | Electron micrographs of immunopositive and immunonegative presynaptic terminals. Representative electron micrographs illustrating immunopositive and immunonegative terminals. Both postsynaptic dendrites are AVP-positive (DAB-positive, outlined in green). (A) An immunonegative terminal (outlined in blue) forms an asymmetrical synaptic contact (arrows). (a) A segment of the terminal is enlarged to show 12-nm immunogold particles (small arrows), synaptic vesicles (arrowheads) and synaptic contact (dotted line). (B) An immunopositive terminal (outlined in orange) forms a symmetrical contact (arrows). (b) A portion of the terminal is enlarged to show 12-nm gold particles (small arrows), synaptic vesicles (arrowheads) and synaptic contact (dotted line). Mito: mitochondria.

Effect of Ethanol on Relative Glutamate Immunogold Density

No group differences were found in glutamate density within either terminal type (Table 2, page 91). Although the range of glutamate density in ethanol animals was similar to controls, the glutamate density in immunopositive CRH terminals was linearly related to average daily ethanol intake ($r = -0.91$, $p = 0.012$), Figure 22A (page 94). Conversely, glutamate density in immunoreactive AVP terminals was not correlated with 12-month average daily ethanol intake (g/kg; $r = 0.65$, $p = 0.23$, Figure 22B, page 94, page 94), despite a trend for higher immunogold density in animals with ethanol access. The glutamate density in immunoreactive AVP and CRH axon collaterals was significantly correlated ($r = -0.78$, $p = 0.04$; Figure 22C, page 94), suggesting a coordinated balance of excitatory input onto these principal cell populations. Among immunoreactive AVP terminals there was a trend for glutamate and GABA density to be correlated ($r = 0.72$, $p = 0.07$), however this was not the case for CRH ($r = 0.09$, $p = 0.82$).

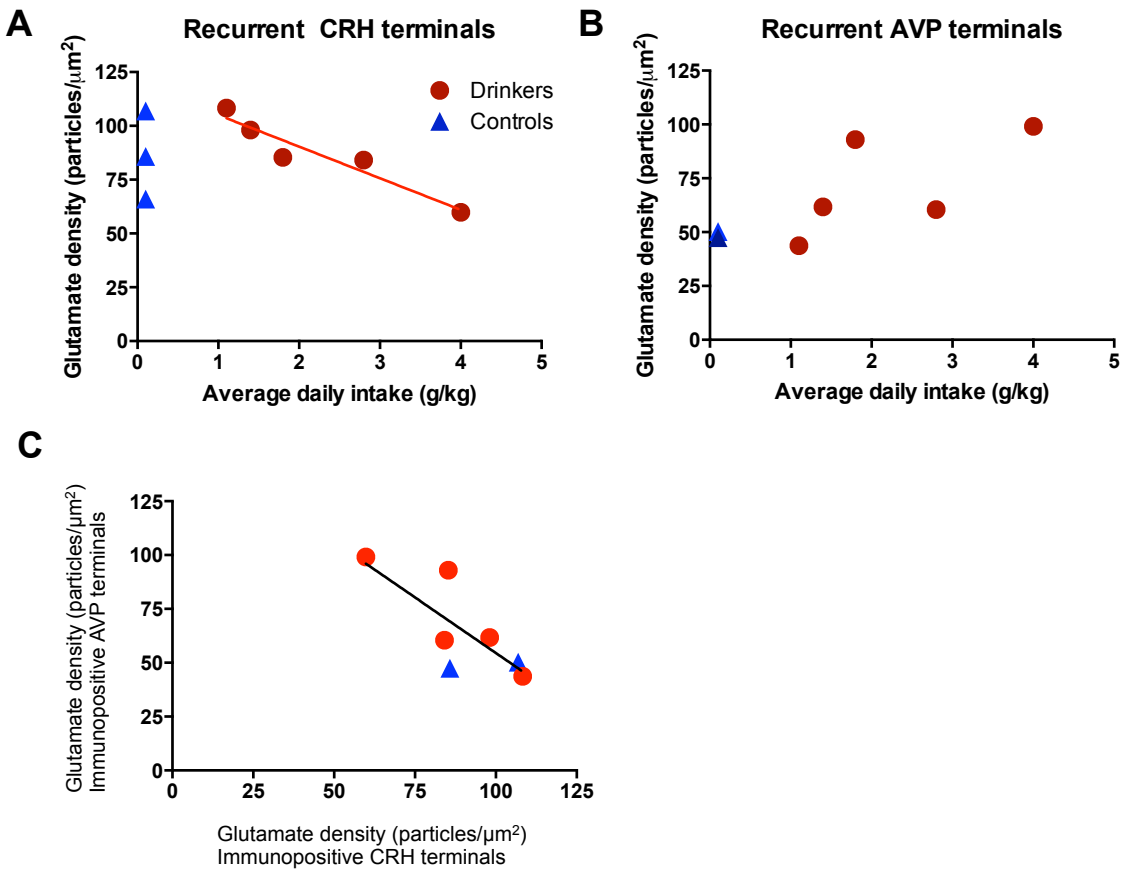

Figure 22 | Immunogold Density in Immunopositive Terminal (Rhesus females). The relative glutamate immunogold density in (A) immunopositive CRH terminals was highly correlated with the average daily ethanol intake over open-access conditions ($r = -0.91$, $p = 0.01$); however there was no group difference $p = 0.97$. (B) The relative immunogold density in immunopositive AVP terminals did not correlate with average daily ethanol intake ($r = 0.65$, $p = 0.23$), but there was a weak trend towards an increase in in immunogold density in animals consuming ethanol ($p = 0.10$). (C) Glutamate density in CRH and AVP immunopositive terminals were correlated ($r = -0.78$, $p = 0.04$). Blue triangles: controls; red circles: drinkers (Jimenez et al., 2015)

Effect of Ethanol Self-Administration on CRH- and AVP-immunoreactivity:

Monkeys chronically consuming ethanol showed a trend towards elevated AVP-immunoreactivity (controls (n = 3): 6.5 ± 2.0 O.D., drinkers (n = 4): 10.9 ± 2.6 O.D., p = 0.06) and no difference in CRH (controls (n = 3): 2.5 ± 2.2 O.D., drinkers (n = 3): 5.6 ± 3.1 O.D., p = 0.22), Figure 23, page 96. Glutamate density in immunopositive AVP terminals weakly correlated with the OD of AVP (r = 0.71, p = 0.06).

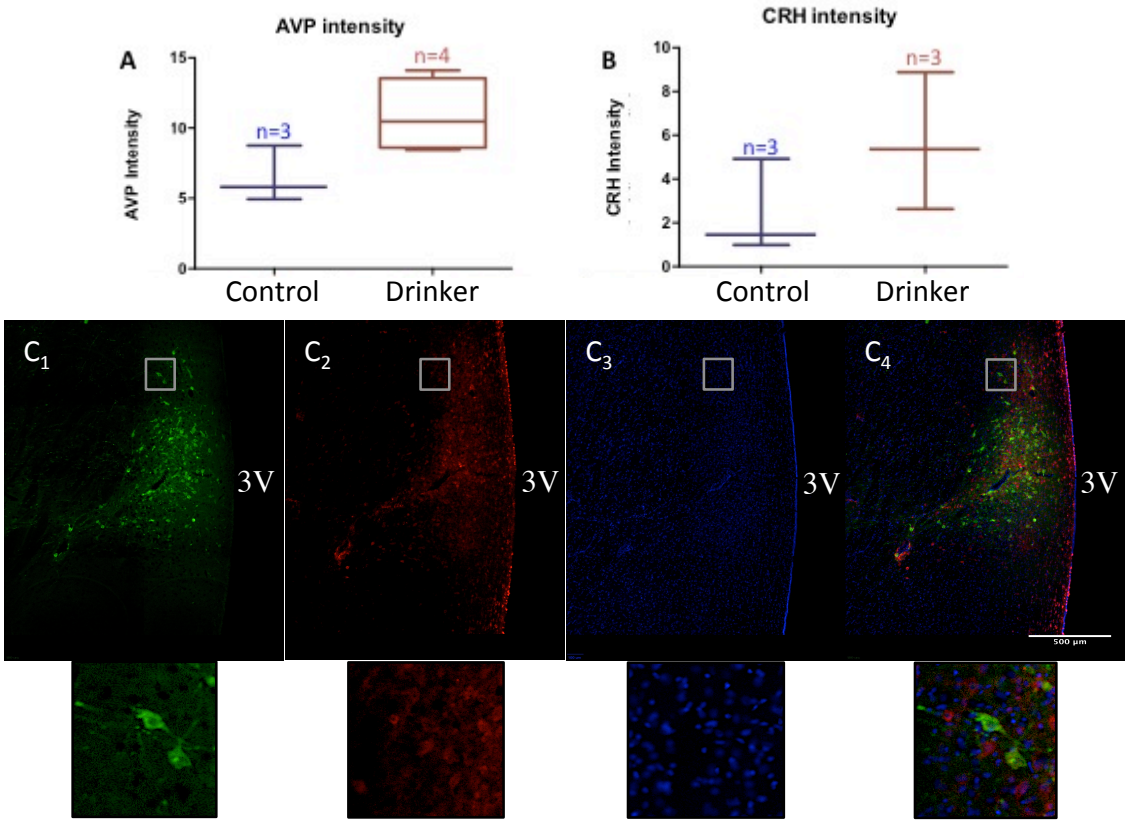

Figure 23 | CRH and AVP Immunohistochemistry (Rhesus females) AVP (A) and CRH (B) immunofluorescence intensity (optical density, arbitrary units). A trend towards increased AVP optical density in animals consuming ethanol (red) compared to ethanol naïve controls (blue) was found ($p = 0.06$). No difference between drinkers and controls was found in CRH immunoreactivity ($p = 0.22$). ($C_{1\text{-}4}$) Representative example of immunostaining for AVP, CRH, DAPI and the merged image, respectively. The boxed region is enlarged beneath the respective image to show individual neurons. 3V: third ventricle. Scale bar = 500 µm.

Summary and Conclusions:

The most important finding presented here is the unique relationship of chronic (12-months) ethanol intake and the relative glutamate density in immunopositive axon terminals of the hypothalamic PVN. Few regions, particularly those expressing CRH and AVP, have direct glutamatergic projections to neurons in the PVN. Instead, information is relayed primarily via GABAergic and glutamatergic peri-PVN and hypothalamic regions to the PVN (Herman et al., 2005; also see Figure 20, page 87). Recurrent axon collaterals (locally synapsing terminals originating from parvocellular neurons in the PVN) have been reported from parvocellular neurons in the PVN (van den Pol, 1982; Ray and Choudhury, 1990) and may contribute to these peptide-immunoreactive terminals found here, particularly those that are co-labeled with glutamate. These putative recurrent axon collaterals indicate that parvocellular neurons may be uniquely related to ethanol intake, supporting the effect of long-term voluntary ethanol self-administration directly in the PVN. Specifically, glutamate density in putative recurrent CRH collaterals was highly negatively correlated with average daily ethanol intake. Because the range of glutamate density measured after chronic ethanol drinking matches the range in ethanol-naïve controls, one interpretation is that glutamate density in putative recurrent collaterals is antecedent to ethanol exposure and may serve as a predictive factor in the development of a heavy drinking phenotype. Evidence for altered glutamate and GABA signaling after ethanol has been disproportionally focused on post-synaptic receptor changes following chronic ethanol consumption (see Mihic and Harris, 1997; Chandrasekar, 2013 for review) although more recently presynaptic effects have been gaining attention (see Siggins et al., 2005 for review). These data provide initial evidence for interactions of

ethanol with distinct populations of pre-synaptic terminals in the hypothalamic PVN. As the downstream stress hormones did not show gross disruption following ethanol self-administration in this cohort, appropriate HPA response may be maintained by altered glutamate signal integration within the PVN. Specifically, AVP in the PVN is hypothesized to rescue the stress response following stress-induced loss of CRH neurons (Volpi et al., 2004, Tanoue et al., 2004). Importantly, the relationship between ethanol intake and presynaptic neurotransmitter density measured with immunogold density was specific to glutamate. Contrary to the hypothesis that presynaptic GABA density would reflect changes in postsynaptic receptor expression or function, no relationship was found between average daily ethanol self-administration and the relative GABA immunogold density. It is important to note that GABAergic terminals immunoreactive for either AVP or CRH are unlikely to belong to parvocellular neurons of the PVN. These terminals likely belong to projections from the BNST, CeA and/or the SCN (see Figure 20, page 87). Additionally, the lack of a significant relationship between presynaptic GABA immunogold and ethanol self-administration is not conclusive. Given the number of regions with GABAergic projections to the PVN, even those with CRH and/or AVP immunoreactivity, it is impossible to determine whether a subset of terminals are uniquely affected. For example, previous studies in male rhesus macaques found a subset of BNST neurons had an increased inhibitory tone in animals with ethanol access, but not controls (Pleil et al., 2015). It is unknown whether these are PVN-projecting neurons, and if so, whether they co-express CRH. However, if these were PVN-projecting neurons, the decreased inhibitory tone in the BNST would be hypothesized to decrease the relative GABA immunogold density in the terminals within the PVN.

It is unclear what the functional implications of immunogold density are in the primate PVN. It is reasonable to hypothesize that an increase in glutamate immunogold density may indicate nerve terminals that are more active, thus having a larger readily-releasable pool of synaptic vesicles. Conversely, as was shown in the rodent striatum (Meshul et al., 1999), glutamate immunogold density may be negatively correlated with release. The following experiments sought to pair a functional measure of neuronal activity, patch-clamp electrophysiology, with ultrastructural measures of presynaptic glutamate density in two cohorts of macaques that participated in the ethanol self-administration protocol. Because the ultrastructural analysis suggested glutamate, but not GABA, may be related to ethanol intake, the following analyses focused on presynaptic glutamate in the PVN.

4.3.2 Medium-term Ethanol Self-Administration:

Twelve cynomolgus males (cohort 13: 3 controls and 9 drinkers) with open-access conditions for approximately 7.5 months (188 ± 1 consecutive sessions) were sent to necropsy in the open-access phase of the protocol (see experimental timeline, Figure 4, page 24).

Effect of Ethanol on Relative Glutamate Immunogold Density:

Although eight of nine subjects with ethanol access were categorically defined as either heavy or very heavy drinkers, no group differences were found in glutamate density when compared to ethanol-naïve controls (Table 3; Figure 24, pages 101 and 102, respectively). Due to the limited between-subject variability in average daily intake,

assessing the relationship between average daily intake and immunogold density is unfairly weighted by a single subject when using a linear approach, such as a Pearson correlation. For this reason, a Spearman rank correlation was used. Average daily intake was not related to average immunogold density in AVP-immunopositive ($R_s = 0.20$, $p = 0.61$) or immunonegative terminals ($R_s = 0.60$, $p = 0.10$). Additionally, these data did not support a relationship between glutamate immunogold density within immunopositive or immunonegative AVP and CRH terminals (immunopositive: $R_s = 0.29$, $p = 0.56$; immunonegative: $R_s = -0.57$, $p = 0.20$).

Neurotransmitter	Postsynaptic Immunoreactivity	Presynaptic Immunoreactivity	Mean density (particles/μm^2) Controls	Mean density (particles/μm^2) Drinkers	p-value
Glutamate	AVP	AVP	76.6 (14.7)	80.0 (7.3)	0.85
		Non-AVP	73.9 (25.9)	83.3 (7.8)	0.76
	CRH $^{\Phi}$	CRH	78.4 (16.1)	87.0 (8.0)	0.67
		Non-CRH	74.4 (13.1)	87.5 (8.4)	0.45

Table 3 | Mean (± SEM) glutamate density in control (n = 3) and ethanol-drinking monkeys (n = 9). $^{\Phi}$ Two drinkers were excluded from the analysis due to insufficient glutamate immunogold density.

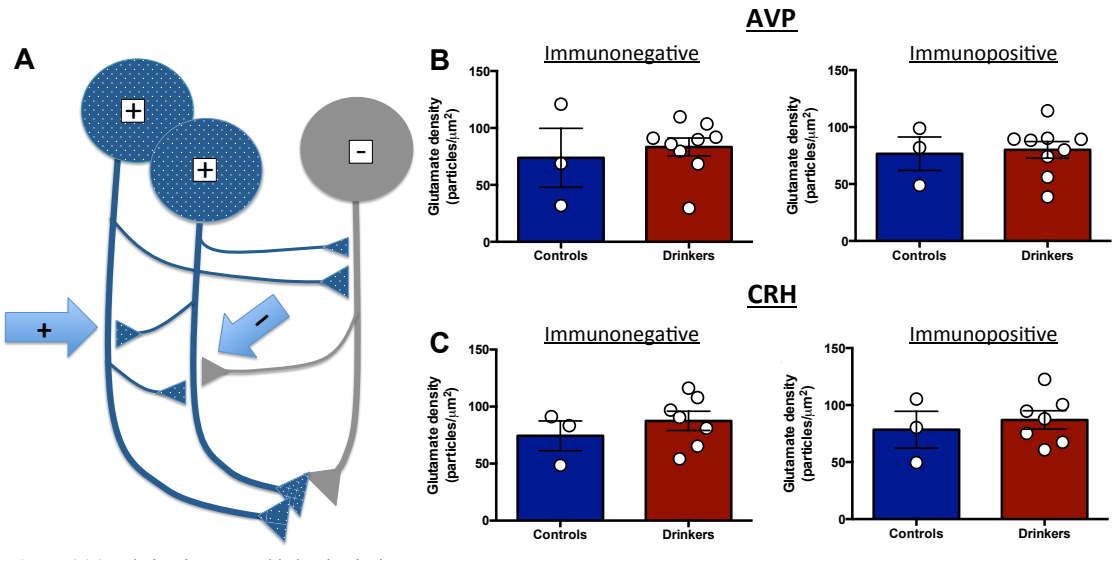

Figure 24 | Relative Immunogold Density (Cyno males). Relative immunogold density in immunonegative and immunopositive terminals contacting AVP- and CRH-immunoreactive postsynaptic structures. Schematic representation of the two types of terminals (A), where immunoreactivity is indicated by blue and "+" in the cell body, a non-immunoreactive neuron is illustrated in grey with a "–" in the soma. The "+" arrow points to an immunopositive terminal while the "–" points to an immunonegative terminal. No differences were found between the relative glutamate immunogold density for AVP-immunonegative (B, left) or AVP-immunopositive (B, right) terminals. No differences were found between the relative glutamate immunogold density for CRH-immunonegative (C, left) or CRH-immunopositive (C, right) terminals. Data points represent the average for individual animals, bars represent group mean ± SEM.

Effect of Ethanol on Glutamate Terminal Area and Synaptic Contact Length

In addition to changes in presynaptic neurotransmitter density, changes in the size of the terminal and the length of the synaptic contact may be harbingers of ethanol-sensitivity in the PVN (Bentea et al., 2017). However, among immunonegative presynaptic terminals contacting AVP-immunopositive dendrites neither terminal size (drinkers: 0.70 ± 0.05 μm^2; controls: 0.74 ± 0.17 μm^2; p = 0.84) nor the length of the synaptic contact (drinkers: 0.53 ± 0.04 μm; controls: 0.52 ± 0.07 μm, p = 0.91) differed between groups. Among immunopositive AVP terminals, terminal area did not differ (drinkers: 0.92 ± 0.05 μm^2; controls: 1.01 ± 0.62 μm^2, p = 0.59) but drinkers had significantly smaller synaptic contacts (0.52 ± 0.03 μm) when compared to controls (0.61 ± 0.02 μm, p = 0.04). Among immunonegative terminals contacting CRH-immunoreactive dendrites, drinkers had smaller terminals (drinkers: 0.80 ± 0.02 μm^2; controls: 0.91 ± 0.02 μm^2, p = 0.03) but no difference in synapse length (drinkers: 0.54 ± 0.04 μm; controls: 0.59 ± 0.04 μm, p = 0.51). In immunopositive CRH terminals no group differences were found in terminal size (drinkers: 1.03 ± 0.05 μm^2; controls: 1.12 ± 0.09 μm^2, p = 0.37) or contact length (drinkers: 0.54 ± 0.02 μm; controls: 0.53 ± 0.05 μm, p = 0.84).

Effect of Ethanol on Glutamate Signaling in the PVN

To follow up on the preliminary findings from rhesus females suggesting glutamate, but not GABA, within putative recurrent terminals was uniquely related to ethanol intake, whole-cell patch clamp electrophysiology was used to measure glutamate signaling onto parvocellular neurons in the primate PVN. In rats, parvocellular neurons

have been differentiated from magnocellular neurons by the presence of a low threshold spike and absence of a transient outward rectification in response to a depolarizing current step (See Figure 7, page 42; Luther and Tasker, 2000; Stern, 2001; Tasker and Dudek, 1991; Hoffman and Tasker, 1991). This is the first demonstration of these electrophysiological signatures in the primate PVN. Neurophysin staining has been used to support the differential electrophysiological response in rats (Hoffman & Tasker, 1991). Although this technique was not used here, the difference in capacitance between magnocellular and parvocellular neurons (see Table 4, page 105) suggests these two cell populations differ in size, which has been reported in both rats (Tasker and Dudek, 1991) and monkeys (Rafols et al., 1987). Blood and cerebral spinal fluid were collected at the time of necropsy to measure ethanol concentration. No ethanol was detected in either sample type for any of the subjects. The membrane properties for parvocellular and magnocellular PVN neurons are shown in Table 4 (page 105). These are averaged over 2-8 cells/animal. No significant differences were found between drinkers (n = 9 animals) and controls (n = 3 animals) within magnocellular or parvocellular membrane properties ($p > 0.05$ for all comparisons).

Parvocellular			
	Rm (GΩ)	Cm (pF)	Vm (mV)
Drinker	1.99 (0.21)	14.90 (1.07)	-46 (1)
Control	1.79 (0.20)	17.88 (1.11)	-49 (2)
Magnocellular			
	Rm (GΩ)	Cm (pF)	Vm (mV)
Drinker	1.82 (0.24)	21.58 (3.27)	-52 (2)
Control	2.45 (0.07)	20.33 (0.24)	-49 (1)

Table 4 | Membrane properties of putative parvocellular and magnocellular neurons recorded from cynomolgus males. These subjects were in an active drinking state, however there was no measurable ethanol in blood or CSF at the time of death. Data presented as mean (SEM). Rm: membrane resistance; Cm: membrane capacitance; Vm: resting membrane voltage.

Picrotoxin (100 μM, a $GABA_A$ antagonist) was added to the perfusate solution to isolate spontaneous excitatory transmission (i.e. basal). The frequency of spontaneous EPSCs were measured in voltage-clamp mode and compared between groups. The frequency of postsynaptic events are representative of presynaptic release probability, rather than postsynaptic receptor expression or subunit composition (Siggins et al., 2005; Weiner and Valenzuela, 2005). Spontaneous EPSC frequency did not differ between groups (drinkers: 4.08 ± 0.96 Hz; controls: 3.11 ± 0.80 Hz, p = 0.46; Figure 25A, page 107). No group differences were found in event characteristics that would indicate postsynaptic receptor differences (amplitude: drinkers: 35.3 ± 9.6 pA, controls: 24.3 ± 0.7 pA, p = 0.27; rise time: drinkers: 3.2 ± 1.4 ms, controls: 1.9 ± 0.0 ms, p = 0.38; decay time: drinkers 2.7 ± 1.1 ms, controls 1.9 ± 0.1 ms, p = 0.48; half-width: drinkers: 2.8 ± 1.1 ms, controls: 1.9 ± 0.1 ms, p = 0.45).

To test the hypothesis that parvocellular neurons adapt to the presence of

intoxicating concentrations of ethanol, spontaneous EPSCs were recorded the application of 20mM ethanol. For comparison, the range of average BECs measured in this cohort during the active open-access phase was 23 – 172 mg/dl (minimum of 34 BEC samples/animal). 20mM (approximately 92 mg/dl) is just below the group average BEC of 115 mg/dl measured seven hours into the drinking sessions. Slices from ethanol-naïve animals did not respond to 20mM ethanol bath application (frequency: 100.7 ± 20.5 % of baseline; $t(2) = 0.03$, $p = 0.98$). Compared to the pre-ethanol basal frequency, animals that had been drinking ethanol daily for over six months had a significant decrease (70.2 ± 8.1 % of baseline; $t(8) = 3.70$, $p = 0.0061$), although this difference was not significantly different from the ethanol-naïve controls ($p = 0.12$; Figure 25B, page 107). Furthermore, the range of suppression in spontaneous EPSC frequency was not related to average daily intake ($r = 0.01$, $p = 0.50$).

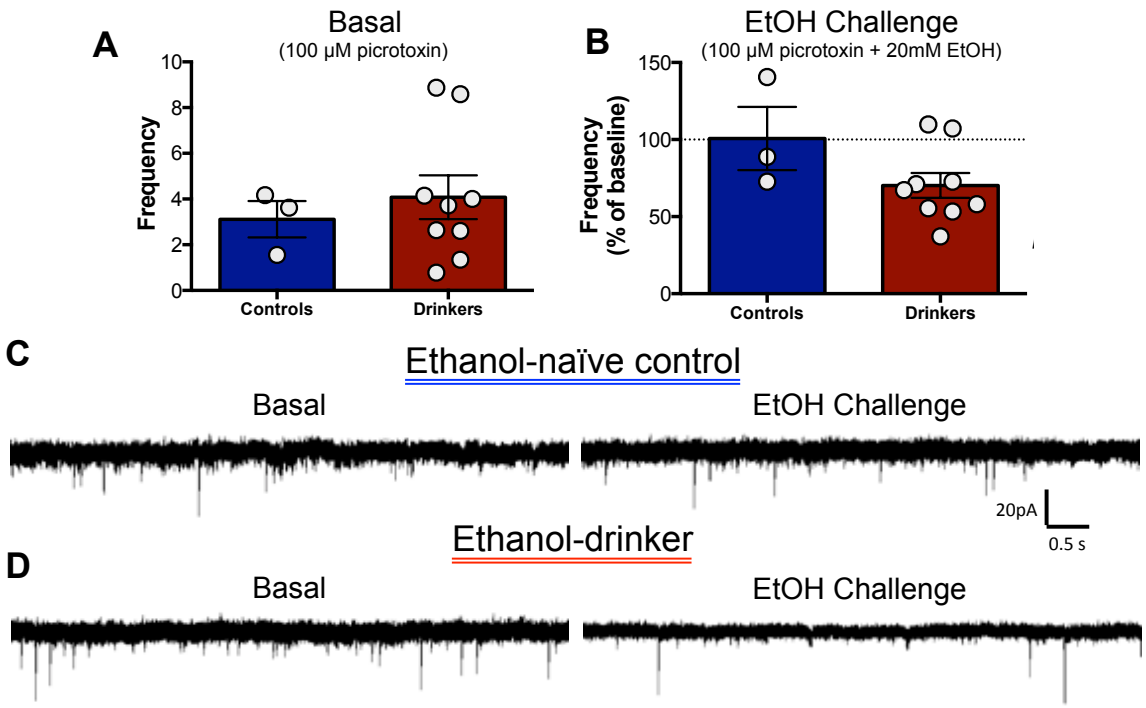

Figure 25 | Summary of Electrophysiology (Cyno males). Summary of spontaneous EPSC frequency under basal (A) or ethanol-challenged (20mM, B) conditions. No significant differences in frequency were found under the basal condition. In the presence of ethanol, ethanol-drinkers had a significantly lower frequency when compared to aCSF-baseline (* $p = 0.0061$), but no significant difference was found between groups. Representative traces of a single parvocellular neuron under basal (left) and ethanol-challenged (right) conditions from an ethanol-naïve control (C) and a ethanol-drinker (D). Data points represent the average for individual animals, bars represent group mean ± SEM.

Summary and conclusions:

To the best of our knowledge, these are the first electrophysiological recordings of the primate hypothalamic PVN. These neurons displayed electrophysiological signatures demonstrated previously in rats (Luther and Tasker, 2000; Stern, 2001; Tasker and Dudek, 1991; Hoffman and Tasker, 1991) allowing reliable differentiation between the two primary neuronal populations. Although the populations were not confirmed using a secondary technique, such as immunohistochemistry or polymerase chain reaction, the difference in capacitance is in agreement with these characteristic responses to a depolarizing current step.

No differences were found in the frequency of spontaneous EPSCs under basal conditions. However, when a physiologically relevant concentration of ethanol was applied, drinkers but not controls, had a decrease in spontaneous EPSC frequency compared to the basal activity. No significant differences were found in event characteristics such as amplitude or half-width that would suggest differences in post-synaptic receptor expression or subunit composition were found between groups. Thus, because a change in frequency of spontaneous events are attributed to changes in presynaptic release, the basal frequency being similar between drinkers and controls is in agreement with the ultrastructural data where glutamate immunogold density in both immunopositive and immunonegative terminals did not differ between drinkers and controls. Only when the system was challenged with ethanol was a significant difference in the frequency of spontaneous EPSCs observed. The ultrastructural analysis included comparisons of the size of the presynaptic terminals and the length of the synaptic contact. The size of glutamatergic terminals in the thalamus and cortex are related to their

response to electrical stimulation, axonal branching and whether they contact proximal or distal dendrites (Petrof and Sherman, 2013). Furthermore, electron microscopy studies have found relationships between the active zone area and glutamate release probability in the hippocampus (Schikorski and Stevens, 1997). In the data reported here, immunonegative CRH terminals were smaller in ethanol drinkers than in controls. These results are difficult to interpret on their own, primarily because it is unknown where these projections originate. Additionally, without serial sectioning it is not possible to know the location of the synaptic contacts (i.e., distal or proximal), thus the relative contribution to action potential generation. An interesting possibility is the crosstalk between AVP and CRH terminals, given the hypothesis that chronic stress, including ethanol, increases AVP in parvocellular PVN neurons. Evaluating this relationship at the ultrastructural level is possible with three unique electron-dense immuno-labels.

The ultrastructural analyses presented so far have not revealed consistent results. There are several possible explanations for these differences. First, the duration of ethanol self-administration may be a critical component. The duration-dependent effects of ethanol on the stress system have not been studied in the primate. However, although the drinking paradigm used here results in relatively stable self-administration patterns, there are categorical changes that occur over the experimental timeline (Baker et al., 2014). Specifically, animals changing from light to binge or heavy to very heavy drinkers may determine which homeostatic mechanisms are challenged. In fact, adrenal steroid hormones were found to be significantly different when comparing the first and second six-months of ethanol self-administration in heavy and non-heavy macaques (Helms et al., 2014). Second, the activation of the HPA axis in response to a variety of stressors

may be sex-dependent (Uhart et al., 2006). The combination of sex and duration of ethanol self-administration are important considerations. In rodents, sex-dependent HPA axis responses to long-term ethanol and withdrawal have been reported (Silva et al., 2009) and the effects of long-term alcohol use has sex-dependent effects in the human brain (Pfefferbaum et al., 2001). Finally, there may be species differences in PVN vulnerability to long-term ethanol self-administration. This last consideration seems less likely given the similarities between rhesus and cynomolgus macaques in the changes in circulating stress hormones over the experimental timeline.

Overall, the ultrastructural results were not replicated in this cohort of cynomolgus males. This may be due to differences in the duration of open-access, the species or sex. However, the current dataset did reveal that parvocellular neurons from monkeys with access to ethanol were more sensitive to a moderate dose of ethanol, showing a decrease in spontaneous glutamatergic events onto parvocellular neurons. Because action potentials were not blocked in the electrophysiological recordings presented here, there is still the possibility that this effect is partially mediated by projections to the PVN. This is unlikely, however, as the slices used for recordings contained only a portion of the lateral hypothalamus, which represents only a fraction of the glutamatergic projections to the PVN.

4.3.3 Long-term Ethanol Self-Administration with Repeated Forced Abstinence

Twelve rhesus males (cohort 10: 4 controls, 8 drinkers) had open-access conditions for approximately 14-months (425 consecutive sessions) before going through three forced abstinence phases (each 28-34 days) with approximately three months of ethanol self-administration in between. These subjects were sent to necropsy at the end of the third forced abstinence phase, 28-34 days after their last ethanol self-administration session (see experimental timeline, Figure 4, page 24).

Subject ID	12-month[ß] average ethanol intake (g/kg/day)	Final 3-month[ß] average ethanol intake (g/kg/day)
10208	2.3	2.8
10209	2.4	3.0
10210	1.6	2.0
10211	2.1	2.5
10212	2.3	4.6
10213	1.3	2.8
10214	4.2	5.0
10215	3.2	5.3

Table 5 | Average Daily Ethanol Intake During the First (12-months) and Last (3-months) Open-Access phases (Rhesus males). Average daily ethanol self-administered during the first and final open-access phases. [a]Approximation of the length of open-access phases, see text for number of open-access sessions and description of exclusion criteria.

Effect of Ethanol on Relative Glutamate Immunogold Density:

As shown above in Table 5 (page 111), the range of average daily intake (dark blue points) for these subjects was 1.3 – 4.2 g/kg/day during the first twelve-months of ethanol open-access. Abstinence differentially influenced heavy (heavy and very heavy) and non-heavy (binge and light) drinkers (Allen et al., under review). After repeated abstinence, heavy drinkers had a sustained increase in consumption while non-heavy drinkers had a transient increase but returned to pre-abstinence levels (Allen et al., under review). During the final three months of ethanol open-access the range of average daily intake was 2.0 – 5.3 g/kg/day (Table 5, page 111).

Similar to the rhesus females with approximately 13-months of ethanol open-access and cynomolgus males with approximately 7.5 months of ethanol open-access, no group differences were found in glutamate density in rhesus males with approximately 18-months of ethanol open-access and three forced abstinence periods when compared to ethanol-naïve controls (Table 6, Figure 26, pages 113 and 114, respectively). Average daily intake was not related to average immunogold density in immunopositive AVP (12-month: $r = -0.27$, $p = 0.52$; 18-month: $r = -0.19$, $p = 0.66$) or immunonegative AVP terminals (12-month: $r = -0.36$, $p = 0.38$; 18-month: $r = -0.47$, $p = 0.24$). Additionally, these data did not support a relationship between glutamate immunogold density within immunopositive (12-month: $r = 0.45$, $p = 0.27$; 18-month: $r = 0.40$, $p = 0.33$) or immunonegative (12-month: $r = 0.05$, $p = 0.91$; 18-month: $r = 0.34$, $p = 0.41$) CRH terminals.

Neurotransmitter	Postsynaptic Immunoreactivity	Presynaptic Immunoreactivity	Mean density (particles/μm^2) Controls	Mean density (particles/μm^2) Drinkers	p-value
Glutamate	AVP $^{\phi}$	AVP	88.6 (6.8)	75.0 (6.3)	0.20
		Non-AVP	95.6 (1.3)	84.8 (10.1)	0.32
	CRH	CRH	73.3 (13.1)	72.1 (9.4)	0.94
		Non-CRH	64.4 (4.4)	67.8 (7.5)	0.71

Table 6 | Relative Immunogold Density (Rhesus males). Mean (±SEM) glutamate immunogold density in control (n = 4) and ethanol-drinking (n = 8) monkeys. $^{\phi}$ n = 3

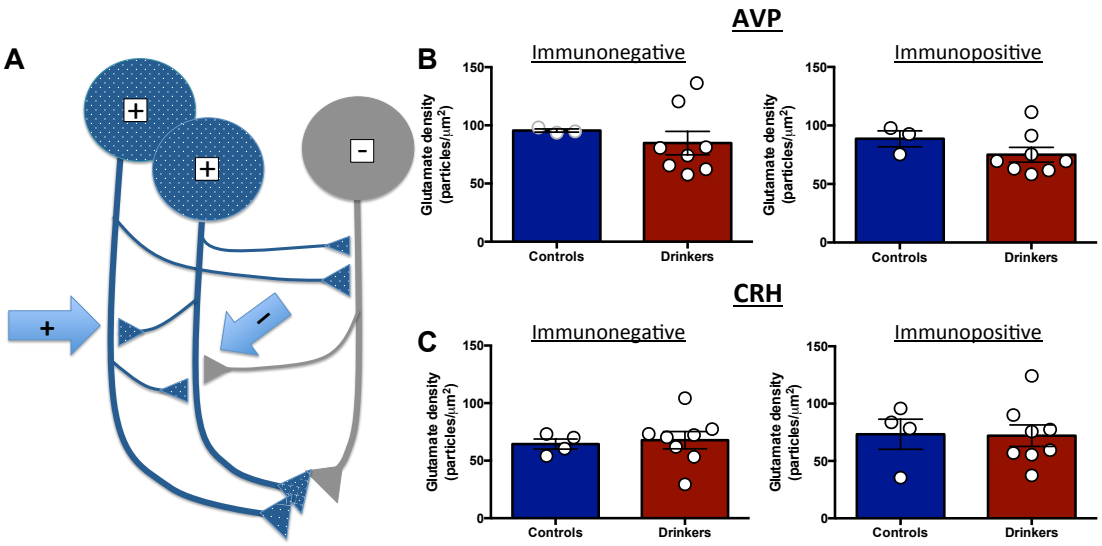

Figure 26 | Immunogold Density (Rhesus males). Relative immunogold density in immunonegative and immunopositive terminals contacting AVP- and CRH-immunoreactive postsynaptic structures. Schematic representation of the two types of terminals (A), where immunoreactivity is indicated by blue and "+" in the cell body, a non-immunoreactive neuron is illustrated in grey with a "–" in the soma. The "+" arrow points to an immunopositive terminal while the "–" points to an immunonegative terminal. No differences were found between the relative glutamate immunogold density for AVP-immunonegative (B, left) or AVP-immunopositive (B, right) terminals. No differences were found between the relative glutamate immunogold density for CRH-immunonegative (C, left) or CRH-immunopositive (C, right) terminals. Data points represent the average for individual animals, bars represent group mean ± SEM. Note: AVP analysis included 3 ethanol-naïve controls.

Effect of Ethanol on Glutamate Terminal Area and Synaptic Contact Length

Among AVP terminals, the size of the presynaptic terminal did not differ between groups for immunonegative terminals (drinkers: 0.84 ± 0.05 μm^2; controls: 0.82 ± 0.18 μm^2; p = 0.92) or immunopositive (drinkers: 1.05 ± 0.05 μm^2; controls: 1.11 ± 0.04 μm^2; p = 0.35) terminals. Similar for CRH terminals, neither immunonegative (drinkers: 0.81 ± 0.04 μm^2; controls: 0.88 ± 0.10 μm^2; p = 0.53) nor immunopositive (drinkers: 1.01 ± 0.05 μm^2; controls: 0.87 ± 0.08 μm^2; p = 0.20) terminals significantly differed by group. Furthermore, no differences were found in the length of synaptic terminals (drinkers: 0.44 ± 0.01 μm; controls: 0.41 ± 0.01 μm, p = 0.71).

Effect of Ethanol on Glutamate Signaling in the PVN

The membrane properties for parvocellular and magnocellular PVN neurons are shown in Table 7 (page 116). These are averaged over 2-6 cells/animal, 14-34 cells/group. The capacitance of magnocellular neurons showed a trend-level difference between drinkers and controls (p = 0.053). No other significant differences were found between drinkers and controls within magnocellular or parvocellular membrane properties (p > 0.05 for all comparisons).

Parvocellular			
	Rm (GΩ)	Cm (pF)	Vm (mV)
Drinker	1.36 (0.16)	18.39 (1.36)	-48 (2)
Control	1.48 (0.22)	16.14 (1.73)	-46 (1)
Magnocellular			
	Rm (GΩ)	Cm (pF)	Vm (mV)
Drinker	1.21 (0.06)	20.54 (1.77)	-49 (1)
Control	3.24 (0.96)	14.87 (1.14)	-45 (2)

Table 7 | Membrane properties of putative parvocellular and magnocellular neurons recorded from rhesus males. These subjects were in abstinence (28 – 34 days since the last ethanol self-administration session). Data presented as mean (SEM). Rm: membrane resistance; Cm: membrane capacitance; Vm: resting membrane voltage.

Picrotoxin (100 μM) was applied to isolate excitatory transmission. The frequency of spontaneous EPSCs were measured in voltage-clamp mode and compared between groups. Spontaneous EPSC frequency was higher in drinkers (4.79 ± 0.97 Hz) compared to controls (2.05 ± 0.54 Hz, p = 0.03, Figure 27A, page 118). No group differences were found in event characteristics that would indicate postsynaptic receptor differences between groups (amplitude: drinkers: 30.31 ± 1.44 pA, controls: 30.25 ± 3.51 pA, p = 0.98; rise time: drinkers: 1.67 ± 0.04 ms, controls: 1.66 ± 0.04 ms, p = 0.83; decay time: drinkers 1.86 ± 0.16 ms, controls 1.86 ± 0.08 ms, p = 1.0; half-width: drinkers: 1.82 ± 0.13 ms, controls: 1.76 ± 0.04 ms, p = 0.69). A weak trend was found where the average daily intake during the final three months of ethanol access was negatively correlated with basal frequency (r = -0.63, p = 0.095), but not with 12-month average (r = -0.33, p = 0.42) or total lifetime intake (r = -0.52, p = 0.19) indicating repeated ethanol self-administration and abstinence might have increased the activity of glutamate release onto parvocellular neurons.

To test the hypothesis that parvocellular neurons adapt to the presence of intoxicating concentrations of ethanol, 20mM ethanol was added to the picrotoxin. This concentration of ethanol is equivalent to 92 mg/dl, which is within the range of average BEC measured during open-access ethanol self-administration, 8 - 167 mg/dl. Both controls and drinkers had a decrease in spontaneous EPSC frequency with 20mM ethanol bath application (controls: 51.0 ± 8.4% of baseline; $t(2) = 5.83$, $p = 0.028$; drinkers: 57.1 ± 11.2% of baseline; $t(7) = 3.84$, $p = 0.0064$; Figure 27B, page 118). No significant difference in suppression of sEPSC frequency was found between groups ($p = 0.76$).

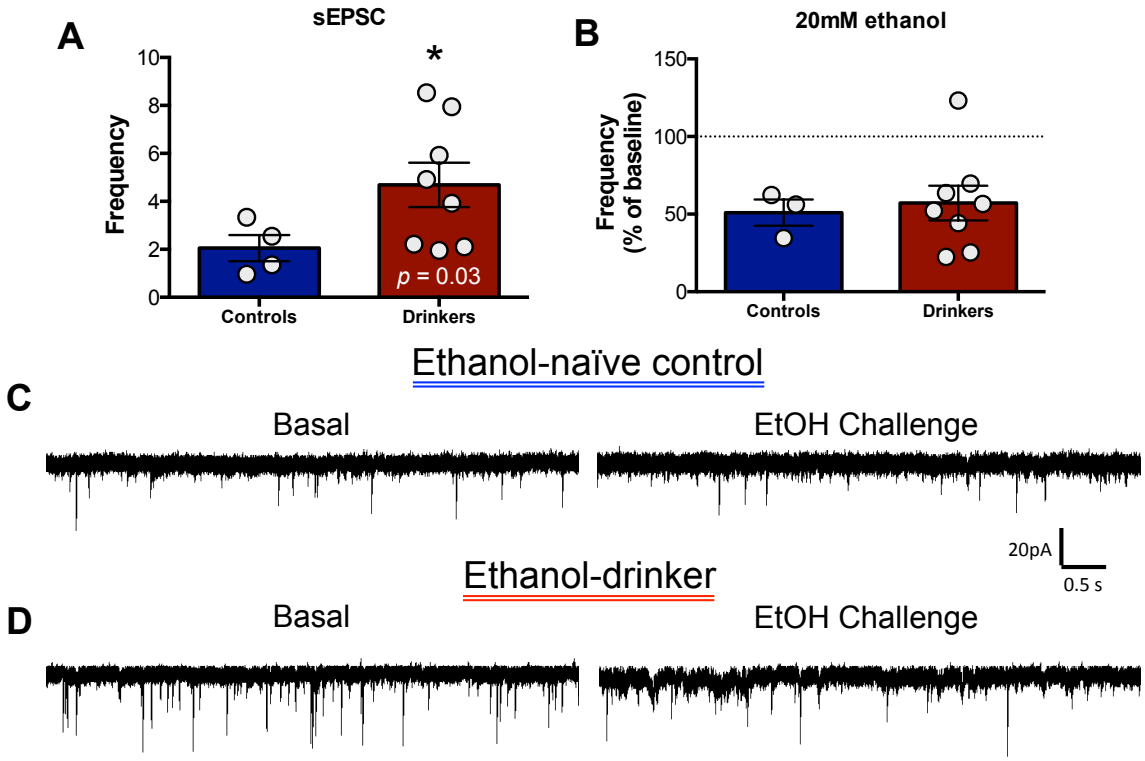

Figure 27 | Summary of Electrophysiology (Rhesus males). Baseline frequency of spontaneous EPSC (A) differed significantly between ethanol-naive controls and drinkers. Both groups had a significant reduction in frequency of spontaneous EPSCs with application of 20mM ethanol (B), but no difference between groups (note: one control animal did not have an ethanol challenge (n = 3)). Representative traces of a single parvocellular neuron under basal (left) and ethanol-challenged (right) conditions from an ethanol-naïve control (C) and a ethanol-drinker (D). ^ $p < 0.05$ compared to controls, * $p < 0.05$ compared to baseline. Data points represent the average for individual animals, bars represent group mean ± SEM.

Basal spontaneous EPSC frequency onto putative parvocellular neurons was positively correlated with cortisol ($r = 0.68$, $p = 0.01$, Figure 28, page 119), but not ACTH ($r = 0.44$, $p = 0.15$) under the low stress condition during the third abstinence. No relationship was found with ACTH or cortisol under mild stress conditions ($r = 0.14$, $p = 0.69$; $r = -0.07$, $p = 0.83$, respectively).

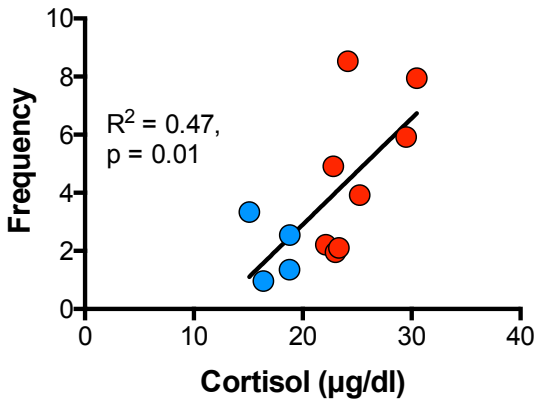

Figure 28 | Correlation between low stress cortisol during the third abstinence and frequency of sEPSCs in male rhesus. Spontaneous EPSC frequency under basal (aCSF + 100µM picrotoxin) conditions positively correlates with cortisol under the low stress condition in abstinence 3. Blue: ethanol-naïve controls, red: drinkers.

Summary and Conclusion:

In both cynomolgus (cohort 2: Cuzon Carlson et al., 2011) and rhesus (cohort 10: Allen et al., under review) males, repeated forced abstinence has been shown to influence both ethanol self-administration behavior and stress hormones. Interestingly, the changes were not always consistent. Among the similarities, 28-days of forced abstinence significantly increased cortisol in the low stress condition for both species. Additionally, ethanol self-administration increased when ethanol open-access was reinstated (Cuzon Carlson et al., 2011; Allen et al., under review). However, the cynomolgus males showed markedly reduced variation in their average daily intake following each abstinence phase, while the rhesus males did not. In addition, heavy and very heavy drinkers in the rhesus cohort demonstrated a sustained elevation in their ethanol intake while this was transient in the cynomolgus males.

Among the ethanol-naïve controls, both the cynomolgus males reported above (cohort 13) and the rhesus males shown here (cohort 10) had similar basal glutamatergic frequency (cynomolgus: 3.14 ± 0.80 Hz, n = 3; rhesus: 2.05 ± 0.54 Hz, n = 4), perhaps suggesting similar basal circuitry of excitatory input across these two species. In the repeated forced abstinence protocol, rhesus males with ethanol access had a higher frequency of spontaneous EPSCs, which positively correlated with cortisol under low stress conditions during the final abstinence. Importantly, the longitudinal analysis of cortisol under the low stress condition revealed that drinkers and controls responded similarly to the experimental phases, with the exception of the final abstinence where the ethanol-naïve controls no longer exhibited a significant increase in cortisol. These data bring to light the potential role of the maltose-dextran solution. During open-access

conditions each ethanol-naïve control animal is yoked to a drinker with similar body weight and receives a daily maltose-dextran solution to match the calories consumed in the ethanol. During abstinence, the activation of the HPA axis in ethanol-naïve animals may be due to an anticipatory response due to the absence of this high value commodity. However, after repeated experience with the abstinence phase the ethanol-naïve controls no longer have elevated cortisol while the ethanol-drinking animals continue to show this response. These data help to parse apart the effect of repeated stress (removal of highly palatable solution) from the additive effect of ethanol self-administration by showing that the ethanol-naïve controls are able to adapt to the repeated abstinence, while the drinkers may be experiencing a physiologic response due to the allostatic state established by long-term ethanol self-administration.

The drinkers had significantly higher basal spontaneous EPSC frequency than controls. Interestingly, although the 20mM ethanol challenge decreased the frequency of glutamatergic activity to a similar degree in both drinkers and controls (approximately 55% reduction), it is notable that this would reduce the frequency of spontaneous EPSCs under the basal condition to that of the ethanol-naïve controls. Further support can be found in the average BEC in early post-abstinence (defined as the first 28-days of open-access following an abstinence period) where after the first and second abstinence phases the average BEC for 6 of 8 drinkers was above 90 mg/dl (Allen et al., under review). Allen and colleagues additionally show that within one week into open-access following abstinence cortisol concentrations are significantly reduced, while ACTH concentration remains elevated.

No differences were found in glutamate immunogold density, presynaptic terminal size or synaptic contact length between drinkers and controls. One possible explanation is that if there had been changes in presynaptic glutamate related to ethanol self-administration, these may have recovered during the 28-34 days of abstinence, although this seems unlikely given the significantly higher basal (aCSF) spontaneous EPSC frequency in drinkers compared to controls (Figure 27A, page 118). An important consideration is that although the electrophysiological recordings were made from putative parvocellular neurons, the molecular identity of these neurons could be non-CRH or non-AVP, such as vasoactive intestinal peptide, enkephalin, cholesystokinin, angiotensin II, neurotenin, among others. Future efforts should be made to identify the molecular identity following electrophysiology recordings to better understand the unique contributions of this diverse population of neurons.

These data indicate that while GABA and glutamate make up the majority of fast synaptic signaling in the PVN, their role in regulating the activity of parvocellular neurons is complex. Retrograde signaling, membrane channel activation and intra-PVN signaling via recurrent collaterals and/or dendritic peptide release also influence the activity of parvocellular neurons (see Figure 3, page 14). The complex circuitry is important to consider for the results presented here. First, several of the modulatory roles of monoamines or neuronal crosstalk were examined in preautonomic neurons. This population of parvocellular neurons projects to the brainstem and incorporates the sympathetic limb of the autonomic nervous system. Splanchnic nerve activation and increased norepinephrine concentration sensitize the adrenal cortex to ACTH. The monkey model of ethanol self-administration routinely finds a dissociation between

circulating concentrations of ACTH and cortisol. A possible mechanism for this finding could be that the decrease in ACTH under low stress conditions is a consequence of long-term ethanol self-administration on neuroendocrine parvocellular neurons, while cortisol concentration is maintained due to a compensatory shift towards activation of preautonomic parvocellular neurons, upregulation of splanchnic nerve activation and plasma concentration of norepinephrine which sensitizes the adrenal cortex to ACTH (Edwards and Jones, 1987). Future studies should incorporate longitudinal measures of sympathetic tone to better understand its role. Second, it is currently unknown if the modulatory mechanisms described above are also involved in regulation of GABA and glutamate onto neuroendocrine parvocellular neurons and if so, to what extent. Finally, the effect of ethanol on these mechanisms within the PVN is currently unknown. The electrophysiological analysis of glutamatergic activity onto neuroendocrine parvocellular neurons presented here did not reveal any changes suggestive of postsynaptic differences (amplitude of spontaneous events or decay time, for example). This is consistent with the ultrastructural findings and suggests that in the PVN, presynaptic mechanism contribute to the dysregulation of the HPA axis.

The ultrastructural relationship between the relative glutamate immunogold density in putative recurrent CRH and AVP terminals and average daily ethanol self-administration found in female rhesus macaques with long-term ethanol self-administration was not found in male cynomolgus with medium-term ethanol self-administration or rhesus males with long-term ethanol self-administration and repeated forced abstinence. It is important to consider the “knowns and unknowns” with electron microscopy and electrophysiology. In electron microscopy, there is a good amount of

certainty that the immunopositive CRH and AVP terminals are recurrent terminals belong to parvocellular neurons. Recurrent collaterals have been found among parvocellular PVN neurons in both rodents and monkeys (Van den Pol, 1982; Rafols et al., 1987), but further evidence is drawn from the limited number of direct glutamatergic projections that express CRH or AVP immunoreactivity (see Figure 20, page 87 for circuitry). Although CRH-immunoreactive terminals originating in other brain regions, such as the BNST or amygdala project to the PVN (Herman et al., 2003 and Figure 20, page 87) and could contribute to the present results indirectly, these projections are primarily GABAergic thus represent a separate population from the terminals that were found to be related to ethanol self-administration. However, in ultrastructural analyses the post-synaptic dendrite is largely unknown. Aside from whether or not it is immunoreactive for CRH or AVP, the population (magnocellular, autonomic parvocellular or neuroendocrine parvocellular) is unknown. In electrophysiology, these "knowns and unknowns" are reversed. The postsynaptic neuron is reliably identified with a signature response following a depolarizing current step. However the presynaptic terminals responsible for the frequency of excitatory events (whether they are recurrent terminals, peri- or extra-PVN) are unknown. These are important considerations when trying to hypothesize a connection between ultrastructural and functional techniques.

In female rhesus macaques a relationship between glutamate immunogold density and ethanol intake was found, but the functional correlates of this are unknown. One hypothesis is that basal glutamatergic activity (frequency) would correlate with immunogold density, indicating that synaptic terminals have a higher relative immunogold density due to increased activity. If this were the case, the highest EPSC

frequency would be expected in animals with the lowest average daily intake, as they had the highest immunogold density overall (immunopositive CRH and AVP terminals). On the other hand, if the relative glutamate immunogold density were increased due to a lack of release (i.e., the synaptic vesicles are "backed-up"), then animals with the highest average daily intake would be expected to have higher spontaneous EPSC frequencies than those with lower levels of self-administration. Future studies are required to determine which of these hypotheses are at play in the macaque PVN.

CHAPTER 5: Discussion

5.1 Summary of findings

This dissertation represents an investigation into the consequences of daily ethanol self-administration on the HPA axis of macaques. Chapter three was devoted to the evaluation of the principal stress hormones, ACTH and cortisol, in response to two distinct stress conditions. These analyses compared baseline (pre-ethanol) to open-access (post-ethanol), using a within-subject design. The cohorts included in these analyses varied in the duration of open-access ethanol self-administration; ranging from medium-term (approximately 7.5 months) and long-term (approximately 13-months) to long-term with repeated forced abstinence (20 months of ethanol self-administration with three 28-35 day forced abstinence phases; see Figure 4 (page 24) for experimental timeline and Table 1for cohort details, page 25). The results show a strong negative correlation between ACTH, measured under low stress conditions following medium- and long-term ethanol self-administration, and average daily ethanol self-administration while ACTH under the mild stress condition did not significantly differ from baseline. Cortisol, under both the low and mild stress conditions, did not significantly differ between baseline and following medium- or long-term ethanol self-administration. An extended self-administration protocol with three repeated forced abstinence phases interwoven with months of ethanol access, revealed that cortisol, but not ACTH, in the low stress condition was elevated during forced abstinence. This effect was initially present in both ethanol-drinking and ethanol-naïve animals in the first abstinence phase, but only in the ethanol-drinking animals at the last abstinence phase. In response to mild stress, the

ACTH response was persistently dampened following the first abstinence phase, while cortisol did not significantly change in relation to the availability of ethanol. Overall, these data show that the HPA axis response to low and mild stress conditions are responsive to long-term ethanol self-administration as well as periods of forced abstinence.

Chapter four focused on the structure and function of hypothalamic paraventricular neurons. The structural analysis revealed that glutamatergic and GABAergic immunogold density in presynaptic terminals immunopositive for CRH and AVP were uniquely related to ethanol and water self-administration in rhesus females with approximately 13-months of open-access. These ultrastructural findings were not replicated in cynomolgus males with approximately 7.5-months of open-access ethanol self-administration or in rhesus males with approximately 20-months of open-access ethanol self-administration and repeated forced abstinence. However, measures of glutamatergic neurotransmission onto the parvocellular neurons of the PVN revealed ethanol-specific changes in glutamatergic activity. Under basal (aCSF + 100μM picrotoxin) conditions, drinkers and ethanol-naïve controls did not differ in the frequency of spontaneous EPSCs onto parvocellular neurons measured in cynomolgus males who were sent to necropsy during the open-access phase of the experimental timeline. However, a physiologically relevant concentration of ethanol (20mM, approximately 96 mg/dl) significantly decreased the frequency of spontaneous EPSCs onto parvocellular neurons in male cynomolgus macaques with a history of ethanol self-administration, but had no effect on the ethanol-naïve controls. In contrast, rhesus males who were sent to necropsy 28-35 days after the last ethanol self-administration session had a greater

frequency of excitatory events onto parvocellular neurons when compared to ethanol-naïve controls under basal (aCSF) conditions. Similar to the cynomolgus males, application of the binge-concentration of ethanol significantly decreased the frequency of excitatory events equally in drinkers but also revealed a significant decrease in the ethanol-naïve controls that was not present in the cynomolgus control subjects. These ultrastructural and functional analyses support a role for presynaptic glutamatergic signaling in HPA axis dysregulation following daily ethanol self-administration. The results of chapters three and four are summarized in Figure 29:

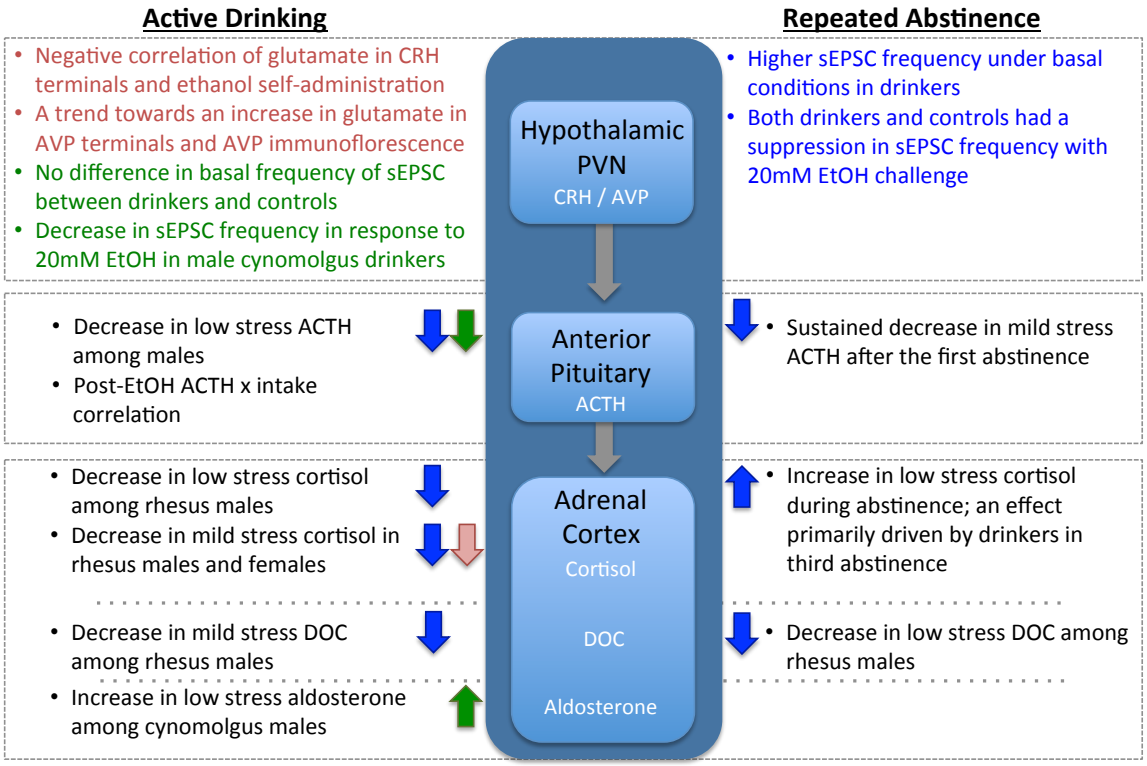

Figure 29 | Summary of Results. A summary of the findings for each level of the HPA axis during active drinking (left) and repeated abstinence (right). Red, blue and green text indicates rhesus females (cohort 6b), rhesus males (cohort 10) and cynomolgus males (cohort 13), respectively. For the pituitary and adrenal results, cohorts are indicated by arrows representing increases or decreases and are colored red, blue or green to indicate female and male rhesus or male cynomolgus, respectively.

5.2 Evidence of Allostasis

Understanding the unique consequences of long-term ethanol consumption on the HPA axis in humans has been a challenge. Having control over key experimental variables (for example how much and how often ethanol is consumed, the age when drinking began, family history, and environmental conditions) in an animal model with similar propensity to consume ethanol and shared neuroendocrine physiology offers an unparalleled opportunity to understanding this complex relationship. As outlined in Chapter 1, the HPA axis response in alcoholic subjects is largely dependent on whether the participants are actively drinking or in acute or protracted abstinence, and the evidence indicating that the HPA axis recovers after several weeks of abstinence is mixed. The data presented here suggests that in actively drinking monkeys with no history of abstinence, the HPA axis is functioning appropriately under low and mild stress conditions. While low stress concentrations of ACTH were dampened following open-access conditions, this is not believed to represent a pathological state. Basal ACTH concentrations below 50 pg/ml from rhesus macaques have been previously reported (Williams et al., 2003; Pascoe et al., 2008). Importantly, these earlier studies utilized indwelling catheters, thus avoiding the use of anesthesia or handling-induced activation of the HPA axis. More importantly in the data presented here, is that despite lower ACTH concentration under the low stress condition, the mild stress condition stimulated an elevation in both ACTH and cortisol indicating that the HPA axis maintained appropriate reactivity to changing conditions. Finally, the concentration of ACTH decreased in both drinkers and controls suggests that these animals continued to acclimate to the home-cage blood draw procedure after the baseline sample had been

collected. The extended ethanol self-administration protocol with alternating phases of ethanol access and forced abstinence, however, revealed disruptions in both low and mild stress response. Given that cycles of abstinence and relapse are a hallmark of AUDs, this extended protocol is highly translational in capturing a key feature of this disorder. It is important to note that during open-access self-administration, each ethanol-naïve control was given a maltose-dextran solution to match the calories from ethanol. During forced abstinence, this was withheld and may account for the increase in cortisol reported here as maltose-dextran is a highly palatable solution that is rapidly consumed and is believed to be of high value (unpublished observation). Loss of control and a decrease in predictability are known to potentiate the stress response (Weiss, 1968; Weiss, 1971) and these principles likely extend beyond noxious stimuli. By the third forced abstinence phase the ethanol-naïve animals no longer had elevated cortisol under the low stress condition, while the ethanol-drinking animals continued to show this response. Importantly, this acute effect of forced abstinence on cortisol concentration (measured three days after the last ethanol session) was correlated with a sustained difference (measured 28-35 days after the last ethanol session) in the frequency of spontaneous EPSCs onto parvocellular neurons in the PVN. In this same cohort Allen et al. (under review) has shown that when ethanol is reintroduced after the first and second abstinence phases, there is an increase in average daily ethanol self-administration and a decrease in cortisol concentration. These findings are consistent with the results of the 20mM ethanol challenge. This binge-concentration of ethanol resulted in approximately 50% reduction in the frequency of excitatory events which normalized the frequency of excitatory events measured in drinkers to the range found in controls under basal (aCSF + 100μM

picrotoxin) conditions, supporting the development of an allostatic state. The difference between drinkers and controls in the basal frequency of spontaneous EPSCs was not found with medium-duration ethanol self-administration, suggesting the additive stress of repeated forced abstinence contributes to the dysregulation of glutamate activity at the level of the PVN. The effect of this ethanol challenge was similar in rhesus and cynomolgus males with a history of ethanol self-administration. However, the in-vitro ethanol challenge influenced the frequency of excitatory events uniquely in the ethanol-naïve controls in these cohorts, having no effect in the cynomolgus males with no history of abstinence and mirroring the decrease in the frequency of excitatory events found in the drinking rhesus males in the repeated forced abstinence protocol. Again, the control subjects in the repeated abstinence protocol showed an elevated cortisol response to the first two abstinence phases, demonstrating that even in the absence of ethanol the forced abstinence phase was a stressor but suggesting that these animals were able to adapt to the conditions as they did not have elevated cortisol during the final abstinence phase. This suggests that repeated stress alters the glutamatergic activity onto parvocellular neurons of the PVN resulting in a sensitization to the effects of ethanol. Additional studies are needed to confirm this, but it is in line with data showing that repeated stress alters neurotransmitter signaling in the PVN (Miklos and Kovaks, 2005; Flak et al., 2009) and that exposure to stress increases ethanol self-administration (Sinha, 2012; Becker, 2012). If this hypothesis is correct, this could be an important mechanism in the relationship between a history of stress and risk for developing an AUD.

In male cynomolgus and rhesus macaques, ACTH decreased for both drinkers and naïve animals following medium- and long-term self-administration protocols. The

concentration of ACTH following open-access conditions (both medium- and long-term) negatively correlated with average daily intake, while no relationship was found between ACTH at baseline and future ethanol self-administration. As discussed above, the decrease in ACTH found in males may reflect a habituation of the stress response to the blood collection procedure that continued after the baseline sample was collected. Regardless, these data show a strong relationship between post-ethanol ACTH concentration under the low-stress condition and average daily ethanol self-administration in both males and females. Importantly, it has previously been demonstrated that a moderate dose of ethanol (0.75 g/kg, ingested over 15 minutes) blunted the ACTH response to o-CRH (Waltman et al., 1993). In the current studies, BECs were not routinely analyzed in the morning or during the low and mild stress procedures, leaving the possibility that the heavier drinkers may have had ethanol in their system which may contribute to the negative between ACTH and average daily intake.

Given the relationship between stress and alcohol intake, one hypothesis generated from the results presented here is that the stress response elicited by the schedule-induced polydipsia procedure may be predictive of future heavy drinking. Because both drinkers and controls experience this induction procedure for approximately four months, the HPA axis response to this long-term stressor may set the stage for future heavy drinking by similarly "priming" parvocellular neurons to the effects of ethanol. Previous work in rodents and monkeys has shown that schedule-induced polydipsia elicits an activation of the HPA axis even under the induction of water (Brett and Levine, 1981; Helms et al., 2013) and that adrenal steroids are required for acquisition of SIP (Levine and Levine, 1989). However, in monkeys cortisol

concentrations are generally stable while ACTH levels are more variable during SIP (Helms et al., 2013), indicating important species differences in the involvement of the HPA axis in SIP. Although outside the scope of this dissertation, future studies should investigate the stress response to SIP as a risk factor.

While the focus of this dissertation was on ACTH and cortisol, additional adrenal steroids from each layer of the adrenal cortex were analyzed. These data are particularly important as the role of neurosteroids in alcohol use and dependence continues to evolve. Like the relationship between ethanol self-administration and the HPA axis, the relationship between neurosteroids and ethanol self-administration is complex. Data suggests that neurosteroids can influence ethanol self-administration (for review, see Morrow et al., 2006) while adrenal steroids and their neuroactive metabolites are regulated, at least partially, by the HPA axis and also influence the PVN (Porcu et al., 2006; Womack et al., 2006; Gunn et al., 2015; Jimenez et al., 2017a). The regulation of PVN activity by neuroactive steroids represents another possible mechanisms for ethanol-modulated feedback. The current data show that the layers of the adrenal cortex are uniquely affected during the self-administration and repeated abstinence protocols that suggest sex- and species-specific alterations. Specifically, following the self-administration protocol, male rhesus had reduced dexamethasone suppression of DOC while cynomolgus males had increased suppression, suggesting an important species difference. Importantly, data from cohort 10 included the DOC response to dexamethasone following 6-months of ethanol self-administration, showing a significant reduction in dexamethasone suppression at the same experimental timepoint which further supports the importance of species rather than duration of open-access.

Furthermore, no differences were found in female rhesus between baseline and post-open-access, indicating that this may be a sex- and species-specific effect. From the zona reticularis, males (rhesus and cynomolgus) but not females (rhesus) had blunted DHEAS suppression following dexamethasone while aldosterone from the zona glomerulosa had not changed. Previous research has shown that DHEA supplementation in humans may interact with a subset of GABAergic receptors to increase AVP in response to exercise stress (Deuster et al., 2005) and that glutamatergic receptors are positively modulated by DHEAS in the hippocampus and frontal cortex (see Perez-Neri et al., 2008 for review). The effects of adrenal steroids and their neuroactive metabolites on neuronal activity in the PVN during ethanol self-administration and withdrawal are exciting areas for future research that will be critical for integrating ethanol-related changes in adrenal secretions to the larger network of stress physiology.

There is evidence for the development of an allostatic state at each level of the HPA axis. In the PVN, ultrastructural GABA and glutamate immunogold density were uniquely related to fluid self-administration in female rhesus. This relationship was not found in male macaques with medium-term or long-term and repeated forced abstinence. In cynomolgus males with medium-term access to ethanol self-administration, there were no differences in basal frequency of excitatory events between drinkers and ethanol-naïve controls, however a binge-concentration of ethanol significantly reduced the frequency of excitatory events in animals with a history of ethanol self-administration. Finally, in the rhesus males with long-term ethanol self-administration and three cycles of forced abstinence with intervening open-access conditions in between, animals with a history of ethanol self-administration had significantly greater frequency of excitatory events when

compared to ethanol naïve-controls, even though the last ethanol session was 28-35 days prior to necropsy. Additionally, the binge-concentration of ethanol reduced the frequency of excitatory events in the animals with a history of ethanol self-administration similar to the cynomolgus males with medium-term open-access conditions. However, this concentration of ethanol also reduced the frequency of excitatory events in the ethanol-naïve controls, which was not found in the medium-term open-access cynomolgus males. These data suggest that glutamate signaling in the PVN is altered by ethanol and by repeated periods of abstinence of a palatable substance (maltose dextran or ethanol). At the anterior pituitary there was a strong negative correlation between ACTH and average daily ethanol intake, although no overall difference between drinkers and ethanol-naïve controls was found. This may suggest the HPA axis response to stress is associated with intake during open-access. At the adrenal gland, the data show that repeated cycles of ethanol self-administration and forced abstinence disrupts cortisol concentration under the low stress conditions and dampens the ACTH response to mild stress. Each layer of the adrenal cortex showed evidence of species-, sex- and protocol- (open-access or repeated abstinence) specific effects related to ethanol self-administration. Future studies are needed to understand the mechanisms behind these changes and how this contributes to allostasis in the hypothalamus and pituitary.

5.3 Sex Differences in PVN Response to Stress and Ethanol

Sex differences have been reported in the physiological response to stress and in the HPA axis response to drugs of abuse (Uhart et al., 2006; Kudielka and Kirschbaum, 2005; Becker and Hu, 2008; Bale and Epperson, 2015), including alcohol (York and

Welte, 1994). Furthermore, the rate of mood and anxiety disorders are higher in females than males, and females have a greater risk of negative health consequences following alcohol use despite lower rates of alcohol use disorders (WHO, 2017).

The data presented here show that male and female rhesus macaques have baseline (ethanol-naïve) differences in the concentration of stress hormones and in sensitivity to dexamethasone inhibition in select adrenal steroids. Specifically, male and female rhesus macaques differ in basal (low stress) ACTH and DOC concentration and in the sensitivity of DOC and DHEAS to dexamethasone suppression. Furthermore, these data show similar average daily ethanol self-administration among males and females, but differences in average blood ethanol concentration. As discussed in Chapter three, differences in the pattern of ethanol intake over the daily 22-hour sessions is responsible for this finding. The HPA axis interacts with the hypothalamic-pituitary-gonadal (HPG) axis (Viau, 2002). This reciprocal interaction inhibits reproductive behavior and physiology by activation of the HPA axis, while gonadal hormones (i.e. testosterone and estrogen) also regulate the HPA axis. Animal models are a valuable tool in understanding sex differences in the HPA axis response to ethanol. Rodent models of ethanol exposure and withdrawal have revealed sex differences in CRH and AVP within the PVN. An intraparitoneal administration of ethanol for three consecutive days decreased testosterone in males but had no effect on estradiol or progesterone in females (Przybycien-Szymanska et al., 2010). This protocol also increased CRH and AVP mRNA in the medial PVN of male, but not female, rats. Using a 6-month liquid diet paradigm, female rats were found to have a greater decrease in the number of CRH- and AVP-immunoreactive neurons in the medial-PVN compared to males (Silva et al., 2009). Two-

months of abstinence resulted in a partial recovery in males, but further loss in females (Silva et al., 2009). These data highlight that sex differences are present in ethanol-naïve animals, extend beyond the initial exposure to ethanol, and likely contribute to neurobiological changes that occur during abstinence.

Sex differences are important when considering the ultrastructural findings presented here. The ultrastructural findings in putative recurrent CRH and AVP terminals related to ethanol self-administration in the rhesus females were not replicated in either of the male cohorts examined here. In rats, ethanol-naïve males have been reported to have a larger PVN (Silva et al., 2009), a greater number of CRH-immunoreactive neurons (Silva et al., 2009; Przybycien-Syzmanska et al., 2010), and fewer AVP-immunoreactive neurons (Silva et al., 2009) when compared to females. In addition to sex differences in the PVN, extra-PVN regions that regulate the stress response have also show sex-dependent differences. For example, rodent models have revealed higher rates of action potentials in locus coeruleus neurons in females compared to males who overexpress CRH, showing that norepinephrine neurons are more sensitive to CRH over-activation in females due to reduced receptor internalization (Bangasser et al., 2013). Importantly, the locus coeruleus is a major source of norepinephrine and contributes to the stress response. Thus, sex-differences in this region may implicate or recruit sex-specific circuits in the stress response. In the rhesus macaque, supplementing ovarian steroids (estrogen or progesterone) in ovariectomized females decreased the concentration of CRH in the PVN (Bethea and Centeno, 2007) and decreased with GABAergic content in the pituitary stalk and hypothalamus (Mirkes & Bethea, 2001), which may contribute in regulating the PVN that is sex-specific.

5.4 Future Directions

Due to the limited CRH and AVP in glutamatergic projections to the PVN (Figure 20, page 87), the ultrastructural analysis indicates a unique relationship between putative recurrent terminals and fluid intake in rhesus females. Although parvocellular PVN collaterals have been identified previously (Van den Pol, 1982; Rafols et al., 1987), these have not been extensively characterized, particularly in the macaque brain. Verification of recurrent axon collaterals should be made following a microinjection of biotinylated dextran amine (BDA), an anterograde tracer, into the PVN. At the light level, neuronal reconstructions would verify the presence of axonal ramifications within the borders of the PVN. Ultrastructurally, serial sections should be analyzed for unbiased estimates of the density of synaptic contacts (number of labeled terminals as a proportion of all terminals within the test sections) across the rostral-caudal extent of the PVN. A combination of neuronal tracing and immunohistochemistry would further characterize these connections. For example, a retrograde tracer, such as fluorogold, into the brainstem and spinal cord or circulating blood would label preautonomic parvocellular neurons or peripherally-projecting magno- and neuroendocrine parvo-cellular neurons, respectively. In combination with immunohistochemically labeled neurons, such as neurophysin-I or -II to investigate OXY- and AVP-magnocellular neurons, respectively, or a number of proteins expressed in the parvocellular PVN (particularly CRH and AVP) would further characterize the relative contributions to recurrent collaterals as well as identify the postsynaptic population targeted by the collaterals. These analyses would provide the foundation for studying the reorganization of specific recurrent terminals following ethanol self-administration and repeated forced abstinence as well as

understanding the physiological role recurrent collaterals play in maintaining homeostatic function.

It is critical to understand the neuroendocrine adaptations that occur with repeated cycles of drinking and abstinence, and whether these differ between males and females. The data presented here from male rhesus suggests that a history of stress (as indicated by elevated cortisol) and ethanol self-administration may sensitize parvocellular PVN neurons to the effect of acute ethanol. However, there was no evidence for a difference between drinkers and controls at the ultrastructural level. It is possible that in the active drinking phase, rhesus males had a similar relationship between immunogold density in putative recurrent axon collaterals and average daily ethanol, but that the removal of ethanol results in a reorganization of glutamatergic signaling, consistent with recovery of downstream stress hormones during prolonged abstinence (Adinoff et al., 1991; 2005a; 2005b). The timecourse of ultrastructural changes are currently unknown. Alternatively, the relative immunogold density and ethanol self-administration may be female-specific. Adding females to the repeated abstinence study design is necessary to answer these questions.

5.5 Final Comments

The changes in circulating stress hormones presented here are not uncommon following long-term stress. Specifically, the dissociation between ACTH and cortisol has been reported across many physiologic and psychological disorders, as mentioned above and reviewed by Bornstein and colleagues (2008). Furthermore, an increase in glutamatergic and noradrenergic synapses onto CRH neurons has been reported with

chronic variable stress paradigms (Flak et al., 2009). This suggests that the long-term consequences of ethanol self-administration may not be unique, but instead represent a challenge to homeostasis and recruitment of a general physiological adaptation that sustains the functional integrity of the system; i.e. allostasis.

Understanding the relationship between ethanol self-administration and stress should continue towards understanding allostasis at an organismal level, incorporating extra-hypothalamic limbic regions, a wider view of adrenal steroids and how they orchestrate homeostatic processes, and how the sympathetic nervous system is recruited and participates in allostasis. The NHP model described here is built firmly on a foundation of rodent and human research, and it will continue to bridge these two areas of research, extending our knowledge where it is uniquely situated to do so. Ultimately, understanding the mechanisms and interactions of central and peripheral processes of allostasis in an individualized context will accelerate the development of pharmacological interventions and treatment strategies.

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