THE CENTRALLY PROJECTING EDINGER-WESTPHAL NUCLEUS: MIDBRAIN NEUROPEPTIDE CONTROL OF ETHANOL INTAKE

By

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LIST OF ABBREVIATIONS

2-BC – two-bottle choice

5-HT – 5-hydroxytryptamine; serotonin

ABA – Allen Brain Atlas

ACTH – adrenocorticotropin hormone

ANOVA – analysis of variance

B6 – C57BL/6J

BEC – blood ethanol concentration

BNST - bed nucleus of the stria terminalis

CA – continuous access

CART – cocaine- and amphetamine-regulated transcript

CCK - cholecystokinin

CeA – central nucleus of the amygdala

cm - centimeter

CO2 – carbon dioxide

CORT – corticosterone/cortisol

cp - centrally projecting

CPA – conditioned place aversion

CPP – conditioned place preference

CRF – corticotropin-releasing factor

CRF1 – CRF type-1 receptor

CRF2 – CRF type-2 receptor

CRFBP – CRF binding protein

CT – cycle threshold

D2 – DBA/2J

DA – dopamine

DAB - diaminobenzidine

DID – drinking in the dark

dl - deciliter

DNA – deoxyribonucleic acid

DRN – dorsal raphe nucleus

Drd2 – dopamine receptor subtype 2

Drd5 – dopamine receptor subtype 5

E-Bout – ethanol bottle bout

E-Lick – ethanol bottle lick

EPM – elevated plus maze

EtOH – ethanol, alcohol

EW - Edinger-Westphal

EWcp – centrally projecting Edinger-Westphal nucleus

EWpg – preganglionic Edinger-Westphal nucleus

g - gram

GABA – gamma-aminobutyric acid

GC – gas chromatography

Ghsr – growth hormone secretagogue receptor, ghrelin receptor

H - hypothalamus

H2O – water

HPA – hypothalamic-pituitary-adrenal

hr - hour

IA – intermittent access

IACUC – Institutional Animal Care and Use Committee

i.c.v. - intracerebroventricular

IHC - immunohistochemistry

i.p. - intraperitoneal

IR - immunoreactivity

ITF – inducible transcription factor, immediate early gene

JAX – The Jackson Laboratory

kcal - kilocalorie

kg - kilogram

KD - knockdown

KO - knockout

LC - locus coeruleus

LDB – light-dark box

LS – lateral septum

LSO – lateral superior olive

μl – microliter

μm – micrometer

mA - milliamp

min - minute

ml - milliliter

mm - millimeter

MRN – median raphe nucleus

mRNA – messenger ribonucleic acid

NaN3 – sodium azide

Nesfatin-1 – nucleobindin-2; *Nucb2*

NIH – National Institutes of Health

OHSU – Oregon Health & Science University

PACAP – pituitary adenylate cyclase-activating polypeptide; *Adcyap1*

PBS – phosphate buffered saline

PFA - paraformaldehyde

pg – preganglionic

Ptprn - protein tyrosine phosphatase, receptor type N

PVN – paraventricular nucleus of the hypothalamus

qPCR – quantitative polymerase chain reaction

RLi – rostral linear nucleus of the raphe

RM-ANOVA – repeated measures analysis of variance

RNA – ribonucleic acid

RNAi – RNA interference

s.c. - subcutaneous

SEM – standard error of the mean

shRNA – short hairpin RNA

SON - supraoptic nucleus

Ucn1 – urocortin-1

Ucn2 – urocortin-2

Ucn3 – urocortin-3

Ucns – urocortins

VMH - ventromedial hypothalamus

VTA – ventral tegmental area

v/v – volume/volume

WT – wild-type

ZT – zeitgeiber time (ZT-0 = lights-on)

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ABSTRACT

Alcoholism is a disorder characterized by long-term, excessive intake of ethanol (EtOH). Chronic EtOH abuse is maintained by cycles of abstinence and relapse, and stress can induce relapse in dependent alcoholics. Even in non-dependent populations, evidence supports a key role for neural stress networks in high EtOH intake. Research on the neurobiology of stress and EtOH drinking will give insight into future strategies for managing psychiatric disease states. The aim of this dissertation was to identify the mechanisms by which a particular neural stress locus (the centrally projecting Edinger-Westphal nucleus; EWcp) contributes to excessive EtOH intake.

Animal models are required for precise control over genetic and environmental variables that impact behavior, and C57BL/6J (B6) mice are widely-used due to their innate preference for EtOH-containing fluids. EtOH dependence can be modeled using forced vapor or diet EtOH exposure, but these methods are metabolic and psychological stressors capable of impacting neural systems in unexpected ways. Therefore, I implemented several free-choice EtOH drinking models in B6 mice. In some cases, mice voluntarily surpassed the National Institutes of Health criterion for “binge drinking” (blood EtOH concentrations >80 milligrams/deciliter).

In Chapter 1, I characterized the genetic profile of the EWcp and compared it between two inbred mouse strains that serve as models for genetic differences in EtOH-related traits. I identified several EWcp-enriched genes that were upregulated in high oral EtOH-drinking B6 mice relative to low oral EtOH-drinking DBA/2J mice, hinting that these genes could regulate EtOH-related behavior via the EWcp.

In Chapter 2, an electrolytic lesion technique was combined with a genetic knockout approach to determine if a particular stress-related neuropeptide within the EWcp, urocortin-1 (Ucn1), was critical for EtOH consumption. EWcp lesion decreased

EtOH preference in a Ucn1-dependent manner, providing the first functional evidence for EWcp-Ucn1 involvement in EtOH drinking. Further, Chapter 2 showed that genetic deletion of Ucn1 or its receptor (the corticotropin-releasing factor type-2 receptor) attenuated EtOH's conditioned rewarding effects, but deletion of Ucn1 did not alter EtOH's conditioned aversive effects.

By altering several variables across multiple studies in genetic mutant mice, Chapter 3 showed that deletion of Ucn1 decreased EtOH drinking only when experiments lasted longer than four days, and when mice were offered escalating concentrations of EtOH. Chapter 3 also showed that deletion of the Ucn1 gene did not alter caloric intake, tastant drinking, EtOH sedation and tolerance, nor anxiety-like behavior.

Chapter 4 compared the EWcp gene expression profile between EtOH-experienced and naïve mice, identifying genes that were altered either immediately following an EtOH drinking session, or after 24 hours of forced abstinence from EtOH. Genes encoding Ucn1 and other neuropeptide system components were upregulated in EtOH-experienced mice, relative to controls.

Chapter 5 implemented EWcp-specific reduction of Ucn1 levels by viral-mediated gene interference, finding that EWcp-Ucn1 knockdown decreased anxiety-like behavior and long-term EtOH consumption without altering baseline consummatory behavior.

Together, these experiments demonstrated that EWcp-Ucn1 drives voluntary EtOH consumption and related behaviors. Knowledge gained from this research may inform future treatment strategies for neurobiological disorders of stress and addiction.

GENERAL INTRODUCTION

Alcohol and the Brain

Compulsive use of alcohol (ethanol; EtOH) can arise from changes within the brain that lead to dysregulated drug-seeking (Koob and Le Moal, 2008). These persistent adaptations can originate from pre-existing differences in baseline neural circuit function, from the effects of prior drug exposure, or from interactions between the two. Efforts to characterize the maladaptive changes underlying this phenomenon have identified numerous brain regions, neurotransmitter systems, and genes that work in concert to drive voluntary, repeated intake of intoxicating doses of EtOH (Crabbe et al., 2011a; Crabbe et al., 2006; Koob et al., 1998).

EtOH is hypothesized to interact directly with receptors for gamma-amino butyric acid (GABA) and glutamate (Harris et al., 2008; Howard et al., 2011). Consistent with this idea, voluntary EtOH consumption causes plastic changes in GABA and glutamate transmission within the mesolimbic dopamine (DA) reward pathway (including the ventral tegmental area [VTA] and nucleus accumbens [NAcc]) (Seif et al., 2013; Stuber et al., 2008). Furthermore, EtOH dependence alters GABA and glutamate physiology within stress-related circuits of the extended amygdala (including the central nucleus of the amygdala [CeA] and bed nucleus of stria terminalis [BNST]) (Roberto et al., 2012; Silberman and Winder, 2013; Wills et al., 2012). Extrasynaptic GABA-A receptors mediate behavioral and physiological effects of EtOH (Lobo and Harris, 2008), and these receptors are also modulated by neuroactive steroids. Several lines of evidence support a sex-specific role for endogenous neurosteroids in EtOH sensitivity, drinking, and withdrawal (Finn et al., 2010; Helms et al., 2012).

The conditioned rewarding effects of EtOH rely on DA, GABA, glutamate, and endogenous opioid signaling within mesolimbic, amygdalar, and cortical brain areas (Bechtholt and Cunningham, 2005; Cunningham et al., 2000; Gremel and Cunningham,

2008; Gremel and Cunningham, 2009; Gremel et al., 2011; Young et al., 2013). Perhaps related to their effects on reward, these neurotransmitter systems also drive “excessive” EtOH consumption, in which rodent subjects exceed the National Institutes of Health (NIH) criterion for binge drinking (blood ethanol concentrations [BECs] >80 milligrams per deciliter [mg/dl]) (Rice et al., 2012; Sabino et al., 2013; Tanchuck et al., 2011).

Mapping the Neural Substrates of EtOH

Inducible transcription factors (ITFs) are deoxyribonucleic acid (DNA)-binding proteins that initiate gene transcription following the onset of a stimulus. These “immediate early genes” are widely used as tools to quantify neural activity (i.e., transcriptional events within neurons) (Morgan et al., 1987). Prior experiments implemented immunohistochemistry (IHC) for ITFs following EtOH exposure in order to create an anatomical “map” of the brain areas underlying EtOH’s molecular and behavioral effects.

In the initial neural mapping studies, human experimenters forcibly administered EtOH to rodents, and noted increased protein expression of the prototypical ITF c-Fos in several brain areas (Chang et al., 1995; Knapp et al., 2001; Murphy et al., 2006; Ryabinin et al., 1997). Most of the regions identified in these studies were expected, based on existing hypotheses regarding the involvement of mesolimbic and amygdalar systems in EtOH-related behaviors. However, these preliminary experiments also noted that a previously unidentified candidate region, the Edinger-Westphal nucleus (EW), was uniquely sensitive to EtOH-induced increases in neural activity.

Further experiments by the Ryabinin Laboratory and others improved the face validity of the neural mapping approach by implementing IHC for ITFs following *voluntary* consumption of EtOH. Across several different drinking paradigms, and among numerous rodent strains and species, the EW was the only brain area that consistently displayed significantly elevated c-Fos expression following oral EtOH consumption

(Anacker et al., 2011; Bachtell et al., 1999; Bachtell et al., 2003; Kaur and Ryabinin, 2010; Ryabinin et al., 2001; Ryabinin et al., 2003; Sharpe et al., 2005b; Topple et al., 1998; Weitemier et al., 2001).

In addition to the EW's sensitivity to voluntary EtOH intake, systemic treatments with morphine, cocaine, amphetamine, and methamphetamine also increased neural activity in the EW nucleus (Bachtell et al., 2002a; Chang et al., 1995; Giardino et al., 2011b; Ryabinin et al., 1997; Spangler et al., 2009). These findings suggest that the EW's sensitivity to EtOH might generalize broadly to several classes of abused drugs.

The Edinger-Westphal Nucleus: EWcp vs. EWpg

The EW exists within the ventromedial periaqueductal gray of the midbrain, extending along the midline between the caudal division of the VTA and the rostral division of the dorsal raphe nucleus (DRN). Classically, the EW was defined as the group of parasympathetic cholinergic neurons projecting to the ciliary ganglion to control oculomotor functions. An updated nomenclature was required following the discoveries that certain neurons within the vicinity of the "EW" were highly-enriched in components of stress- and feeding-related neuropeptide systems (Dun et al., 2005; Foo et al., 2008; Koylu et al., 1998; Maciewicz et al., 1984; Ryabinin et al., 2005; Tanaka et al., 2003a; Vaughan et al., 1995; Weitemier et al., 2005; Xu et al., 2011; Zigman et al., 2006). These findings were highly unexpected for a supposed preganglionic cholinergic oculomotor nucleus.

Confirming the existence of two separate cell types within the EW, double-labeled IHC experiments showed that the neuropeptide-containing neurons of the EW did not express choline acetyltransferase (the enzyme required for acetylcholine synthesis) (Ryabinin et al., 2005; Weitemier et al., 2005). These examinations revealed that the EW is comprised of two distinct (yet partially overlapping) nuclei. The centrally-

projecting (cp) neurons of the *EWcp* are enriched in neuropeptides that project broadly throughout the central nervous system, while the preganglionic (pg), cholinergic neurons of the *EWpg* control oculomotor functions (Cavani et al., 2003; Kozicz et al., 2011; Ryabinin et al., 2005; Weitemier et al., 2005). Thus, the EWcp emerged as a recently-identified (and therefore, under-characterized) brain region.

Accumulating evidence now shows that the EWcp is not only sensitive to drugs of abuse, but is also important for the neural response to environmental "stressors" (i.e., challenges to homeostasis). The laboratory of Dr. Tamas Kozicz reported that EWcp neurons display increased neural activity in response to behavioral and physiological stress (Gaszner et al., 2004; Gaszner et al., 2009a; Korosi et al., 2005; Kozicz, 2003; Kozicz et al., 2008a; Okere et al., 2010; Rouwette et al., 2011; Rouwette et al., 2010; Spencer et al., 2012; Sterrenburg et al., 2011; Xu et al., 2010), and in response to food restriction and anorectic hormone (leptin) signaling (Xu et al., 2009; Xu et al., 2011).

Urocortin-1 and the EWcp

In 1995, the laboratory of Dr. Wylie Vale published the discovery and characterization of a novel mammalian peptide hormone related to corticotropin-releasing factor (CRF), named urocortin-1 (Ucn1) (Vaughan et al., 1995). The EWcp is the primary site of Ucn1 expression within the central nervous system (Bittencourt et al., 1999; Kozicz et al., 1998; Vaughan et al., 1995; Vetter et al., 2002; Weitemier et al., 2005). Following the reports of EWcp-Ucn1 expression, the Ryabinin Laboratory repeatedly used double-labeled IHC to show that EtOH-induced c-Fos expression in the EW was restricted to EWcp neurons containing Ucn1 (Bachtell et al., 2002b; Ryabinin et al., 2003; Spangler et al., 2009). The discovery that EWcp-Ucn1 neurons were activated by EtOH was intriguing because Ucn1 is a member of the CRF system, which participates in the negative affective state associated with EtOH dependence, discussed below.

Ucn1 and the CRF System

Ucn1 is one of four endogenous peptide ligands of the CRF system (including CRF, Ucn2, and Ucn3 – each transcribed from distinct genes) (Dautzenberg and Hauger, 2002). CRF displays high affinity for the CRF type-1 receptor (CRF1), while urocortin peptides (Ucns) display high affinity for the CRF type-2 receptor (CRF2) (Bale and Vale, 2004; Fekete and Zorrilla, 2007). However, Ucn1 binds to both G-protein-coupled CRF receptors with high affinity (Hauger et al., 2006; Lewis et al., 2001; Vaughan et al., 1995). The CRF binding protein (CRFBP) interacts primarily with CRF and Ucn1, and is capable of binding Ucn2 in a species-specific manner (Jahn et al., 2004). **Table 1** details the nomenclature of the CRF system, and **Figure 1** illustrates the pharmacological relationships of the CRF system.

Early work indicated that CRFBP abrogated the actions of CRF and Ucns (Potter et al., 1992), but further evidence demonstrated that interactions between CRFBP and CRF/Ucns may be *required* for CRF receptor signaling in some cell types (Ungless et al., 2003; Wang et al., 2007). Overall, the data suggest that CRFBP has unique interactions with each individual component of the CRF system. Similarly, CRF and Ucn1 have unique interactions with CRF1, as each ligand can drive a distinct signaling cascade from the same receptor (Beyermann et al., 2007; Stern et al., 2011a; Stern et al., 2011b). This phenomenon, known as “biased agonism,” “ligand-directed signaling,” or “functional selectivity” (Urban et al., 2007) may help explain the complex role of the CRF system in stress- and addiction-related behaviors.

CRF, Ucns, and the HPA-axis

CRF released from the paraventricular nucleus of the hypothalamus (PVN) stimulates the hypothalamic-pituitary-adrenal (HPA) axis, the primary neuroendocrine response to stress (Bonfiglio et al., 2010; Elliott et al., 2010). CRF acting via CRF1 in the anterior

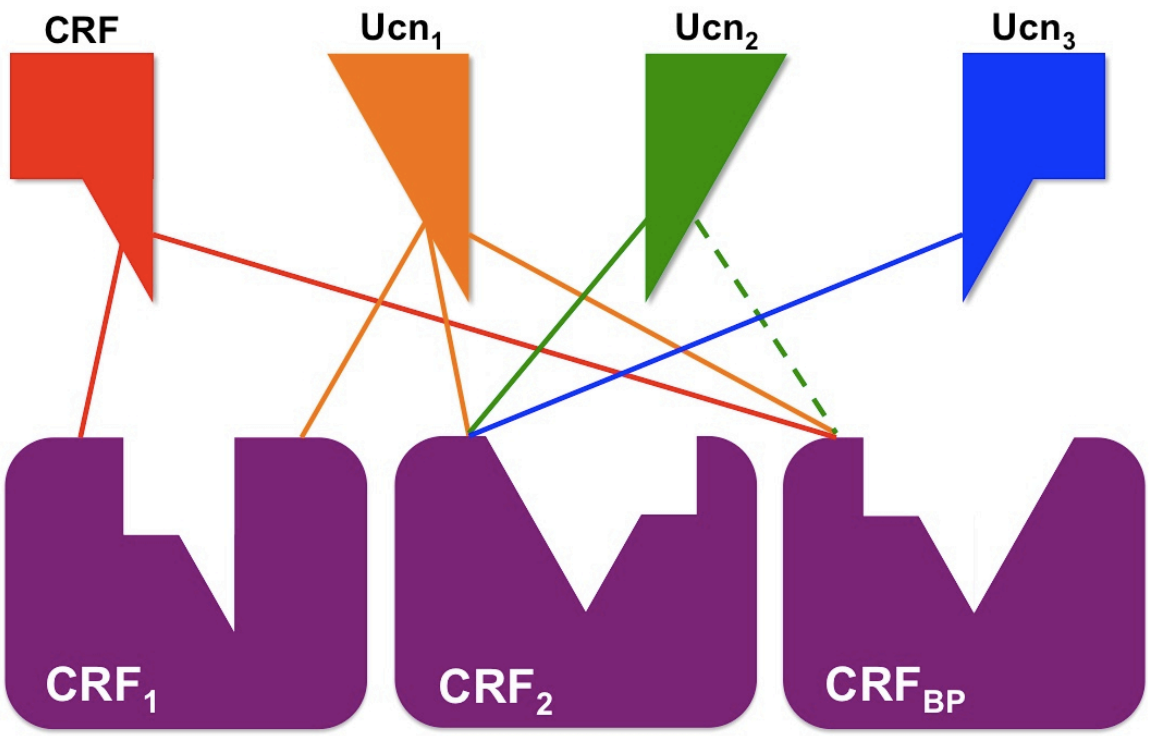
Table 1. CRF System Nomenclature

The CRF system is also known as corticotropin-releasing hormone system. Components of the system have distinct abbreviations when referring to the gene vs. the protein form, although these terms (and several variations) are used interchangeably in the CRF system literature. When discussing mutant mouse lines, I referred to the deleted gene in its abbreviated form, but to the mouse line itself with the protein abbreviation, as to acknowledge that both the gene and protein are dysfunctional in the mutant mice. When discussing viral interference studies, I referred to the component using its protein name, to reflect the fact that downregulation occurring at the transcript level produced corresponding effects on levels of the protein.

Name	***Gene***	**Protein**
Corticotropin-releasing factor	*Crh*	CRF
Urocortin-1	*Ucn*	Ucn1
Urocortin-2	*Ucn2*	Ucn2
Urocortin-3	*Ucn3*	Ucn3
Urocortins (any two, or all three)	*Ucns*	Ucns
CRF binding protein	*Crhbp*	CRFBP
CRF type-1 receptor	*Crhr1*	CRF1
CRF type-2 receptor	*Crhr2*	CRF2

Figure 1. Pharmacological Relationships of the CRF System

While CRF binds primarily to CRF1, and Ucn2 and Ucn3 are selective for CRF2, Ucn1 has high affinity for both receptors and the CRFBP. Solid lines indicate high affinity binding. Dashed line indicates that mouse Ucn2 binds with high affinity to rat CRFBP, but not human CRFBP (Fekete and Zorrilla, 2007; Jahn et al., 2004). Figure adapted from (Giardino and Ryabinin, 2012).



pituitary gland causes secretion of adrenocorticotropin hormone (ACTH), resulting in release of corticosterone or cortisol (CORT) from the adrenal glands. CORT exerts negative and positive feedback on components of the central CRF system in a site-specific manner via mineralocorticoid and glucocorticoid receptor signaling (Makino et al., 2002). Furthermore, central Ucns are influenced by HPA-axis tone. Levels of Ucn2 and Ucn3 in the hypothalamus (H) and amygdala are directly regulated by stress and glucocorticoids (Jamieson et al., 2006; Tanaka et al., 2003b). In addition, levels of EWcp-*Ucn* messenger ribonucleic acid (mRNA) fluctuate in a circadian rhythm opposite to plasma CORT levels, suggesting HPA regulation (Gaszner et al., 2009b).

Outside of the HPA-axis, the CeA and BNST are two key sites of CRF expression that influence stress- and addiction-related behavior (Koob, 2010; Regev et al., 2011; Regev et al., 2012). Extrahypothalamic CRF neurons coordinate the neurobehavioral response to stress via projections to the locus coeruleus (LC), raphe nuclei, and extended amygdala (Koob, 1999; Reul and Holsboer, 2002).

CRF, Ucns, and Stress

Early studies found differing roles for CRF receptors in anxiety-like responses, with CRF1 and CRF2 producing anxiogenesis and anxiolysis, respectively (Bale and Vale, 2004). However, CRF2 signaling can also promote anxiogenesis (Land et al., 2008; Vuong et al., 2010), consistent with the bi-directional effects of Ucns on stress-related behaviors (Kuperman et al., 2010; Land et al., 2008; Neufeld-Cohen et al., 2010a; Neufeld-Cohen et al., 2010b; Pelleymounter et al., 2002; Telegdy and Adamik, 2013; Zhao et al., 2007). Effects of Ucns tend to differ depending on the particular ligand, brain target site, and stress state of the subject (Bakshi et al., 2007; Bakshi et al., 2002; Henry et al., 2006; Todorovic et al., 2007).

In the past decade, several authors suggested that CRF/CRF1 signaling mediates the activating effects of stress, while Ucns/CRF2 signaling mediates stress adaptation or recovery (Bale and Vale, 2004; Neufeld-Cohen et al., 2010a; Neufeld-Cohen et al., 2010b; Reul and Holsboer, 2002). While this conceptual framework provides a convenient starting point, it may offer an over-simplified view (Janssen and Kozicz, 2012). Emphasizing a more nuanced view of the roles of individual CRF system components, recent studies revealed that the same CRF system ligand or receptor may have differing roles depending on the cell type and prior stress history of the subject (Kuperman et al., 2010; Muller et al., 2003; Refojo et al., 2010; Regev et al., 2011; Regev et al., 2012; Sztainberg et al., 2011; Sztainberg et al., 2010).

In the EWcp, stressors are capable of increasing levels of Ucn1 at both the mRNA and protein levels (Cespedes et al., 2010; Derks et al., 2012; Korosi et al., 2005; Okere et al., 2010; Rouwette et al., 2011; Xu et al., 2009). However, stress can also decrease expression of EWcp-Ucn1 (Kozicz et al., 2008a; Kozicz et al., 2008b) or have no effect (Gaszner et al., 2004; Gaszner et al., 2009a; Sterrenburg et al., 2011). Regardless, previous studies confirmed that stress is not the primary factor underlying EtOH-induced c-Fos expression in EWcp-Ucn1 neurons (Spangler et al., 2009; Turek and Ryabinin, 2005). In summary, the precise relationship between CRF/Ucns and the stress response is complex, and likely depends on multi-level interactions between anatomical, pharmacological, and environmental factors.

Neuroanatomy of the Ucns

Ucns are traditionally viewed as endogenous ligands of CRF2, but Ucn1 has high affinity for both CRF1 and CRF2. CRF1 is expressed widely throughout the brain, with major sites in the extended amygdala, septal nuclei, H, cortex, and mesolimbic pathway. CRF2 expression is expressed primarily in the lateral septum (LS), raphe nuclei, extended

amygdala, ventromedial hypothalamus (VMH), and brainstem (Chalmers et al., 1995; Reul and Holsboer, 2002; Van Pett et al., 2000).

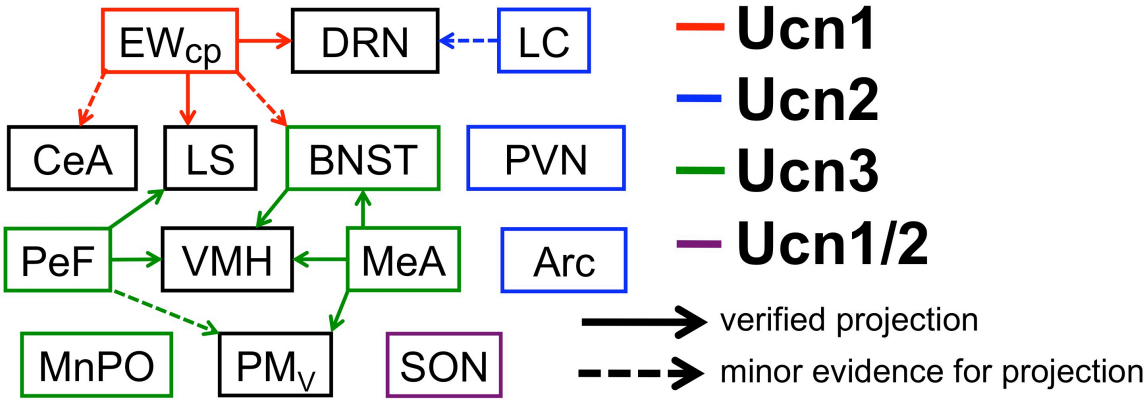
Although Ucn1 is mainly expressed in the EWcp, Ucn1-positive neurons are also present in the lateral superior olive (LSO) and the supraoptic nucleus (SON) (Bittencourt et al., 1999; Kozicz et al., 1998; Vaughan et al., 1995; Vetter et al., 2002; Weitemier et al., 2005). Anatomical tracing studies by Dr. Jackson Bittencourt and lesion studies from the Ryabinin Laboratory revealed that EWcp-Ucn1 neurons project to the LS and DRN, as well as to the spinal cord (Bachtell et al., 2004; Bittencourt et al., 1999) **(Fig. 2)**. These findings complemented earlier work documenting the existence of "EW" neurons that did not project to the ciliary ganglion (Loewy and Saper, 1978; Loewy et al., 1978). Ucn1-positive fibers are also present throughout the amygdala, H, midbrain, and brainstem, which may represent Ucn1 projections from either the EWcp, LSO, or SON (Bittencourt et al., 1999; Kozicz et al., 1998).

Ucn2 is expressed in the LC, PVN, SON, and arcuate nucleus (Reyes et al., 2001; Tanaka et al., 2003b). Ucn2 projections have not yet been characterized, although intracerebroventricular (i.c.v.) Ucn2 administration increased c-Fos expression primarily in the amygdala and VMH (Reyes et al., 2001). Several lines of evidence suggest that Ucn2 regulates the hypothalamic-pituitary-gonadal axis, likely via release from the PVN and/or SON (Nemoto et al., 2007; Nemoto et al., 2010). Although CRF2 is not expressed in pituitary corticotrophs (and therefore, it cannot cause release of ACTH), CRF2 is present in pituitary *gonadotrophs*. Therefore, Ucn2/CRF2 signaling in the pituitary gland likely regulates release of luteinizing hormone and follicle-stimulating hormone, although this mechanism remains understudied (Kageyama et al., 2003; Van Pett et al., 2000).

Ucn3-containing cell bodies are localized mainly to the medial amygdala, BNST, and H (Deussing et al., 2010; Lewis et al., 2001; Li et al., 2002). Ucn3 pathways are somewhat well characterized, with major projections to the BNST, LS, and

Figure 2. Neuroanatomical Pathways of the Urocortin Peptides

Projections of Ucn1 from the EWcp to the LS and DRN are well-established, and additional EWcp-Ucn1 projections require further study. Since the EWcp is the main site of Ucn1 production in brain, and Ucn1 fibers are widely distributed, the number of EWcp-Ucn1-innervated brain regions is likely much greater than depicted. Projections from Ucn2 cell bodies await characterization. Much of the forebrain Ucn3 circuitry has been mapped. All areas indicated contain CRF2 to varied degree, and some also contain CRF1. Median raphe nucleus is not illustrated because it likely shares the same Ucns inputs as the DRN. Brainstem and peripheral sites are not illustrated. Arc, arcuate nucleus; BNST, bed nucleus of the stria terminalis; CeA, central nucleus of the amygdala; DRN, dorsal raphe nucleus; EWcp, centrally projecting Edinger-Westphal nucleus; LC, locus coeruleus; LS, lateral septum; MeA, medial amygdala; MnPO, median preoptic area; PeF, perifornical area of the lateral hypothalamus; PMv, ventral premammilary nucleus; PVN, paraventricular nucleus of the hypothalamus; SON, supraoptic nucleus VMH, ventromedial hypothalamus. Figure adapted from (Schank et al., 2012).



VMH (Cavalcante et al., 2006; Deussing et al., 2010; Wittmann et al., 2009). In summary, the largely distinct (yet partially redundant) patterns of Ucns expression across key limbic brain areas likely underlie the complex contributions of these systems to mobilization and recovery of the stress response.

Effects of Genetic Deletions of CRF System Components

The hypothesized roles of the CRF system in regulation of HPA-axis activity and stress-related behavior were largely confirmed upon examination of mice containing targeted mutations in individual genes of the CRF system. For example, deletion of *Crhr1* produced an anxiolytic behavioral phenotype associated with low basal and stress-induced levels of CORT and ACTH (Smith et al., 1998; Timpl et al., 1998). The results from three independently-generated CRF2 KO mouse lines were varied, with deletions of *Crhr2* causing increased, decreased, or no change in anxiety-like behavior and CORT/ACTH levels (Bale et al., 2000; Coste et al., 2000; Kishimoto et al., 2000). Mice lacking CRFBP displayed increased anxiety-like behavior, despite no change in basal or stress-induced CORT and ACTH levels (Karolyi et al., 1999).

Deletion of *Crh* reduced levels of CORT and ACTH as expected (Muglia et al., 1995), although anxiety-like behavior in CRF KO mice was comparable to wild-type (WT) littermate control mice (Weninger et al., 1999). The results from three independently-generated Ucn1 KO mouse lines were varied, with *Ucn* deletion causing either increased, decreased, or no change in anxiety-like behavior and CORT/ACTH levels (Vetter et al., 2002; Wang et al., 2002; Zalutskaya et al., 2007). Interestingly, Vetter et al.

(2002) described auditory deficits in Ucn1 KO mice, perhaps reflecting a consequence of the loss of Ucn1 in the LSO (an auditory brainstem nucleus).

Deletion of *Ucn2* produced a female-specific antidepressant-like phenotype, despite enhancements in plasma CORT and ACTH (Chen et al., 2006). More recently, an independently generated Ucn2 KO mouse line displayed normal HPA-axis activity and reduced aggressive behavior (Breu et al., 2012). The first neural and behavioral characterization of Ucn3 KO and WT mice focused on the enhanced social recognition abilities observed following deletion of *Ucn3* (Deussing et al., 2010).

Double and triple deletions of *Ucns* (*Ucn*/*Ucn2* and *Ucn/Ucn2/Ucn3*) produced decreases and increases in anxiety-like behavior, respectively. These effects were accompanied by complex effects on the HPA-axis and stress reactivity (Neufeld-Cohen et al., 2010a; Neufeld-Cohen et al., 2010b). Double and triple Ucns KO mice also showed substantial alterations within the 5-hydroxytryptamine (serotonin; 5-HT) system (Kozicz, 2010). Complementing the link between Ucns and 5-HT, a recent report observed decreased EWcp-*Ucn* mRNA in 5-HT transporter KO vs. WT mice (Fabre et al., 2011). These data suggest that Ucn1 signaling in the raphe nuclei is a potential mechanism by which stress regulates 5-HT tone and accompanying mood. Indeed, an extensive literature documents the interactions between CRF/Ucns signaling and raphe nuclei 5-HT transmission within the context of stress and addiction (Bethea et al., 2011; Lukkes et al., 2008; Valentino et al., 2010; Vuong et al., 2010).

Multiple site-specific compensations in expression of CRF system components have been identified in CRF system KO mice. These alterations, as well as KO effects on anxiety-like behavior and HPA-axis activity, are summarized in **Table 2**.

Table 2. CRF System KO Effects

The top row refers to the deleted gene (or genes). The lefthand column lists the behavior, HPA-axis marker, or component of the CRF system assessed. Up arrow: increased in KO vs. WT. Down arrow: decreased in KO vs. WT. Horizontal line: assessed, but no significant effects. n/a = not assessed or not applicable. Red: conflicting effects reported. Blue: direction of effect depends on the sex, circadian timepoint, or stress level. Green: Direction of effect depends on sex, circadian timepoint, or stress levels, but conflicting effects reported.

	Crhr1	***Crhr2***	***Crh***	***Crhbp***	***Ucn***	***Ucn2***	***Ucn3***	***Ucn + Ucn2***	***Ucn + Ucn2 + Ucn3***
Anxiety	↓	↑-↓	-	↑	↑-↓	-	-	↓	↑
CORT	↓	↑-	↓	-	↑	↑-	-	↑	↓
ACTH	↓	↑-	↓	-	↑	↑-	-	n/a	n/a
PVN-*Crh*	↑	↑-	n/a	n/a	-	-	n/a	↑	↑
CeA-*Crh*	↑	↑	n/a	n/a	n/a	↑	n/a	↑	n/a
EWcp-*Ucn*	n/a	↑-	↑	n/a	n/a	-	n/a	n/a	n/a
LS-*Crhr2*	-	n/a	n/a	n/a	↓	↑	n/a	↓	↑
DRN-*Crhr2*	-	n/a	n/a	n/a	n/a	↑	n/a	↑	↑

Animal Models of EtOH Drinking and Dependence

There are currently several different preclinical models of EtOH drinking being used to investigate underlying neurobiological and genetic factors. One widely-used model is the 24-hour (hr) continuous access (CA) two-bottle choice (2-BC) procedure, in which rodents are offered simultaneous access to one bottle containing water (H2O) and one bottle containing EtOH throughout the entire length of the day. Oftentimes, 2-BC procedures begin with low EtOH concentrations (3%-6%) and increase progressively to higher EtOH concentrations (15%-40%), with 1-4 days of CA at each concentration. The 2-BC configuration allows calculation not only of EtOH and H2O intake, but also the EtOH preference ratio, which may be useful in determining the selectivity of effects on motivation for EtOH vs. motivation for fluid consumption in general. One limitation of the 2-BC CA paradigm is that subjects are potentially consuming EtOH throughout the entire day, thereby making it difficult to identify a timeperiod during which they might consume enough EtOH in a short enough amount of time to reach binge-level BECs (80 mg/dl).

To address this need for an animal model of binge drinking, several attempts were made to develop a procedure in which rodents would consume intoxicating doses of EtOH during a brief time window in the circadian dark period, when consummatory behaviors are at their peak (Ryabinin et al., 2003; Sharpe et al., 2005b). Ultimately, the drinking-in-the-dark (DID) method emerged as a favorable model for elucidating the neural and genetic substrates of binge-like drinking (Rhodes et al., 2005). A standard DID experiment lasts four days, during which B6 mice are offered daily limited access (2-4 hrs) to a single bottle containing 20% EtOH during the circadian dark cycle. Importantly, this procedure results in levels of EtOH intake that are sufficient to produce behavioral intoxication and binge-like BECs (Rhodes et al., 2007). Several variations of the DID model have been used, including a 2-BC variation where subjects receive limited access to 15% or 20% EtOH and H2O for 2-4 hrs in the circadian dark cycle. In

this procedure, B6 mice are still capable of reaching near-binge or binge BECs (Giardino and Ryabinin, 2013; Rhodes et al., 2007).

By nature of its “limited access” design, the DID paradigm incorporates intermittent periods of forced EtOH abstinence following binge-like drinking. If repeated over the long-term, one could speculate that this schedule produces neuroadaptations related to EtOH dependence. However, EtOH intake is static across multiple cycles of DID, and multi-cycle DID experience has relatively minor effects on later 2-BC CA preference drinking (Cox et al., 2013; Lowery-Gionta et al., 2012). The lack of escalating intake and the minor impact on preference drinking contrasts with the phenotype that would be expected during the transition to EtOH dependence. Although multiple-cycle DID clearly impacts neural systems, these adaptations appear to differ substantially from those observed following dependence induced by chronic intermittent EtOH vapor (Lowery-Gionta et al., 2012; Roberto et al., 2010).

To model long-term escalations in EtOH intake, Dr. Roy Wise used an intermittent access (IA) procedure in which rats received 24hr sessions of 2-BC access to 20% EtOH and H2O that alternated every other day with 24hr access to H2O-only (Wise, 1973). Using this long-term IA paradigm, Simms et al. (2008) showed that normally low EtOH-drinking Wistar and Long-Evans rat strains progressively increased their EtOH intake and preference to levels that were greater than those observed in standard 2-BC CA controls, and similar to those observed in selectively-bred EtOH-preferring (P) rats (Simms et al., 2008). Although mean BECs did not reach the binge criterion in these studies, further characterization of the long-term IA procedure in a different line of selectively-bred EtOH-preferring (Scr:sP) rats documented mean BECs that slightly surpassed the binge threshold (81.1 mg/dl) (Sabino et al., 2013).

When a slightly-modified long-term IA procedure using escalating concentrations of EtOH (3%-20%) was applied in male B6 mice, subjects reached high levels of daily

20% EtOH intake (~20 g/kg) and preference (~70%) that were greater than those observed in standard 2-BC CA controls (Hwa et al., 2011). In addition, patterns of intake in the long-term IA procedure were associated with mean BECs surpassing the binge threshold (145.34 mg/dl) (Hwa et al., 2013). Of course, the "escalating" nature of intake observed in the mouse version of the long-term IA model is partly an artifact of the escalating concentrations of EtOH used, as intake generally stabilized once 20% EtOH was reached. Furthermore, the role of intermittency differs across genotypes, and intermittency is not *required* to produce high levels of EtOH intake (Crabbe et al., 2012). Nevertheless, Hwa et al. (2011) documented significant physical withdrawal symptoms (handling-induced convulsions) following 6-8hrs of forced abstinence in B6 mice that underwent 16 weeks of IA drinking, suggesting that the long-term IA procedure produces neuroadaptations relevant to the development of EtOH dependence.

Established models of EtOH dependence primarily rely on chronic forced exposure to EtOH vapor or liquid EtOH diet, which produce long-lasting enhancements in operant self-administration and voluntary consumption of EtOH (Heilig and Koob, 2007; Valdez and Koob, 2004). The mouse intragastric model of EtOH dependence has also been used to examine genetic differences in EtOH consumption (Fidler et al., 2011; Fidler et al., 2012), and to investigate the role of withdrawal in driving further EtOH drinking via negative reinforcement processes (Cunningham et al., 2013). By passively exposing animals to EtOH, these manipulations may affect neural systems in unexpected ways, likely causing adaptations distinct from those observed when EtOH exposure is completely voluntary. However, rodents made dependent using these methods showed long-lasting enhancements in EtOH consumption, and are therefore useful for studying mechanisms underlying EtOH dependence-induced excessive drinking (Griffin et al., 2009). For my own studies on the role of neural stress systems in EtOH intake, I chose to focus on voluntary models of 2-BC drinking, as forced EtOH

exposure could be considered a psychological and metabolic stressor itself, thereby complicating issues of interpretation.

Effects of EtOH on the HPA-Axis

Like all drugs of abuse, EtOH can activate the HPA-axis (Armario, 2010). Dr. Catherine Rivier and her colleagues at the Salk Institute performed seminal studies in rats showing that intraperitoneal (i.p.) administration of EtOH (1-3 grams per kilogram body weight [g/kg]) significantly increased CORT and ACTH levels. The effects were attenuated by either i.c.v. immunoneutralization of CRF (Rivier et al., 1984) or electrolytic lesion of the PVN (Rivest and Rivier, 1994), suggesting the importance of PVN-CRF and the HPA-axis. A further study revealed that a non-selective CRF receptor antagonist with minimal actions in the pituitary also reduced the EtOH-induced ACTH response (Rivier et al., 1996). This result indicated that *extrahypothalamic* CRF components were also important for driving the effects of EtOH on the HPA-axis (Rivier, 1996).

Despite the HPA-activating effects of EtOH in naïve rats, rats pretreated with i.p. EtOH showed a blunted ACTH response to acute stress or i.c.v. CRF administration (Rivier and Vale, 1988). Consistent with this finding, rats with prior EtOH vapor exposure also showed an attenuated ACTH response to intravenous CRF and footshock stress (Rivier et al., 1990). Others have replicated the dampened neuroendocrine state observed following EtOH vapor dependence (Richardson et al., 2008a). However, the HPA-blunting effects of EtOH appear to differ across development, as 21 day-old rat pups born to EtOH vapor-exposed dams displayed an *accentuated* ACTH response to stress, relative to control rats (Lee et al., 1990). Indeed, several prenatal exposure studies observed a hyperresponsive HPA phenotype in rodents born to EtOH-treated dams (Hellemans et al., 2010).

In several cases, *Crh* mRNA was elevated in the PVN of EtOH-exposed vs. control rats (Lee et al., 1990; Ogilvie et al., 1997; Rivier et al., 1990). However, other studies reported no influence of EtOH on PVN-CRF expression (Lee and Rivier, 1997; Rivier and Lee, 1996; Wills et al., 2010; Zhou et al., 2000), suggesting that effects differed depending on the method of exposure and the timecourse of analysis.

Effects of EtOH on the Extrahypothalamic CRF System

Elevations in *Crh* mRNA or CRF-immunoreactivity (IR) were observed in the CeA following long-term EtOH vapor or diet exposure (Roberto et al., 2010; Sommer et al., 2008; Zorrilla et al., 2001), and following short-term binge drinking (Lowery-Gionta et al., 2012). However, other studies reported decreases in amygdalar *Crh* mRNA and CRF-IR following voluntary drinking (Falco et al., 2009; Gilpin et al., 2012; Karanikas et al., 2013) or withdrawal from liquid EtOH diet (Wills et al., 2010). In yet a few other cases, authors reported no significant effects of EtOH on CeA-CRF expression (Ogilvie et al., 1997; Walker et al., 2010).

Following chronic EtOH vapor, *Crhr1* levels in the amygdala were increased, while amygdala *Crhr2* levels were decreased (Sommer et al., 2008). In contrast, voluntary EtOH drinking reduced *Crhr1* mRNA in the CeA and the NAcc of selectively-bred high-preferring rats (Hansson et al., 2007). Although repeated i.p. EtOH or voluntary EtOH intake had no effect on EWcp-Ucn1 protein expression, EtOH drinking significantly reduced the number of Ucn1 fibers present in the LS (Bachtell et al., 2002b; Bachtell et al., 2003). Furthermore, repeated i.p. EtOH increased CRF2 binding in the LS and DRN, which could reflect either increased or decreased release of Ucn1 from the EWcp (Weitemier and Ryabinin, 2005a).

Given the dynamics of gene transcription, and the differing rates of peptide synthesis, release, and binding, it can be difficult to interpret mRNA findings without

corresponding data at the protein level, and vice-versa. Nevertheless, the overall picture indicates that acute EtOH exposure reduces CRF system activity, while abstinence from chronic EtOH increases it. Supporting the interpretation of enhanced CRF release during EtOH abstinence, *in vivo* microdialysis studies found increased extracellular CRF content in the amygdala and BNST following 6-12 hrs of forced abstinence from liquid EtOH diet (Merlo Pich et al., 1995; Olive et al., 2002).

Effects of CRF System Manipulations on EtOH Drinking

Several of the EtOH drinking and exposure paradigms described above were used to investigate the involvement of the CRF system in EtOH consumption. Disruption of CRF1 by pharmacological and genetic methods attenuated the enhancements in EtOH drinking and operant self-administration observed in EtOH-dependent rodents (Chu et al., 2007; Funk et al., 2007; Gehlert et al., 2007; Richardson et al., 2008b; Roberto et al., 2010). Additional studies that performed intra-CeA microinfusions of a non-selective CRF receptor antagonist found similar effects (Finn et al., 2007; Funk et al., 2006a).

After publication of these findings, some argued that CRF1 antagonists might selectively inhibit EtOH intake in dependent subjects (Heilig and Koob, 2007). However, short-term studies provided evidence that CRF1 signaling drives binge drinking even in a non-dependent state (Giardino and Ryabinin, 2013; Kaur et al., 2012; Lowery et al., 2010; Sparta et al., 2008), possibly via the CeA (Lowery-Gionta et al., 2012). Disruption of CRF1 signaling by pharmacological blockade or genetic deletion also decreased EtOH drinking in the long-term IA procedure (Cippitelli et al., 2011; Hwa et al., 2013; Simms et al., 2013) and the 2-BC CA procedure (Lodge and Lawrence, 2003; Pastor et al., 2011b).

Others have argued that CRF1 manipulations are effective at decreasing drinking only when subjects reach binge levels of EtOH intake (Lowery and Thiele, 2010; Thiele,

2012), but data supporting this hypothesis may be confounded by floor effects. While most results suggest that CRF1 signaling facilitates EtOH intake, central administration of CRF and Ucn1 unexpectedly *decreased* EtOH drinking (Bell et al., 1998; Ryabinin et al., 2008; Thorsell et al., 2005). These findings are perhaps related to CRF and Ucn1's abilities to decrease food and H2O intake when administered i.c.v. (Spina et al., 1996).

With regard to the CRF2 receptor, deletion of *Crhr2* increased EtOH intake in one limited-access procedure, but had no effect in the standard DID paradigm (Kaur et al., 2012; Sharpe et al., 2005a). Studies focusing on Ucn3 found that i.c.v. administration decreased EtOH drinking in non-dependent mice (Lowery et al., 2010; Sharpe and Phillips, 2009), but intra-CeA administration had bi-directional effects on EtOH intake in dependent vs. non-dependent rats (Funk and Koob, 2007; Valdez et al., 2004). Overall, the data indicate that CRF1 facilitates EtOH consumption while CRF2 inhibits it. However, there are several exceptions to this framework that require further study.

CRF System, Stress, and EtOH Drinking

Effects of stress on EtOH drinking are bi-directional, depending on the stressor length and the developmental timepoint, reviewed elsewhere in detail (Becker et al., 2011). With respect to the CRF system, either genetic KO or pharmacological blockade of CRF1 blunted the increased EtOH intake observed following forced swim stress or social defeat stress (Lowery et al., 2008; Molander et al., 2012; Pastor et al., 2011b). Furthermore, mice lacking both CRF1 and CRF2 receptors showed a blunted response to the delayed effects of repeated swim stress on increased EtOH drinking in the 2-BC CA procedure (Pastor et al., 2011b). One study found that mice lacking CRF1 displayed an *enhanced* stress-induced increase in EtOH drinking (Sillaber et al., 2002), although this effect was later explained by loss of CRF1 in the pituitary, rather than the brain (Molander et al., 2012). Overall, these data suggest that stress can be a motivating

factor for excessive EtOH drinking, and that the underlying mechanisms likely rely on complex contributions from the CRF system.

CRF System, Stress, and EtOH Dependence

In 2002, the laboratory of Dr. George Koob reported that relative to air-exposed controls, EtOH vapor-dependent rats displayed increased anxiety-like behavior at both acute (2hr) and protracted (5 weeks) timepoints of withdrawal (Valdez et al., 2002). This state of heightened anxiety was also observed 6 weeks following cessation of a liquid EtOH diet, and was reversed by i.c.v. administration of either a non-selective CRF receptor antagonist or the CRF2 agonist Ucn3 (Valdez et al., 2004; Valdez et al., 2003). Observing similarities between the effects of CRF system manipulations on enhanced EtOH drinking and enhanced anxiety-like behavior in the post-dependent state, Dr. Koob hypothesized that EtOH dependence produces adaptations in the CRF system that allow a negative affective state to predominate during EtOH withdrawal. In this model, EtOH dependence recruits CRF and other anxiogenic neuropeptide systems, thereby producing a stress-like state that permits negative reinforcement processes to drive compulsive EtOH-seeking (Koob, 2008; Koob, 2010; Koob and Le Moal, 2008).

CRF System, Stress, and EtOH Relapse

Attempts to study EtOH relapse-like behavior in rodents focused on the reinstatement model, in which EtOH-seeking was assessed following acquisition and extinction of operant EtOH self-administration. Studies in rats from the laboratory of Dr. A.D. Le revealed that behavioral (electric footshock) or pharmacological stressors (i.c.v. CRF or i.p. yohimbine [alpha-2 adrenoreceptor antagonist]) increased non-reinforced operant behavioral responding on an active lever previously associated with EtOH availability (Le and Shaham, 2002)

Stress-induced reinstatement of operant EtOH-seeking was significantly reduced by blockade of CRF1, but not by removal of the adrenal glands (Le et al., 2002; Le et al., 2000; Marinelli et al., 2007), suggesting limited involvement of the HPA-axis. Focused on extrahypothalamic CRF systems, the Le group observed significant decreases in stress-induced EtOH reinstatement following i.c.v. and intra-median raphe nucleus (MRN) administration of a non-selective CRF receptor antagonist (Le et al., 2011; Le et al., 2002; Le et al., 2000).

Footshock, CRF, and yohimbine all increased CRF mRNA in the BNST (Funk et al., 2006b), and the BNST projects to the MRN (Behzadi et al., 1990). Therefore, one could speculate that the BNST and MRN are critical nodes in the circuit underlying stress-induced reinstatement of EtOH-seeking. However, neurons in the MRN express both CRF1 and CRF2 (Van Pett et al., 2000), and intra-MRN CRF2 antagonists have not been tested in the stress-induced reinstatement model. Thus, it remains possible that stress-induced EtOH relapse is mediated via Ucns/CRF2 signaling in the MRN. Even if MRN-mediated effects on reinstatement were mediated entirely by CRF1, the underlying ligand could be either CRF or Ucn1, as both bind CRF1, and both directly innervate the MRN (from the BNST and EWcp, respectively) (Bachtell et al., 2004; Dong and Swanson, 2006; Weitemier et al., 2005).

CRF System, EtOH, and Neuroplasticity

The appearance of physiological adaptations in neural circuit function (i.e., neuroplastic changes) may characterize the transition to EtOH dependence (Koob and Le Moal, 2005; McCool, 2011). Dr. Marisa Roberto and her colleagues characterized the effects of chronic EtOH exposure on neuropeptide-mediated plasticity in the CeA (Gilpin and Roberto, 2012). CRF increased GABA release from CeA interneurons, and this effect was potentiated in EtOH vapor-dependent rats via a CRF1-dependent mechanism

(Roberto et al., 2010). Furthermore, baseline GABA release was enhanced in the CeA of high EtOH-preferring vs. control rats, and retrograde tracing revealed that EtOH specifically activated CRF1-containing, BNST-projecting neurons in the CeA (Herman et al., 2013a; Herman et al., 2013b). In the BNST, several studies observed effects of EtOH on electrophysiological interactions between CRF, DA, and glutamate (Kash et al., 2008; Silberman et al., 2013; Silberman and Winder, 2013).

Genetic Relationships Between EtOH, Stress, and the CRF System

The CRF system can influence EtOH-related behavior via inherent genetic differences in the function of its components and their interactions with stress-related environmental factors. The first identification of a *Crhr1* variant associated with binge drinking in humans was described in 2006 (Treutlein et al., 2006). Further studies revealed *Crhr1* genotypes that interacted with prior stress history to influence excessive EtOH intake (Blomeyer et al., 2008; Nelson et al., 2010; Ray et al., 2013; Schmid et al., 2010).

In a non-human primate model, Dr. Christina Barr and colleagues reported that early life stress (maternal separation) increased EtOH consumption during adulthood, but only in monkeys with a functional variant in the promoter of the *Crh* gene that conferred increased sensitivity to glucocorticoids and stress-induced HPA-axis activation (Barr et al., 2009). In a similar vein, the laboratory of Dr. Markus Heilig identified an allelic variant in the *Crhr1* promoter that differed in frequency between rats genetically selected for high EtOH preference and their control line (Hansson et al., 2006). The variant was associated with *Crhr1* expression in the NAcc and amygdala (upregulated in high-preferring rats vs. controls), and conferred increased sensitivity to the effects of CRF1 blockade on stress- and EtOH-related behaviors (Ayanwuyi et al., 2013). Accordingly, Dr. Heilig advocated a pharmacogenetic approach, in which genetic

sequence analysis may aid in selection of appropriate pharmacotherapy for clinical management of alcoholic patients (Heilig et al., 2011).

Genetic Associations Between the EWcp and EtOH Drinking

Following the IHC co-localization studies implicating EWcp-Ucn1 neurons in the neural response to EtOH, the Ryabinin Laboratory hypothesized that EWcp-Ucn1 neurons contribute to behavioral phenotypes relevant to alcoholism. This idea was supported by evidence from comparisons of EWcp-Ucn1 protein expression between rodent lines that differ in behavioral and physiological responses to EtOH. Stronger IHC expression of the EWcp-Ucn1 protein was associated with a genetic predisposition toward higher EtOH intake (Bachtell et al., 2003; Fonareva et al., 2009; Turek et al., 2005) and heightened sensitivity to some EtOH-related phenotypes (reward, hypothermia, sedation), but not all (locomotor stimulation) (Bachtell et al., 2002b; Kiianmaa et al., 2003; Ryabinin and Weitemier, 2006; Turek et al., 2008).

The EWcp and EtOH Drinking

To test a functional role for the EWcp in voluntary 2-BC CA EtOH drinking, previous studies in the Ryabinin Lab measured EtOH consumption in high-drinking B6 mice that received either electrolytic lesion of the EWcp or sham control surgery. Surgical ablation of the EWcp attenuated intake of and preference for 3%, 6%, and 10% EtOH (but not sucrose, quinine, saccharin, or saline) (Bachtell et al., 2004; Weitemier and Ryabinin, 2005b), supporting the hypothesis that EWcp neurons play an important and selective role in voluntary EtOH consumption (Ryabinin and Weitemier, 2006). Among lesioned mice, Ucn1-IR fibers were decreased in the LS and DRN, suggesting that these areas might mediate Ucn1's effects on preference drinking.

However, Ucn1 neurons are intermingled with DAergic neurons of the adjacent rostral linear nucleus of the raphe (RLi), suggesting that the EWcp could be regulated by local DA release (Bachtell et al., 2002a; Kozicz, 2001). Indeed, a recent report from the Ryabinin Lab showed that inhibition of VTA neurons via site-specific activation of the GABA-A receptor or the autoinhibitory dopamine type-2 receptor (Drd2) receptor increased c-Fos expression in the EWcp (Ryabinin et al., 2013). Behavioral effects of EWcp lesions could therefore be mediated indirectly via disruption of RLi-DA transmission, and further evidence was required to form a link with Ucn1.

Ucn1 and EtOH Drinking

The DRN is a major target of EWcp-Ucn1 neurons, and prior studies hypothesized that DRN-Ucn1 signaling mediates voluntary EtOH intake. In contrast to the expected finding, Ucn1 microinfusions into the DRN had no effect on EtOH intake in the standard CA 2-BC procedure, although they significantly decreased food and H2O consumption (Weitemier and Ryabinin, 2006). The LS is also a major target of EWcp-Ucn1 neurons that was hypothesized to mediate the facilitative effects of Ucn1 on voluntary EtOH intake. Also in contrast to the expected result, Ucn1 microinfusions into the LS selectively *reduced* EtOH drinking in the standard DID paradigm (Ryabinin et al., 2008). In my view, these studies remain difficult to interpret, as exogenous administration of Ucn1 may produce behavioral effects via supraphysiological actions at either CRF1 or CRF2, which are both expressed in the LS and DRN (Van Pett et al., 2000). Because the LS and DRN receive co-innervation by CRF and multiple Ucns (**Fig. 2**), CRF receptor blockade in either of these brain regions would also be unable to produce any meaningful data about the function of endogenous Ucn1. Thus, genetic approaches were required to specifically isolate the role of Ucn1 in EtOH intake. In the first published account of the effects of genetic manipulations of the Ucn1 system on EtOH intake, the Ryabinin Laboratory

tested Ucn1 KO and WT mice in the standard DID paradigm (Kaur et al., 2012). Four days of limited-access consumption of 20% EtOH failed to reveal significant differences in EtOH intake between large cohorts of Ucn1 KO and WT mice (n = 19-29 per sex, per genotype). Further research was clearly needed to thoroughly characterize the role of Ucn1 in voluntary EtOH preference.

Dissertation Studies

The data described above clearly implicated components of the CRF system in voluntary EtOH drinking, but results varied depending on the specific paradigm implemented, the anatomical substrates examined, and the environmental conditions under which the experiments were performed. Although these studies highlighted a role for the EWcp in driving EtOH preference, the EWcp remained understudied. While these data provided associative evidence for contributions of EWcp-Ucn1 neurons to EtOH-related phenotypes, causal evidence was lacking. Therefore, I designed a series of experiments that set out to answer several questions raised by prior studies, namely: which genes are enriched in the EWcp, which are modified by EtOH drinking, and what in particular is the role of EWcp-Ucn1 with regard to EtOH consumption and related traits?

In Chapter 1, I used a small-scale bioinformatics approach based on literature searches and public information available in the Allen Brain Atlas (ABA) to identify several mRNA transcripts that were particularly enriched within the EWcp. The identified genes included several that encode stress-related neuropeptide transmitters (Pituitary adenylate cyclase-activating polypeptide, or PACAP [*Adcyap1*], cocaine- and amphetamine-regulated transcript, or CART [*Cart*], cholecystokinin, or CCK [*Cck*], nucleobindin-2, or Nesfatin-1 [*Nucb2*], and Ucn1 [*Ucn*]). Identified genes also included several that encode proteins responsible for neuropeptide processing, packaging, release, and signaling (growth hormone secretagogue receptor, or ghrelin receptor

[*Ghsr*], leptin receptor [*Lepr*], melanocortin receptor accessory protein 2 [*Mrap2*], proprotein convertase subtilisin/kexin type 1 [*Pcsk1*], phospholipase D, family member 3 [*Pld3*], protein tyrosine phosphatase, receptor type N [*Ptprn*], and secretogranin-II [*Scg2*]).

Enhanced activity of these neuropeptide systems in response to environmental factors could provide a mechanism by which the EWcp underlies the relationship between stress and EtOH drinking. Additionally, genetic differences in basal levels of these systems within the EWcp could potentially explain genetic differences in EtOH-related traits. Therefore, studies in Chapter 1 compared the quantitative levels of these (and several other) genes between EWcp samples taken from naïve male B6 and D2 mice, two inbred strains known to differ in stress- and addiction-related behaviors (Lewis et al., 2007; Ryabinin et al., 1999; Yoneyama et al., 2008).

Following the initial EWcp lesion studies, a functional link between Ucn1 expression and EtOH drinking remained to be determined, as Ucn1 is only one of several neuropeptide system components that are highly-enriched within the EWcp. Thus, additional techniques were required in order to establish whether EWcp lesions affected EtOH drinking specifically via disruption of Ucn1 function. Chapter 2 used a combinatorial EWcp lesion and Ucn1 genetic KO approach to determine whether the effects of EWcp lesion on 2-BC CA EtOH preference were dependent on Ucn1 expression. The hypotheses for this study were that lesions would only be effective at decreasing EtOH drinking in mice expressing Ucn1, and that KO of Ucn1 would only be effective at decreasing EtOH drinking in mice with an intact EWcp. The 2-BC CA paradigm was chosen in order to facilitate comparison to the prior EWcp lesion study (Bachtell et al., 2004).

Chapter 2 also explored the relationships between Ucn1, CRF2 signaling, and EtOH reward by testing Ucn1 and CRF2 KO and WT mice for EtOH-induced conditioned

place preference (CPP). To determine whether the aversive effects of EtOH were related to Ucn1 function, and whether EtOH-related learning was generally affected by the absence of Ucn1, Ucn1 KO and WT mice were also tested for EtOH-induced conditioned place aversion (CPA). Based on findings that similar neurotransmitter systems are involved in the conditioned rewarding effects of EtOH and voluntary EtOH drinking (discussed above), I hypothesized that deletion of *Ucn* would reduce EtOH-induced reward. I also hypothesized that deletion of *Crhr2* would attenuate EtOH-induced reward, as major target sites of EWcp-Ucn1 neurons are relatively enriched in CRF2 vs. CRF1. With regard to the effects of Ucn1 KO on CPA, I had no specific hypotheses about Ucn1's role in conditioned aversion, and I had no reason to believe that Ucn1 was required for EtOH-related learning *per se*.

To better define the contribution of the Ucn1 system to EtOH consumption, I applied the Ucn1 KO model to several different EtOH drinking paradigms in Chapter 3. By comparing fixed vs. escalating concentrations of EtOH, and incorporating various schedules and lengths of EtOH access, I was able to determine the relative contributions of several experimental variables to Ucn1-dependent EtOH preference. I also assessed the effects of Ucn1 KO on several additional EtOH- and stress-related behavioral traits that can influence the propensity for EtOH consumption, discussed further in Chapter 3.

Although EWcp neurons are transcriptionally activated by EtOH intake, and are genetically associated with EtOH-related phenotypes, it remained to be determined whether EtOH significantly altered expression of EWcp-enriched genes of interest within the EWcp. In Chapter 4, I used the long-term IA procedure to identify the effects of EtOH consumption on the EWcp gene expression profile. I chose the long-term IA procedure for these studies because experiments in Chapter 3 identified a specific timeperiod in this paradigm during which expression of Ucn1 impacted the likelihood of whether mice achieved binge-level BECs. Based on the results from Chapters 1-3, I hypothesized that

long-term IA EtOH exposure would upregulate the expression of several neuropeptide-related genes of interest within the EWcp, including *Ucn*.

Technological advances in molecular biology and neuroscience now allow researchers to perform brain region-specific genetic manipulations of anatomically-defined neuron populations via stereotaxic viral delivery. In one approach, called viral-mediated RNA interference (RNAi), neurons that are infected with a virus driving expression of a small interfering RNA (or short hairpin RNA; shRNA) undergo transcript-specific downregulation. In Chapter 5, I aimed to determine if knockdown (KD) of EWcp-Ucn1 by viral-mediated RNAi altered long-term IA EtOH intake. Based on the results from Chapters 1-4, I hypothesized that EWcp-specific KD of Ucn1 would blunt EtOH consumption in the long-term IA paradigm. I also examined the effects of EWcp-Ucn1 KD on baseline anxiety levels, as there is evidence for a relationship between neural stress systems, anxiety-like behavior, and excessive EtOH drinking (Valdez et al., 2002; Valdez et al., 2004; Valdez et al., 2003). However, given the mixed results of Ucn1 KO on such behavioral tasks, I had no directional prediction of how EWcp-Ucn1 KD might alter anxiety-like behavior in these virally-manipulated mice.

CHAPTER 1: Genetic Profiling of the Edinger-Westphal Nucleus

(This chapter has been reformatted for inclusion in this dissertation from: Giardino WJ, Cote DM, Li J, Ryabinin AE. Characterization of genetic differences within the centrally projecting Edinger-Westphal nucleus of C57BL/6J and DBA/2J mice by expression profiling. *Frontiers in Neuroanatomy.* 2012;6:5. EPub 2012 Feb 14.)

INTRODUCTION

Several previous studies identified differences in EWcp-Ucn1 protein expression between inbred and selectively-bred strains of mice and rats (Ryabinin and Weitemier, 2006; Spangler et al., 2009; Turek et al., 2008). It remained unclear whether these observations at the protein level resulted from differences in the magnitude of gene expression, differences in peptide release, or a difference in the total number of EWcp neurons. The goals of Chapter 1 were to identify additional genes besides *Ucn* that are highly-enriched within the EWcp, and then to determine whether the mRNA levels of these transcripts differed between mouse strains that differ in the response to EtOH.

B6 and DBA/2J (D2) are two well-characterized inbred mouse strains that differ in several alcohol-, stress-, and feeding-related phenotypes (Lewis et al., 2007; Ryabinin et al., 1999; Yoneyama et al., 2008). In a previous comparison of the high EtOH-drinking B6 and low EtOH-drinking D2 mice, the Ryabinin Laboratory reported that cells within the proximity of the EW were more numerous (and larger in size) in B6 vs. D2 (Bachtell et al., 2002b). However, this experiment did not differentiate between Ucn1 neurons of the EWcp, cholinergic neurons of the EWpg, and DAergic neurons of the adjacent RLi. Another study found that Ucn1-IR within individual EWcp neurons was greater in B6 vs. D2 mice (Weitemier and Ryabinin, 2005a). Thus, in addition to being driven in part by differences in the total number of neurons, observed strain differences in EWcp-Ucn1 protein expression could also be due to differences in either *Ucn* mRNA expression, or the rate of Ucn1 peptide synthesis, release, or degradation.

In order to identify additional transcripts that were EWcp-enriched, I used publicly-available resources present in the ABA (http://mouse.brain-map.org) (Lein et al., 2007). In order to assess genotypic differences in *Ucn* and newly-identified EWcp genes of interest, RNA was isolated from microdissected EWcp samples of B6 and D2 mice, and mRNA levels were obtained using quantitative real-time polymerase chain reaction

(qPCR). After finding several genes that were significantly upregulated within the EWcp of B6 vs. D2 mice, IHC and *in silico* analyses were used as additional confirmation tests for these findings. As such, these experiments highlighted several genes that may be integral for addiction- and stress-related behaviors regulated via the mammalian EWcp.

MATERIALS AND METHODS

Animals

Male B6 and D2 mice (8-10 weeks old) were delivered from The Jackson Laboratory (JAX; Sacramento, CA) and housed four per cage in our colony. B6 and D2 are inbred strains that were not selectively-bred for EtOH-related traits. Therefore, without causal evidence, it remains possible that any strain differences in EWcp gene expression are unrelated to the behavioral effects of EtOH. This possibility could be minimized by performing comparisons between rodent lines that were selectively-bred for EtOH-related traits. In fact, the Ryabinin Laboratory has now performed EWcp gene expression profile comparisons between several lines of mice selectively-bred for high vs. low EtOH drinking and withdrawal, and results from these studies are forthcoming. However, B6 and D2 mice were chosen for these studies because they are the prototypical rodent strains used to compare EtOH-related traits, and their behavioral and molecular characteristics are well-described and publicly-available in online databases. Therefore, performing these experiments in B6 and D2 mice facilitated comparison of results to other studies, and allowed complementary analyses of relevant data sets.

All mice in these studies received *ad libitum* access to food (LabDiet 5001; Richmond, IN) and H2O, and were maintained on a 12hr/12hr light-dark schedule (lights ON/zeitgeiber-0 [ZT-0] = 0600h). All experiments were approved by the Oregon Health & Science University (OHSU) Institutional Animal Care and Use Committee (IACUC) and

performed with adherence to the NIH Guidelines for the Care and Use of Laboratory Animals.

Identification of EWcp-Enriched Transcripts

Resources available from the ABA identified transcripts that were preferentially expressed within the EWcp. The initial goal of the ABA project was to perform and publish *in situ* hybridization for every protein-coding gene in the mouse genome. The ABA group successfully documented the brain expression of several thousand mRNA transcripts, and made this information publicly available online ([Internet], © 2004 ; Lein et al., 2007).

The ABA includes a “Fine Structure” search feature allowing the search for genes expressed in smaller brain structures. I browsed the expression patterns of the 50 genes defined as being expressed within the “Edinger-Westphal.” Spatial resolution of this feature is relatively low and does not represent the vast coronal span of the EWcp. Thus, I verified that only 27 of 50 transcripts were clearly enriched within the EWcp. Reasons for exclusion included: a pattern of expression that was not within the EWcp, a non-specific pattern of expression that included the EWcp as well as several other structures, or low expression within the EWcp.

Next, I used the ABA “Neuroblast” feature to find additional EWcp-enriched genes with expression patterns similar to those identified by the initial Fine Structure search. Finally, I used the AGEA Gene Finder, another ABA tool that identifies genes with expression patterns that are highly correlated with a user-defined seed region (i.e. any given voxel in the mouse brain). I placed seed regions in five different voxels throughout the mouse midbrain (centered around the EWcp), identifying additional EWcp-enriched genes that had not been identified by prior methods.

After beginning with 7-10 candidate transcripts (based on prior studies and on literature searches), I identified 68 EWcp-enriched genes. By relying on ABA features, this method was prone to false negatives (i.e., *in situ* hybridization probe failure), therefore this list is likely a conservative underestimate of the total number of EWcp-enriched genes. The 68 identified genes were further interrogated by the qPCR array approach, as described below.

Additional Transcripts of Interest

Five categories of additional transcripts that were not necessarily EWcp-enriched were also included in the analysis: 1) three immediate early genes encoding ITFs that are well-established markers of neuronal activity, to assess differences in basal transcriptional activity; 2) eight genes related to the DA system, included because microdissections of the EWcp may include small quantities of DAergic neurons of the adjacent RLi, which intermingle with EWcp-Ucn1 neurons (Bachtell et al., 2002a; Fonareva et al., 2009; Gaszner and Kozicz, 2003); 3) four genes showing robust expression in the VTA, included because the VTA is neurochemically similar to the RLi; 4) three CRF system genes, included because they are targets of the Ucn1 peptide, and their expression is expected in the vicinity of the EWcp if Ucn1 is released locally; and 5) five housekeeping genes, to control for potential loading issues. See **Table 3** for a list of housekeeping genes and **Table 4** for a list of genes of interest.

Gene Expression Analyses

After habituation to the colony, naïve mice (n = 5-7 per strain) were euthanized by carbon dioxide (CO2) inhalation, and dissected brains were immediately placed inside a pre-chilled coronal brain matrix. A 1 millimeter (mm)-thick tissue punch containing the EWcp was isolated with a chilled 18-gauge blunt needle (**Fig. 3**), incubated in 50

Table 3. Chapter 1: List of Housekeeping Genes

Only *Gapdh* cycle thresholds (CTs) differed between strains (t_{10} = 3.49; p < .01). Thus, *Gapdh* was excluded from the list of housekeeping genes used to quantify the genes of interest. Asterisks indicate genes used to normalize expression levels of genes of interest.

Gene Symbol	Gene Name
*Actb**	Beta-actin
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
*Gusb**	Beta-glucuronidase
*Hprt1**	Hypoxanthine-guanine phosphoribosyltransferase
*Hsp90ab1**	Heat shock protein 90-beta

Table 4. Chapter 1: List of Genes of Interest

In addition to five housekeeping genes and five wells dedicated to genomic DNA-, reverse transcriptase-, and PCR-controls, the 96-well qPCR array included 68 EWcp-enriched genes, three ITFs, eight DA-related genes, four VTA-related genes, and three CRF-related genes.

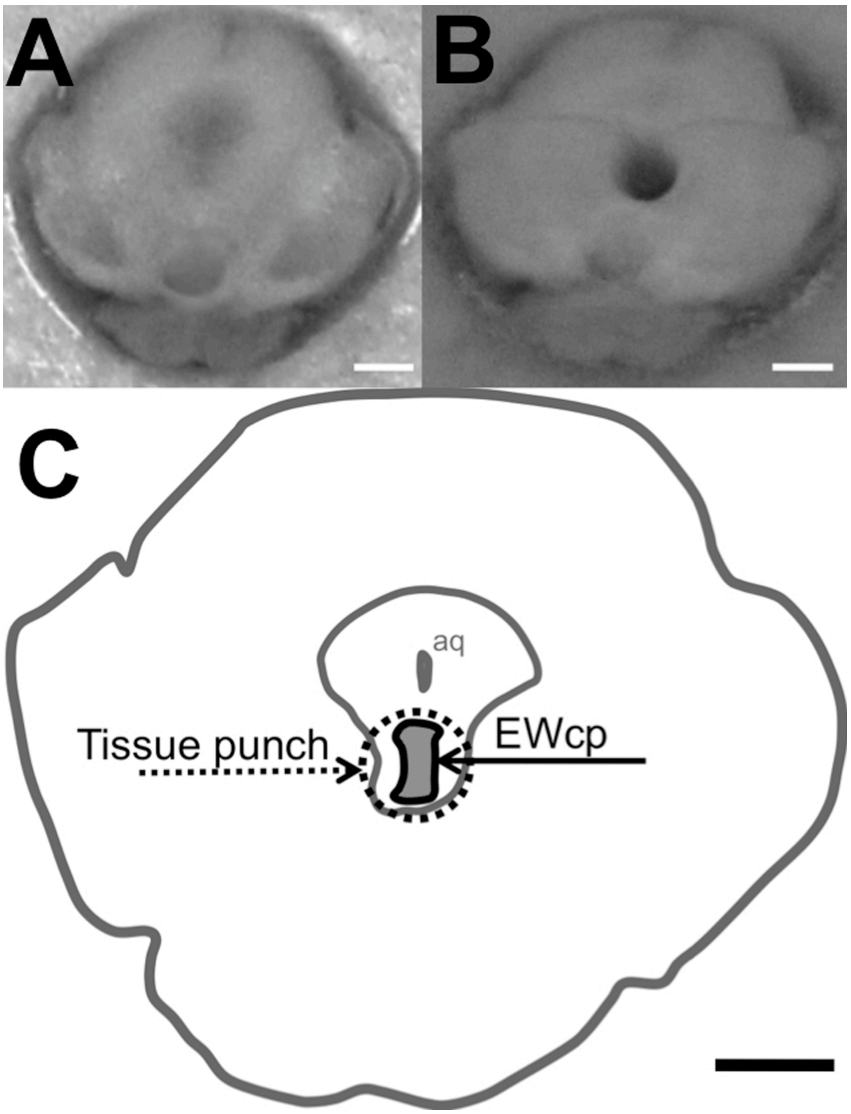
Gene Symbol	Gene Name	Category
A730017C20Rik	RIKEN gene A730017C20	EW-Enriched
Adcyap1	Pituitary adenylate cyclase-activating polypeptide	EW-Enriched
Arhgdig	Rho GDP dissociation-inhibitor 3	EW-Enriched
Arl10	ADP-ribosylation factor –like 10	EW-Enriched
BC023892	Family-with-sequence-similarity-46, member A	EW-Enriched
Brunol6	Bruno-like 6	EW-Enriched
Btg3	BTG family, member 3	EW-Enriched
Bves	Blood vessel epithelial substance	EW-Enriched
C530008M17Rik	RIKEN gene C530008M17	EW-Enriched
Cart	Cocaine- and amphetamine-regulated transcript	EW-Enriched
Cck	Cholecystokinin	EW-Enriched
Cds2	Phosphatidate cytidylyltransferase 2	EW-Enriched
Cpeb1	Cytoplasmic polyadenylation element-binding 1	EW-Enriched
Cthrc1	Collagen triple helix repeat containing 1	EW-Enriched
Ctxn1	Cortexin 1	EW-Enriched
Dlk1	Delta-like homolog 1	EW-Enriched
Dnajc12	DnaJ (Hsp40) homolog, subfamily C, member 12	EW-Enriched
Erp29	Endoplasmic reticulum protein 29	EW-Enriched
Fxyd6	FXYD domain ion transporter regulator 6	EW-Enriched
Gabre	GABA-A receptor subunit epsilon	EW-Enriched
Gabrq	GABA-A receptor subunit theta	EW-Enriched
Gap43	Growth associated protein 43	EW-Enriched
Ghsr	Growth hormone secretagogue (ghrelin) receptor	EW-Enriched
Gpx3	Glutathione peroxidase 3	EW-Enriched
Hap1	Huntington-associated protein 1	EW-Enriched
Itgb1	Integrin beta 1	EW-Enriched
Klhl1	Kelch-like family member 1	EW-Enriched
Mlec	Malectin	EW-Enriched
Mrap2	Melanocortin accessory protein 2	EW-Enriched
Ly6h	Lymphocyte antigen 6 complex	EW-Enriched
Mesdc2	Mesoderm development candidate 2	EW-Enriched
Ndn	Necdin	EW-Enriched
Nenf	Neuron-derived neurotrophic factor	EW-Enriched

Neto1	Neuropilin and tolloid-like 1	EW-Enriched
Npc2	Niemann-Pick disease, type 2	EW-Enriched
Nucb2	Nucleobindin-2 (Nesfatin-1)	EW-Enriched
Pcdh11x	Protocadhedrin 11 X-linked	EW-Enriched
Pcsk1	Proprotein convertase subtilisin/kexin type 1	EW-Enriched
Peg10	Paternally-expressed gene 10	EW-Enriched
Peg3	Paternally-expressed gene 3	EW-Enriched
Pgr15l	G-protein coupled receptor 15-like	EW-Enriched
Pld3	Phospholipase D, family member 3	EW-Enriched
Postn	Periostin, osteoblast specific factor	EW-Enriched
Prmt2	Protein arginine methyltransferase 2	EW-Enriched
Psme1	Proteosome activator subunit 1	EW-Enriched
Psme2	Proteosome activator subunit 2	EW-Enriched
Ptprn	Protein tyrosine phosphatase, receptor type N	EW-Enriched
Rbp4	Retinol binding protein 4, plasma	EW-Enriched
Rcn1	Reticulocalbin-1	EW-Enriched
Rps12	Ribosomal protein s12	EW-Enriched
Rps5	Ribosomal protein s5	EW-Enriched
Rgs4	Regulator of G-protein-signaling 4	EW-Enriched
Scg2	Secretogranin-2, secretoneurin	EW-Enriched
Sidt1	SID1 transmembrane family, member 1	EW-Enriched
Slc39a6	Solute carrier family 39, member 6	EW-Enriched
Sncg	Gamma-synuclein	EW-Enriched
Spint2	Serine peptidase inhibitor, Kunitz-type 2	EW-Enriched
Ssr1	Signal sequence receptor, alpha	EW-Enriched
Syt4	Synaptogamin IV	EW-Enriched
Syt5	Synaptogamin v	EW-Enriched
Tacr2	Tachykinin receptor 2	EW-Enriched
Tmed3	Transmembrane emp24 protein transport domain 3	EW-Enriched
Tmem22	Transmembrane protein 22	EW-Enriched
Tppp3	Tubulin polymerization-promoting protein 3	EW-Enriched
Trpc6	Transient receptor potential action channel C 6	EW-Enriched
Ucn	Urocortin-1	EW-Enriched
Vat1	Vesicle amine transport protein 1 homolog	EW-Enriched
Zcchc12	Zinc finger, CCHC domain containing 12	EW-Enriched
Egr1	Early growth response 1	ITFs
Fos	FBJ osteosarcoma oncogene	ITFs
Fosb	FBJ osteosarcoma oncogene B	ITFs
Th	Tyrosine hydroxylase	DA-Related
Ddc	Dopamine decarboxylase	DA-Related
Slc6a3	Dopamine reuptake transporter	DA-Related
Drd1a	Dopamine receptor 1	DA-Related
Drd2	Dopamine receptor 2	DA-Related
Drd3	Dopamine receptor 3	DA-Related

Drd4	Dopamine receptor 4	DA-Related
Drd5	Dopamine receptor 5	DA-Related
Ntsr1	Neurotensin receptor 1	VTA-Related
Chrna5	Nicotinic acetylcholine receptor subunit alpha-5	VTA-Related
Chrna6	Nicotinic acetylcholine receptor subunit alpha-6	VTA-Related
Chrnb3	Nicotinic acetylcholine receptor subunit beta-3	VTA-Related
Crhr1	CRF receptor 1	CRF-Related
Crhr2	CRF receptor 2	CRF-Related
Crhbp	CRF binding protein	CRF-Related

Figure 3. Diagram of the Tissue Punch Microdissection Technique

(A) Photograph of a 1 mm-thick coronal slice of the adult male B6 mouse midbrain fresh after dissection, in which the EWcp is still intact. **(B)** Photograph of a frozen midbrain slice in which the EWcp was punched out with an 18-gauge blunt needle and removed by making a horizontal cut with a razor blade just dorsal to the EWcp and sliding the tissue sample along the metal plate for harvesting. **(C)** Schematic of the EWcp within a coronal midbrain slice (appx. -3.5 mm from bregma), indicating the area dissected by the tissue micropunch. Each scalebar = 1 mm. aq; cerebral aqueduct.



microliters (μl) of extraction buffer (Arcturus PicoPure RNA Isolation Kit; Applied Biosystems) at 42°C for 30 minutes (mins), briefly vortexed, and stored at -80°C.

RNA was isolated according to the Arcturus PicoPure kit manual, as previously reported by the Ryabinin Laboratory (Cservenka et al., 2010). RNA purification columns were conditioned with 250 μl conditioning buffer for 5 mins. 50 μl of 70% EtOH was added to each sample, mixed thoroughly, transferred to the conditioned column, and centrifuged to collect the RNA. Columns were washed with 100 μl Wash Buffer #1 and DNAse treated (5 μl DNAse I + 35 μl RDD Buffer per sample). Columns were washed again with 40 μl Wash Buffer #1 and twice with 100 μl Wash Buffer #2. Each column was transferred to a new microcentrifuge tube and RNA was eluted using 15 μl elution buffer. Samples were frozen at -80°C until RNA quality readings were obtained.

To determine RNA quality, samples were thawed, spectrophotometer readings were obtained, and samples meeting criterion (260/280 values between 1.80 and 2.20) were diluted to match the RNA concentration of the least concentrated sample. Samples were DNase-treated at 42°C for five mins and then underwent first strand cDNA synthesis upon addition of the reverse transcriptase cocktail from the RT^2 First Strand kit (primer and external control mix, reverse transcriptase enzyme mix, reverse transcriptase buffer, and H2O, in ratios of 1:2:4:3). Synthesized cDNA samples were diluted with a cocktail containing the RT^2 SYBR Green Master Mix (Qiagen), and 25 μl of the mixture was deposited into each well of a custom-designed RT^2 Profiler Array for analysis by a MX3000P real-time thermal cycler (Stratagene).

A qPCR approach was taken instead of microarray because a microarray would require amplification of the small amount of RNA harvested from EWcp, and amplification may be subject to disproportional distortion of quantitative gene amounts. In addition, mouse microarrays are designed based on the B6 genome, yet several

single nucleotide polymorphisms exist between B6 and D2 strains (Walter et al., 2009), complicating this analysis. qPCR analyses were done as biological but not technical replicates, due to the high number of housekeeping genes and additional controls already included on each array.

The mean cycle thresholds (CTs) for the five housekeeping genes included on the qPCR array were first compared between B6 and D2 mice by t-test, and CTs of four housekeeping transcripts that did not show significant strain differences were then averaged and used to normalize the quantitative expression of all genes of interest included on the array. For each individual gene of interest, CT values were normalized by the equation $2^{-\Delta CT}$, where ΔCT = the CT for the gene of interest subtracted from the mean CT value of the housekeeping genes. Additionally, data were analyzed by the $2^{-\Delta\Delta CT}$ method, in order to determine fold change levels of mRNA in B6 mice expressed relative to D2 mice ("calibrator" strain). The mean $2^{-\Delta\Delta CT}$ values were compared by t-test between the two strains (significance threshold at $p < .05$). Bonferroni correction for multiple comparisons was not applied, as I aimed to identify as many differentially-expressed genes as possible. Such an approach relies on confirmation studies. Therefore, genes highlighted in qPCR studies were also analyzed *in silico* using publicly-available microarray data, and at the protein level by IHC performed in the Ryabinin Lab.

In Silico *Analyses*

Following identification of genes exhibiting strain differences in EWcp expression, GeneNetwork (http://genenetwork.org) (Chesler et al., 2004; GeneNetwork, © 2001) was used to test the findings. Analysis of several microarray data sets determined whether the transcripts showing genotype-dependent expression within EWcp also differed in expression throughout whole brain, cerebellum, striatum, hippocampus, H, neocortex, and amygdala.

For each of the genes highlighted by qPCR analysis, I compared the reported values for B6 and D2 mice from the following GeneNetwork data sets: UCHSC BXD Whole Brain M430 2.0 (Nov06) RMA, SJUT Cerebellum mRNA M430 (Mar05) RMA, HQF BXD Striatum ILM6.1 (Dec10v2) RankInv, Hippocampus Consortium M430v2 (June06) PDNN, INIA Hypothalamus Affy MoGene 1.0 ST (Nov10), HQF BXD Neocortex ILM6.1 (Dec10v2) RankInv Database, and INIA Amygdala Cohort Affy MoGene 1.0 ST (Mar11) RMA (Overall et al., 2009; Saba et al., 2006). Data are presented as mean ± standard error of the mean (SEM), and analyzed by t-test. Significance threshold was set at $p < .05$.

IHC Analyses

IHC was performed on the protein products of three genes identified as being differentially expressed between B6 and D2 mice by the qPCR array. Selection of products was based on available commercial antibodies. Ucn1 and Fos were not included in these analyses because previous studies already identified differences in Ucn1-IR and Fos-IR between B6 and D2 mice (B6 > D2 for both proteins) (Bachtell et al., 2002b; Weitemier and Ryabinin, 2005a; Weitemier et al., 2005).

After habituation to the colony, mice (n = 8 per strain) were euthanized by CO2 inhalation and underwent transcardial perfusion with 2% paraformaldehyde (PFA) dissolved in phosphate buffered saline (PBS). Brains were rapidly dissected and placed in 2% PFA for storage overnight, followed by cryoprotection in 20% and 30% sucrose dissolved in PBS containing 0.1% sodium azide (NaN3). Coronal sections were sliced into 30-micrometer (μm) sections on a Leica CM1850 cryostat, and slices were collected in PBS containing 0.1% NaN3.

For each gene product, 6-8 slices containing the EWcp (evenly spaced along the rostral-caudal axis, from -3.2 to -3.8 mm from bregma) were chosen from each animal.

Examinations of CCK- and Ptprn-IR were preceded by an antigen retrieval process. However, antigen retrieval was not necessary for examination of CART-IR, which stains heavily within mouse EWcp even without this additional step (Cservenka et al., 2010; Kozicz, 2003). For IHC procedures examining CCK and Ptprn in the EWcp, antigen retrieval consisted of rinsing the sections in PBS and then boiling the tissue in sodium citrate buffer (10 mM sodium citrate, .05% Tween 20, pH 6.0) followed by cooling to room temperature. For all IHC procedures, slices underwent a standard diaminobenzidine (DAB) staining protocol nearly identical to previous reports from our lab (Giardino et al., 2011b; Spangler et al., 2009). Primary antibodies directed against either CART 55-102 (H-003-60, Phoenix) CCK 26-33 (H-069-04, Phoenix), or Ptprn (HPA-007179, Sigma-Aldrich) were used at concentrations of 1:20,000, 1:30,000, and 1:1000, respectively.

The number of CART-, CCK-, or Ptprn-positive neurons within EWcp was counted manually using a Leica DM4000 microscope. A single value per animal was calculated by averaging the cell counts across all slices from that subject, and mean cell counts for the two strains were compared by t-test separately for each of the three gene products. One data point was excluded from the analysis of CART-IR in B6 mice, because the value was greater than 2.5 standard deviations below the mean. Data are presented as mean ± SEM, and significance threshold was set at $p < .05$.

RESULTS

Analysis of Housekeeping Genes

Preliminary analysis of five housekeeping genes revealed that *Gapdh* CT values were significantly greater in D2 vs. B6 mice ($t_{10} = 3.49$; $p < .01$). Therefore, all genes of interest were normalized to the average of the remaining four housekeeping genes (*Actb, Gusb, Hprt1, Hsp90ab1*).

Gene Expression Analyses

Following normalization, 14/86 genes of interest differed significantly between strains: *BC023892* (also known as *Fam46a*), *Btg3*, *Bves, Cart*, *Cck*, *Ghsr*, *Neto1*, *Postn*, *Ptprn*, *Rcn1*, *Ucn*, *Egr1* (also known as *zif268*), *Fos*, and *Drd5* (**Fig. 4**). In each case, expression was greater in B6 mice, relative to D2 mice (all $t_{10} > 2.44$; all $p < .05$) (**Table 5**). Of these 14 genes, 11 were EWcp-enriched (**Figs. 5-6**). Two were ITFs (*Egr1* and *Fos*), which are not necessarily EWcp-enriched, but are induced within EWcp following certain environmental stimuli (Bachtell et al., 1999; Ryabinin et al., 2001). The remaining gene, *Drd5* (DA receptor subtype 5) may reside on either EWcp neurons, or on the DAergic neurons of the adjacent RLi. Although *Ddc* (dopamine decarboxylase; enzyme involved in DA synthesis) barely missed reaching statistical significance ($p = .0501$), mRNA levels of this gene were upregulated in D2 vs. B6 mice.

In Silico *Analyses*

Of the 14 transcripts demonstrating expression differences, *in silico* analyses confirmed that six (*Btg3, Bves, Cart, Cck, Egr1,* and *Rcn1*) also differed significantly between B6 and D2 mice across whole brain and/or other brain regions (cerebellum, striatum, hippocampus, H, neocortex, amygdala) (**Table 6**). Consistent with qPCR array results, all expression levels were greater in B6 vs. D2 mice, with the exception of *Rcn1*, whose genotype-dependent regulation in whole brain, cerebellum and amygdala was opposite to that in the EWcp.

Figure 4. Gene Categories and Proportions of Expression Differences

(A) Pie chart on left indicates relative proportions of gene categories included on the qPCR arrays. Pie chart on right indicates relative proportions of gene categories in which strain differences were identified. **(B)** Pie chart showing relative proportions of gene categories in which strain differences were identified, as compared to proportion of genes in which no differences were identified. Numbers indicate number of genes in each category (86 genes of interest total + 5 housekeeping genes + 5 control wells for each 96-well plate). While *Ucn* is both CRF-related and EWcp-enriched, it was included only in the EWcp-enriched category for these analyses.

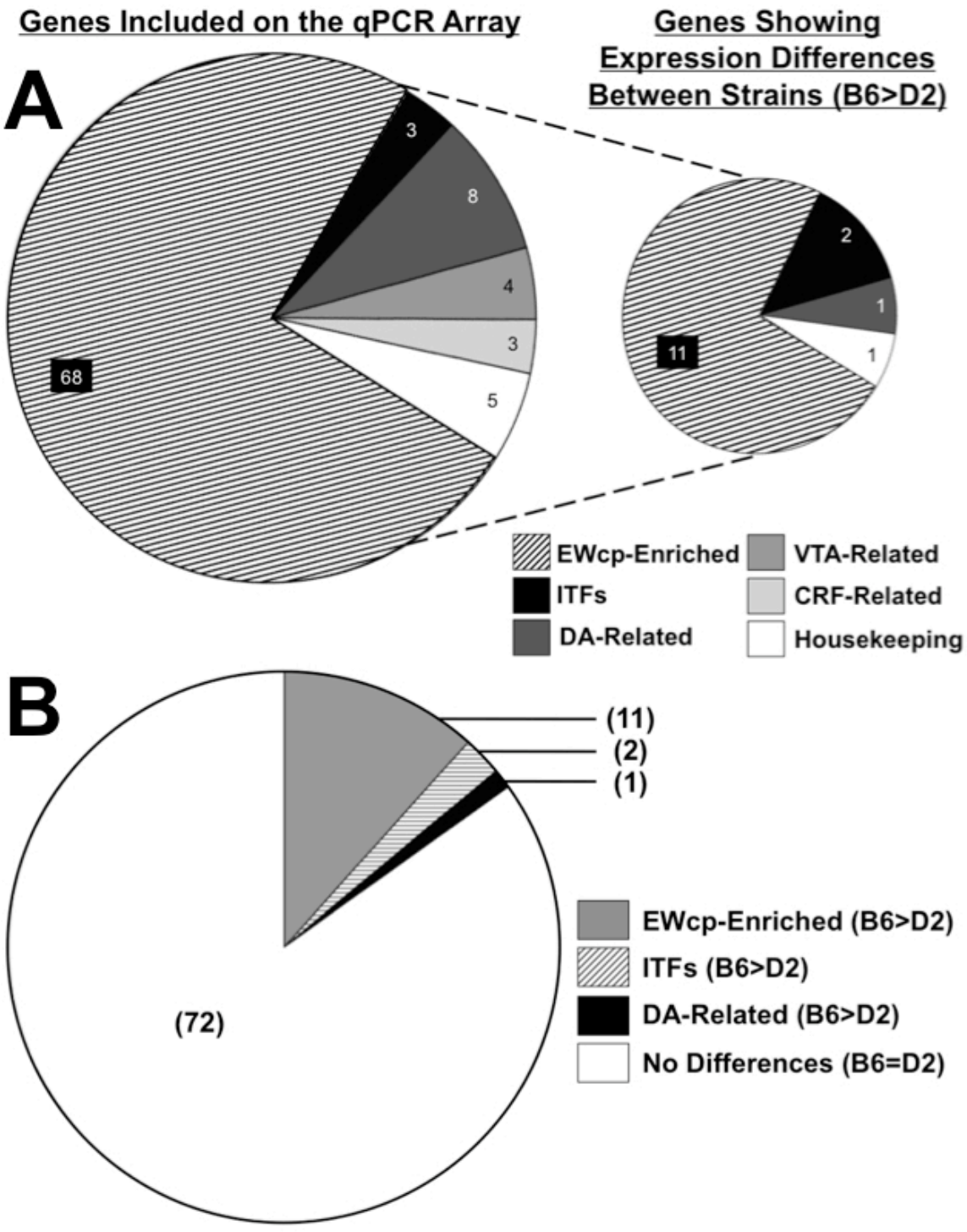

Genes Included on the qPCR Array
Genes Showing Expression Differences Between Strains (B6>D2)
A
68
3
8
4
3
5
11
2
1
1
EWcp-Enriched
ITFs
DA-Related
VTA-Related
CRF-Related
Housekeeping
B
(72)
(11)
(2)
(1)
EWcp-Enriched (B6>D2)
ITFs (B6>D2)
DA-Related (B6>D2)
No Differences (B6=D2)

Table 5. List of Genes Differentially Expressed Between B6 and D2 Mice

All expression differences were in the direction of B6>D2, with the exception of *Ddc*, which demonstrated a near-significant increase in expression in D2 mice, relative to B6 mice (p = .0501).

Gene	**Category**	***t*-value**	***p*-value**	**Fold**	**Effect**
BC023892	EWcp-Enriched	5.720	.0002	+2.53	B6>D2
Btg3	EWcp-Enriched	2.565	.0281	+1.43	B6>D2
Bves	EWcp-Enriched	2.440	.0349	+3.25	B6>D2
Cart	EWcp-Enriched	4.430	.0013	+4.12	B6>D2
Cck	EWcp-Enriched	4.167	.0019	+3.26	B6>D2
Ghsr	EWcp-Enriched	6.668	<.0001	+2.41	B6>D2
Neto1	EWcp-Enriched	3.712	.0040	+1.77	B6>D2
Postn	EWcp-Enriched	4.821	.0009	+2.76	B6>D2
Ptprn	EWcp-Enriched	2.301	.0442	+1.68	B6>D2
Rcn1	EWcp-Enriched	8.581	<.0001	+13.00	B6>D2
Ucn	EWcp-Enriched	4.576	.0010	+4.29	B6>D2
Egr1	ITF	3.263	.0085	+1.96	B6>D2
Fos	ITF	3.676	.0043	+3.69	B6>D2
Drd5	DA-Related	3.316	.0078	+1.93	B6>D2
Ddc	DA-Related	2.227	.0501	-4.40	D2>B6

Figure 5. Visual Examples of Gene Expression (Coronal)

Shown are coronal slices at appx. -3.5 mm from bregma in adult male B6 mice following *in situ* hybridization to reveal EWcp-enriched expression of *BC023892* **(A)**, *Btg3* **(C)**, *Bves* **(E)**, *Cart* **(G),** *Cck* **(I)**, *Neto1* **(K)**, and *Rcn1* **(M).** Close-up images **(B, D, F, H, J, L, N)** show the area within the dotted line of the corresponding figure, indicating that *BC023892*, *Btg3*, *Bves*, *Cart*, *Cck*, *Neto1*, and *Rcn1* demonstrate EWcp-enriched patterns of expression. Scalebar = 100 μm, valid for all close-up images. Images courtesy of the ABA, used with permission.

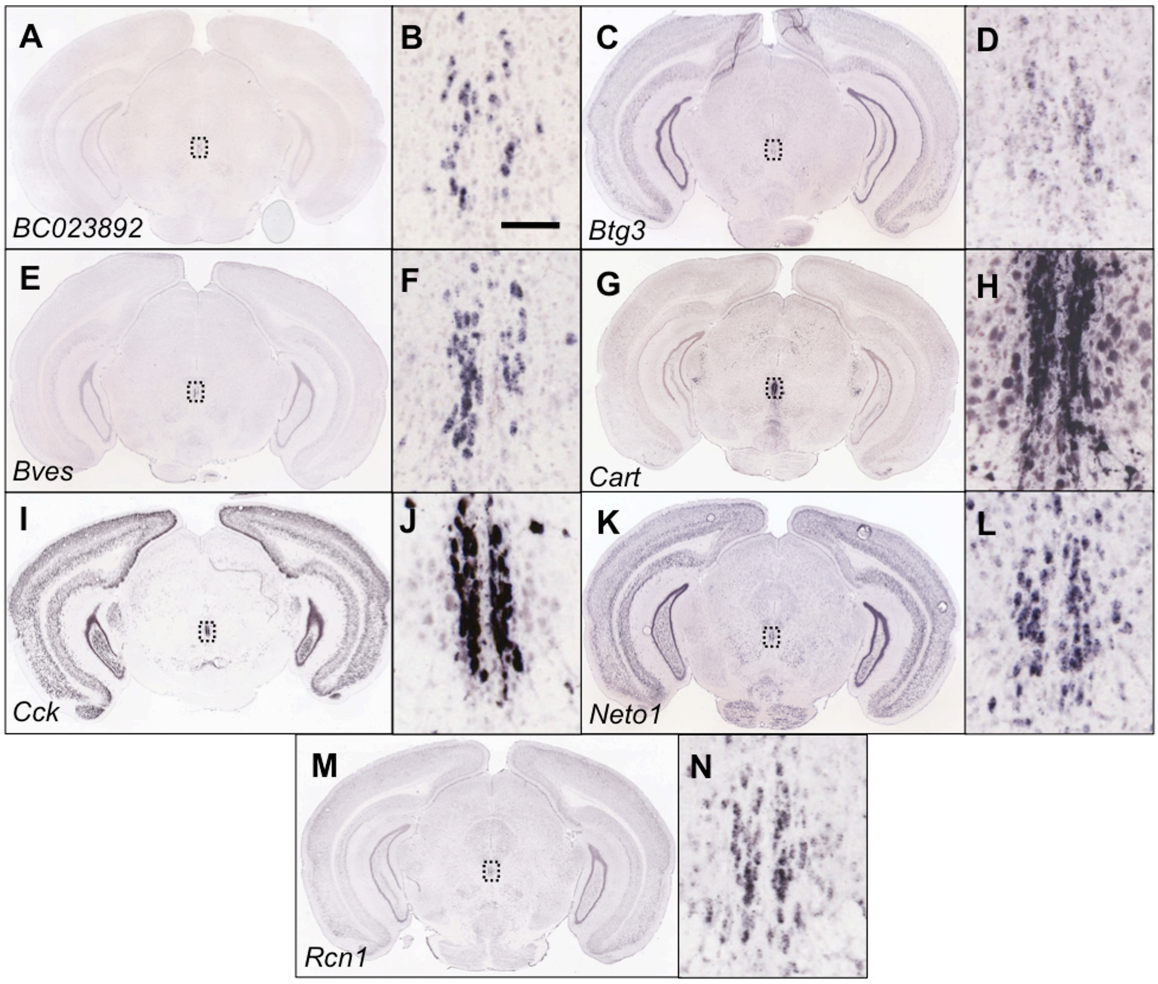


Figure 6. Visual Examples of Gene Expression (Sagittal)

Shown are sagittal slices at the midline from adult male B6 mice following *in situ* hybridization to reveal EWcp-enriched expression of *Ghsr* **(A)**, *Postn* **(C)**, *Ptprn* **(E)**, and *Ucn* **(G).** Close-up images **(B, D, F, H)** show the area within the dotted line, indicating that *Ghsr*, *Postn*, *Ptprn*, and *Ucn* demonstrate EWcp-enriched expression. Each scalebar = 500 μm. Images courtesy of the ABA, used with permission.

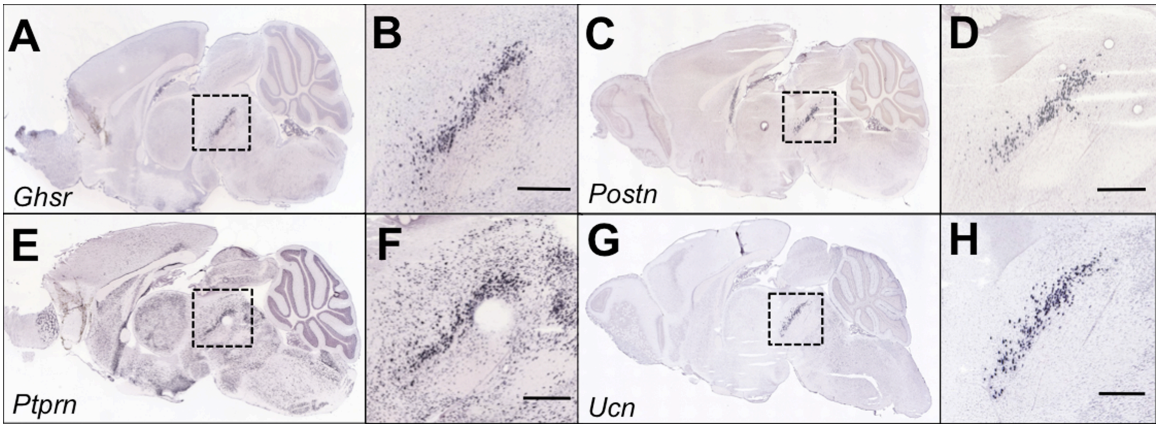


Table 6. *In Silico* Analysis of Gene Expression Differences

Values are mean arbitrary units, with SEM in parentheses, retrieved from publicly-available database sets on www.genenetwork.org. Retrieval date: October 2011.

Gene	Region	C57BL/6J	DBA/2J	*t*	*p*	Effect
Btg3	Whole Brain	10.223 (± .019)	9.987 (± .018)	9.017	<.0001	B6>D2
Btg3	Cerebellum	9.552 (± .049)	9.231 (± .076)	3.550	.0238	B6>D2
Btg3	Striatum	6.724 (± .037)	6.547 (± .016)	4.391	.0482	B6>D2
Bves	Cerebellum	8.848 (± .054)	8.644 (± .048)	2.824	.0477	B6>D2
Cart	Hippocampus	7.539 (± .203)	6.666 (± .101)	3.202	.0493	B6>D2
Cck	Hypothalamus	9.323 (± .091)	9.040 (± .037)	2.881	.0164	B6>D2
Cck	Neocortex	15.227 (± 020)	15.008 (± .039)	4.997	.0378	B6>D2
Egr1	Amygdala	10.707 (± .049)	10.490 (± .071)	2.515	.0456	B6>D2
Rcn1	Whole Brain	9.042 (± .066)	9.551 (± .053)	6.013	.0001	D2>B6
Rcn1	Cerebellum	5.606 (± .065)	6.035 (± .111)	3.304	.0298	D2>B6
Rcn1	Amygdala	9.769 (± .018)	9.988 (± .042)	4.793	.0030	D2>B6

IHC Analyses

To determine whether gene expression differences could be replicated at the protein level, IHC was used to visualize CART-, CCK-, or Ptprn-IR within the EWcp. In each case, there were a greater number of IR neurons in B6 mice, relative to D2 mice (all $t_{13\text{-}14} > 5.08$; all $p < .0005$) (**Fig. 7**), consistent with the results from qPCR analyses.

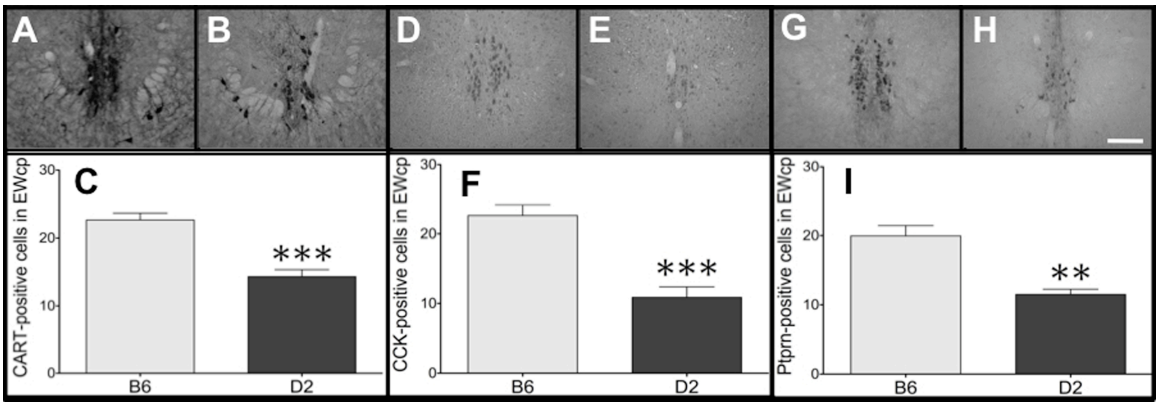
DISCUSSION

This study used publicly available ABA tools to identify genes that were EWcp-enriched. Tissue punch microdissection of the EWcp and array expression profiling quantified those transcripts (along with other genes of interest) in EWcp tissue samples from adult male B6 and D2 mice. Expanding on previous studies that analyzed protein–level expression of ITFs and neuropeptides within the EWcp, the current data confirm that mRNA levels of several EWcp-enriched genes and two ITFs are greater within B6 mice, relative to D2 mice. Interestingly, these findings are paralleled by phenotypic differences between high and low EtOH-drinking B6 and D2 mice (Crabbe et al., 1983; Yoneyama et al., 2008), suggesting that differential levels of neural activity and neuropeptide synthesis/release could explain, in part, genotypic differences in EtOH intake.

Although prior evidence based on lesion studies and comparisons of EWcp-Ucn1 protein expression suggested that EWcp-Ucn1 neurons *promote* alcohol drinking and food consumption (Bachtell et al., 2004; Weitemier and Ryabinin, 2005b), additional studies using intracranial injections showed that Ucn1 also *decreased* alcohol drinking and food consumption (Ryabinin et al., 2008; Spina et al., 1996). Thus, both a decrease in EWcp-Ucn1 tone (via EWcp lesions) and an increase in Ucn1 tone (via intracranial Ucn1 infusions) had similar effects on these two behaviors. One potential explanation for these paradoxical findings was that *higher Ucn1-IR* within the EWcp of EtOH-preferring

Figure 7. IHC Analysis of Gene Expression Differences

Representative EWcp photomicrographs from B6 mice **(A, D, G)** and D2 mice **(B, E, H)** (3.5 mm from bregma)**.** Bar graphs illustrate numbers of IR neurons (mean ± SEM) stained for **(C)** CART, **(F)** CCK, and **(I)** Ptprn in B6 and D2 mice. Scalebar = 500 µm, valid for all representative images. Asterisks indicate significant differences between B6 and D2 by t-test (***p* = .0002; ****p* < .0001).



vs. EtOH-avoiding animals resulted from *lower neuronal activity and less release* of Ucn1 from the EWcp, rather than greater levels of *Ucn* mRNA.

The current data provide a strong argument against a lower rate of release in B6 mice, because levels of *Ucn* mRNA were higher in the EWcp of these animals, mimicking the differences in protein expression. Thus, differences in Ucn1-IR within the EWcp of B6 vs. D2 mice are likely attributed to higher levels of *Ucn* mRNA within individual neurons, rather than lower neural activity and lower rates of peptide release. Because EWcp-Ucn1 protein levels are reflective of EWcp-*Ucn* mRNA levels, these data support the longstanding hypothesis that greater activity of Ucn1 neurons within the EWcp is associated with a genetic predisposition toward greater EtOH intake and heightened EtOH sensitivity (Bachtell et al., 2003; Ryabinin and Weitemier, 2006).

In addition, levels of *Fos* and *Egr1* mRNA (encoding ITFs that drive neural activity) were greater in the EWcp of B6 vs. D2 mice, arguing against the possibility that greater Ucn1-IR in B6 vs. D2 mice was due to less Ucn1 release. Although baseline levels of Fos-IR were not directly compared between strains in the current study, a previous experiment found that the number of Fos-IR cells was greater in B6 vs. D2 mice (Bachtell et al., 2003), consistent with the gene expression data here. Since *Fos* and *Egr1* are well-characterized markers of neural activity, this suggests that basal activity of the EWcp is higher in B6 vs. D2 mice. Given this presumed difference in neural activity, peptide release from the EWcp is likely to be higher in B6 vs. D2 mice, rather than vice-versa.

An additional possibility for the seemingly contradictory relationship between Ucn1 tone and EtOH-related phenotypes was that lesions of the EWcp had the potential to eliminate DA neurons of the RLi, which intermingle with EWcp-Ucn1 neurons (Bachtell et al., 2002a; Fonareva et al., 2009; Gaszner and Kozicz, 2003). However, because there are more DA-synthesizing neurons in the RLi of D2 mice as compared to B6 mice

(D'Este et al., 2007), it remains unclear whether this neuronal population could contribute to voluntary oral EtOH consumption in the expected manner.

The current study detected significantly more neurons IR for CART, CCK, and Ptprn in B6 vs. D2 mice. The protein product of *Cart* is a neuropeptide important for mediating drug reward and regulating food intake (Rogge et al., 2008). CART has an extremely dense pattern of expression within the EWcp, as demonstrated across several mammalian species (Cservenka et al., 2010; Koylu et al., 1998; Kozicz, 2003; Lima et al., 2008). These data are the first to report that EWcp-*Cart* is differentially expressed between B6 and D2 mice at the mRNA and protein levels, suggesting that CART could be involved in similar functions as Ucn1. Indeed, CART is co-localized in 95-100% of EWcp-Ucn1 neurons (Cservenka et al., 2010; Kozicz, 2003).

The protein product of *Cck,* CCK, is a neuropeptide important for several functions, including regulation of food intake, anxiety-like behavior, and drug reward (Beglinger, 2002; Rotzinger and Vaccarino, 2003). Although the presence of CCK in the rat EWcp has been demonstrated previously (Maciewicz et al., 1984; Rattray et al., 1992), this is the first time that *Cck* mRNA and CCK-IR have been reported in the mouse EWcp. It is tempting to speculate that EWcp-CCK is involved in similar functions as EWcp-Ucn1 and EWcp-CART.

Although a suitable IHC procedure for the protein product of *Ghsr* (Ghsr; receptor for the orexigenic hormone ghrelin) was unable to be generated, previous studies from the Ryabinin Laboratory implicate EWcp-Ghsr signaling in the DID model of binge EtOH consumption (Kaur and Ryabinin, 2010), consistent with the hypothesis that greater EWcp-*Ghsr* expression in B6 vs. D2 mice is associated with their differing EtOH intakes.

Ptprn encodes Ptprn (also known as islet antigen 2, or IA-2). Other than the ABA, this is the first report that the gene is expressed in the mammalian EWcp. The function of the gene is not well understood, despite the fact that it is a major auto-antigen in

insulin-dependent diabetes mellitus, and could be involved in mediating dense core vesicle release (Cai et al., 2004; Lu et al., 1996). As such, Ptprn could be involved in release of vesicles from the EWcp. This function, together with the identification of greater *Ptprn* and Ptprn expression in B6 vs. D2 mice, is an additional piece of evidence suggesting that EWcp neuronal activity is greater in B6 vs. D2 mice.

Use of *in silico* analyses as an additional method of confirmation was successful, showing that at least six of the 14 highlighted transcripts showed genotype-dependent expression throughout whole brain and/or cerebellum, striatum, hippocampus, H, neocortex, and amygdala. Strain differences in expression of *Btg3* and possibly *Cck* appear to generalize to several brain areas. However, the majority of identified genes did not differ significantly across other brain regions. The absence of consistent gene expression differences between strains within non-EWcp brain areas strengthens the possibility that the influence of these genes on stress- and addiction-related behavior may be particularly EWcp-specific.

While some of these expression differences could theoretically be confirmed by Western blotting, the difficulties of dissecting relatively large quantities of EWcp from the mouse brain prevented this analysis. I anticipate that the other transcripts expressed higher in B6 vs. D2 mice also have corresponding differences in protein levels. In fact, this would be expected to be the case for nearly all EWcp-enriched proteins that are co-expressed with Ucn1, because there are more Ucn1-positive neurons in the EWcp of B6 versus D2 mice.

The finding that *Drd5* is greater in EWcp microdissections from B6 vs. D2 mice suggests that this transcript might be expressed in EWcp neurons. Drd5 is the least-studied DA receptor, and its potential expression and function in EWcp is an intriguing possibility that awaits further testing.

The conservative use of four housekeeping genes gives confidence to findings of this study. In that respect, it is interesting that *Gapdh* was differentially expressed between B6 and D2 mice. Other studies found that EWcp-*Gapdh* can be regulated by stress (Derks et al., 2008). I hypothesize that the observed differences in *Gapdh* expression might be specific to the EWcp, because other studies did not identify differential expression of *Gapdh* in whole-brain analysis of B6 and D2 mice (GeneNetwork, © 2001; Shirley et al., 2004). Gapdh catalyzes an important energy-yielding step in carbohydrate metabolism, which could serve as another indication of higher neural activity in the EWcp of B6 vs. D2 mice.

Taken together, this study identified at least 11 transcripts that are enriched within the EWcp, and differentially expressed in the EWcp of B6 and D2 mice. Further examination of these genes could shed light on the function of this recently-characterized brain region, and could provide insight into the genetic underpinnings of behavioral differences between B6 and D2 mice. Additional analyses of the EWcp gene expression profile in rodent strains selectively-bred for EtOH-related traits, and in EtOH-exposed subjects, may provide additional information on the roles of these identified transcripts in driving EtOH-related behaviors via the EWcp.

In broader terms, this approach illustrates how a combination of data-mining and genetic techniques can overcome the technical difficulties of analyzing a distinct neuronal population. For example, the tissue punch samples contained a region larger than the EWcp itself, and the search features on the ABA provided fairly low spatial resolution. However, I was conservative in my definition of EWcp-enriched genes by the ABA analysis, which led to successful utilization of the micropunch and expression profiling methods. This combination of tissue-specific gene expression analysis and small-scale bioinformatics may be useful for advancing behavioral neurogenetics.

CHAPTER 2: Urocortin-1 Deletion Alters Ethanol Preference and Reward

(This chapter has been reformatted for inclusion in this dissertation from: Giardino WJ, Cocking DL, Kaur S, Cunningham CL, Ryabinin AE. Urocortin-1 within the centrally projecting Edinger-Westphal nucleus is critical for ethanol preference. *PLoS One.* 2011;6:(10):e26997. EPub 2011 Oct 28.)

INTRODUCTION

Prior studies from the Ryabinin Laboratory revealed that electrolytic lesions of the EWcp decreased 2-BC EtOH preference (Bachtell et al., 2004). Together with the evidence pointing toward a role for Ucn1 in EtOH-related behaviors (Ryabinin and Weitemier, 2006), I hypothesized that electrolytic lesions of the EWcp decreased EtOH consumption by disrupting the function of midbrain Ucn1. However, because several other neuropeptide systems co-exist with Ucn1 in the EWcp (Gaszner et al., 2007; Kozicz, 2003; Xu et al., 2009; Xu et al., 2011; Zigman et al., 2006), the possibility remained that EWcp lesion altered EtOH consumption via a Ucn1-independent mechanism (i.e., through a different neuropeptide or receptor expressed in EWcp). The finding that genetic deletion of *Ucn* did not alter binge-like drinking in the standard single-bottle DID paradigm (Kaur et al., 2012) made it especially important to clarify whether EWcp lesions altered 2-BC EtOH drinking in a Ucn1-dependent manner, because there can be discrepancies in the genetic and neurobiological factors underlying single-bottle DID vs. 2-BC preference drinking. For example, relative to their non-selected founder control line, mice selectively-bred to reach high BECs in a two-day single-bottle DID paradigm showed decreased intake of and preference for 30% EtOH in a 2-BC CA paradigm (Crabbe et al., 2011b).

The studies in Chapter 2 compared the effects of EWcp lesion on 2-BC CA EtOH intake and preference between KO mice lacking Ucn1 and their WT littermates to test the hypothesis that functional Ucn1 expression is required for the EWcp's influence on EtOH consumption. I predicted that Ucn1 KO would decrease EtOH drinking only in mice that received sham surgery, and that lesion of the EWcp would decrease EtOH drinking only in WT mice with normal expression of Ucn1. I further hypothesized that Ucn1 contributed to the rewarding effects of EtOH, and therefore used place conditioning studies to test whether genetic deletion of *Ucn* altered EtOH-induced conditioned

reward. The conditioned aversive effects of EtOH were also examined in order to determine whether Ucn1 KO altered EtOH-induced aversion or EtOH-related learning in general. Finally, in order to determine whether EtOH-mediated behaviors involving Ucn1 were mediated via CRF2, I tested whether genetic deletion of *Crhr2* altered EtOH-induced reward. The expected outcome was that EtOH-CPP would be attenuated in Ucn1 and CRF2 KO mice, relative to WT littermates, but I had no directional hypotheses about EtOH-CPA in Ucn1 KO and WT mice.

MATERIALS AND METHODS

Animals

I used male and female single gene mutant mice created from embryonic stem cells that underwent targeted gene inactivation. Ucn1 KO mice generated on a 129X1/SvJ x B6 background contained a deletion of exon 2 of the *Ucn* gene (Vetter et al., 2002), and CRF2 KO mice generated on a 129X1/SvJ x B6 background contained a deletion of exons 3-4 of the *Crhr2* gene (Coste et al., 2000). The Ucn1 KO line was backcrossed onto a B6 background for 10-12 generations, and the CRF2 KO line was backcrossed onto a B6 background for 14 generations. KO and WT mice used for these studies were littermates, generated by heterozygous matings. Mice were weaned at 28-32 days of age and isosexually housed.

For EtOH drinking, only male mice were used, and underwent surgery at 9-16 weeks of age. For EtOH place conditioning, both male and female mice were tested at 8-14 weeks of age. Importantly, genetic deletion of *Ucn* or *Crhr2* did not alter the rate of EtOH elimination (Kaur et al.; Pastor et al., 2008). All mice remained on a 12hr/12hr light-dark schedule (lights ON/ZT-0 = 0700h) and received *ad libitum* access to food (LabDiet 5001; LabDiet, Richmond, IN) and H2O, with the exception of time spent in the behavioral apparatus (EtOH conditioning experiments).

Mice on a B6 genetic background are known for high voluntary levels of EtOH intake and preference, thus they are a favorable choice for 2-BC EtOH drinking studies (Yoneyama et al., 2008). While mice on a D2 background generally exhibit more robust levels of EtOH place conditioning than B6 mice (Cunningham et al., 1992), I chose to use mice on a B6 genetic background in order to produce data that would be comparable to 2-BC drinking experiments, as well as to avoid the time-consuming and expensive process of backcrossing KO mice onto a D2 background.

All protocols were approved by the OHSU IACUC and were performed with adherence to the NIH Guidelines for the Care and Use of Laboratory Animals.

Surgical Procedures

Electrical rather than chemical lesions were performed in order to facilitate comparisons with previous studies from the Ryabinin Laboratory (Bachtell et al., 2004). EWcp lesion surgery was performed in male Ucn1 KO and WT littermate mice similar to previous reports (Bachtell et al., 2004; Weitemier and Ryabinin, 2005b). Immediately prior, mice were given a subcutaneous (s.c.) injection of Rimadyl (Carprofen; 5 mg/kg). Rimadyl is a non-steroidal anti-inflammatory drug used to treat post-operative pain and inflammation. Mice were placed under isoflurane anesthesia, secured in a stereotaxic apparatus, and received either electrolytic lesion of the EWcp or sham surgery. For both operations, a small hole was drilled through the skull on the midline (-3.4 mm, A/P) and a stainless steel electrode (SNE-300, Rhodes Medical Instruments, Inc., Woodland Hills, CA) was guided down into the EWcp nucleus (-3.9 mm, D/V). The electrode was connected to the positive terminal of a lesion-making device (Model 3500, Ugo Basile, Comerio, Italy). To ground the animal, the negative terminal was attached to the mouse's tail. For sham animals, the electrode remained inactive, but for lesion animals, the electric current (0.4 milliamps [mA]) was activated for five seconds. The electrode was removed, the skin

was sutured, and animals were single-housed in a cage containing fresh bedding and food, and a single bottle containing H2O. Loss of body temperature was avoided by placing the cage on a heating pad for 30-60 mins during the initial recovery period. Following 5-9 days of recovery from surgery, mice received access to two 25 milliliter (ml) glass cylinder bottles (both containing H2O). Importantly, lesions of EWcp did not produce changes in locomotor activity or the rate of EtOH elimination (Bachtell et al., 2004), nor did they produce changes in anxiety-like behavior (Weitemier and Ryabinin, 2005b).

Ethanol Drinking Procedures

Following four days of drinking H2O from two bottles, individually-housed mice underwent a twelve-day 24hr 2-BC CA EtOH drinking experiment during which they received access to two bottles: one containing H2O, and one containing increasing concentrations of EtOH. 95% EtOH was diluted in H2O to reach the desired concentrations, and all concentrations refer to volume/volume (v/v) percentages. The experiment consisted of three phases during which mice had access to either: 3% EtOH and H2O (Days 1-4), 6% EtOH and H2O (Days 5-8), or 10% EtOH and H2O (Days 9-12). Other palatable (or aversive) fluids were not tested here because earlier studies indicated that EWcp lesion did not affect preference (or avoidance) of sucrose, saline, quinine, and saccharin (Bachtell et al., 2004; Weitemier and Ryabinin, 2005b). Mice were weighed and fluid levels from each of the two bottles were recorded on a daily basis between ZT-3 and ZT-5. Location of the bottles on the cage top (left vs. right) was alternated daily to avoid the potential confound of an inherent side preference.

EWcp Lesion Histology

Immediately following the final day of 10% EtOH, mice were euthanized by CO2 inhalation. Brains were rapidly dissected, post-fixed overnight in 2% PFA in PBS, and cryoprotected in 30% sucrose in PBS until saturation. Coronal slices of the midbrain, 30 μm thick, were collected using a CM1850 cryostat (Leica Microsystems) and placed into PBS containing 0.3% NaN3 for storage. Six to eight sections spanning the rostral-caudal extent of the EWcp were selected from each animal and underwent Thionin staining. Sections were mounted on clear glass slides, coverslipped, and viewed on a Leica DM4000 microscope for examination of the location of the lesion (and verification of the absence of damage in sham mice). Images were acquired with the MicroPublisher 3.3 RTV in conjunction with Q-Capture (Q-Imaging, Surrey, BC, Canada). Animals containing lesions that resulted in destruction of a large portion of the EWcp were included in the lesion group, and all sham animals were included in the sham group for statistical analysis of drinking data. Exclusion of mice with missed lesions was performed blinded to genotype.

Statistical Analysis – Ethanol Drinking

EtOH consumption in ml was converted to grams (g) and divided by the animal's body weight to give daily intake scores expressed in g per kilogram (kg). Daily EtOH preference was calculated by dividing EtOH consumption in ml by the total fluid consumption in ml. Total fluid consumption in ml was divided by the animal's body weight to give values expressed in ml/kg. Data points across each of the four days of drinking at the 3%, 6%, and 10% concentrations of EtOH were averaged to produce EtOH intake, preference, and total fluid consumption values for each animal at each EtOH concentration.

Each dependent variable was analyzed by a three-way repeated measures analysis of variance (RM-ANOVA) design with genotype (KO, WT) and surgery (sham, lesion) as the between-subjects factors, and EtOH concentration (3%, 6%, 10%) as the repeated measure. Interactions with EtOH concentration were followed by simple main effect analyses evaluating the impact of surgery and genotype separately across the three EtOH concentrations. Interactions between surgery and genotype were followed by simple main effect analyses evaluating the impact of EtOH concentration and surgery separately between the two genotypes. A priori hypotheses were that lesion would significantly decrease EtOH drinking in WT mice, but not KO mice, and that Ucn1 KO would decrease EtOH drinking in sham mice, but not lesion mice. Following significant or near-significant genotype x surgery interactions, post-hoc comparisons between the four individual groups were made using Bonferroni contrasts corrected for multiple comparisons (significance threshold at $p < .0083$). For all analyses other than post-hoc comparisons, significance threshold was set at $p < .05$.

Conditioning Apparatus

The apparatus for EtOH conditioning consisted of four identical boxes measuring 30 x 15 x 15 centimeter (cm) that contained six detectors placed 2.2 cm above the floor for acquisition of spatial location and locomotor activity data. The conditioned stimuli consisted of two unbiased tactile cues: “grid” and “hole” floors that were interchangeable within the apparatus, allowing for arrangement of the cues in a “split” configuration (for pre-test and test), and a “matching” configuration (for conditioning). The apparatus and conditioned stimuli have been described in detail elsewhere (Cunningham et al., 2006).

Ethanol Conditioning Procedures

In the first set of conditioning experiments (EtOH-CPP), male and female Ucn1 and CRF2 KO and WT littermate mice (n = 8-19 per line, per sex, per genotype) were tested for the conditioned rewarding effects of EtOH using a slight variant of a well-established, unbiased place conditioning protocol in which pre-session exposure to EtOH results in a significant preference for the EtOH-paired environment (Cunningham et al., 2006).

On Day 1 (pre-test), mice were weighed and given a saline injection (12.5 ml/kg, i.p.) before being placed into the apparatus containing the two different tactile floor cues ("split" configuration; one floor on each side of the chamber) for 30 mins. On Days 2-9, mice underwent daily 5-min conditioning trials. Mice in the "Grid+" subgroup were weighed and injected with EtOH (2.0 g/kg, 20% v/v, i.p.) immediately before being placed into the apparatus containing the *grid* floor cue ("matching" configuration; same floor on both sides of the chamber). On alternating days, mice were weighed and injected with saline before being placed into the apparatus containing the *hole* floor cue on both sides of the chamber. Mice in the "Grid-" (or "Hole+") subgroup were treated in a manner opposite from that of Grid+ mice, such that they were weighed and injected with EtOH prior to being placed into the apparatus containing the *hole* floor cues on both sides, while on alternating days, they were weighed and injected with saline prior to being placed into the apparatus containing the *grid* floor on both sides. On Day 10, all mice were weighed and received a saline injection before being placed into the apparatus containing both floor cues (one on each side) for 30 mins.

In the second set of conditioning experiments (EtOH-CPA), male and female Ucn1 KO and WT littermate mice (n = 14-23 per sex, per genotype) were tested for the conditioned aversive effects of EtOH using a slight variant of a well-established protocol in which post-session exposure to EtOH results in significant avoidance of the EtOH-paired environment (Cunningham et al., 2006; Cunningham and Henderson, 2000). The

protocol used for EtOH-CPA was identical to that described for EtOH-CPP, except that mice were weighed and injected with either EtOH or saline immediately *after* being removed from the apparatus on Days 1-9. A post-session injection on Day 10 was unnecessary, because the experiment was complete by the end of the behavioral test session. Importantly, the dose and preparation of EtOH were identical for CPP and CPA experiments.

In order to minimize variation in the conditioning response that could occur based on conditioning subgroup (Grid+ vs. Grid-), conditioning order (EtOH/saline vs. saline/EtOH), and side of the EtOH-paired floor during pre-test and test (left vs. right), all of these variables were fully counterbalanced among all groups in all conditioning experiments.

Statistical Analysis – Ethanol Conditioning

The percent time spent on the grid floor on Day 10 (test) relative to Day 1 (pre-test) was used as the dependent variable (Δ %Time on Grid Floor). Because three-way ANOVA in each conditioning experiment yielded no significant main or interacting effects of sex, analyses were collapsed across sexes, and data were analyzed by two-way ANOVA with between-subjects factors of genotype (KO, WT) and subgroup (Grid+, Grid-). A priori hypotheses were that Ucn1 and CRF2 KO would attenuate EtOH CPP. Significant or near-significant interactions between genotype and subgroup were followed by simple main effect analyses evaluating the impact of subgroup separately across the two genotypes. For all conditioning analyses, significance threshold was set at $p < .05$.

RESULTS

EWcp Lesion Histology

Of the 51 mice that received lesion surgery, Thionin-stained tissue revealed successful targeting and ablation of the EWcp in 29 cases. The percentage of successful surgeries was not significantly different between Ucn1 KO and WT mice (16/27 vs. 13/24). Successful lesions were targeted primarily to the anterior and middle EWcp (-3.4 mm from bregma), and caused destruction of this region with minimal damage to surrounding areas (**Fig. 8A**). Animals that received sham surgery showed no evidence of damage to the EWcp or surrounding tissue (**Fig. 8B**).

Effects of EWcp Lesion and Ucn1 KO on EtOH Intake

RM-ANOVA of EtOH intake indicated differential effects of surgery across the three concentrations (surgery x concentration interaction; $F_{2,120} = 7.14$, $p < .005$; **Fig. 9A**). Follow-up analyses at each concentration revealed that EWcp lesion significantly reduced intake at the 6% and 10% concentrations of EtOH (simple main effects of surgery; both $F_{1,60} > 7.2$, both $p < .01$), but not the 3% concentration of EtOH ($p = .65$), with no significant genotype interactions. Follow-up analyses of group data collapsed across concentrations revealed that Ucn1 KO decreased EtOH intake in sham mice (main effect of genotype; $F_{1,33} = 6.36$, $p < .017$), but not in lesion mice ($p = .82$), with no significant interactions.

Effects of EWcp Lesion and Ucn1 KO on EtOH Preference

Analysis of EtOH preference indicated differential effects of surgery between Ucn1 KO and WT mice (surgery x genotype interaction; $F_{1,60} = 5.55$, $p < .05$), although this did not significantly interact with concentration. Follow-up analyses revealed that EtOH preference was significantly dampened by lesion in WT mice (simple main effect of

Figure 8. Edinger-Westphal Nucleus Lesion Histology

Shown are representative photomicrographs of Thionin-stained sections from anterior, middle, and posterior EWcp (numbers indicate distance from bregma) taken from mice that underwent **(A)** successful EWcp lesion surgery or **(B)** sham surgery. Lesions generally ablated large portions of the anterior and middle EWcp, leaving minimal damage to surrounding tissue. Sham animals displayed no evidence of damage to the EWcp, despite occasional visibility of the electrode tract (posterior sham panel). White arrows point toward intact EWcp observed in sham animals.

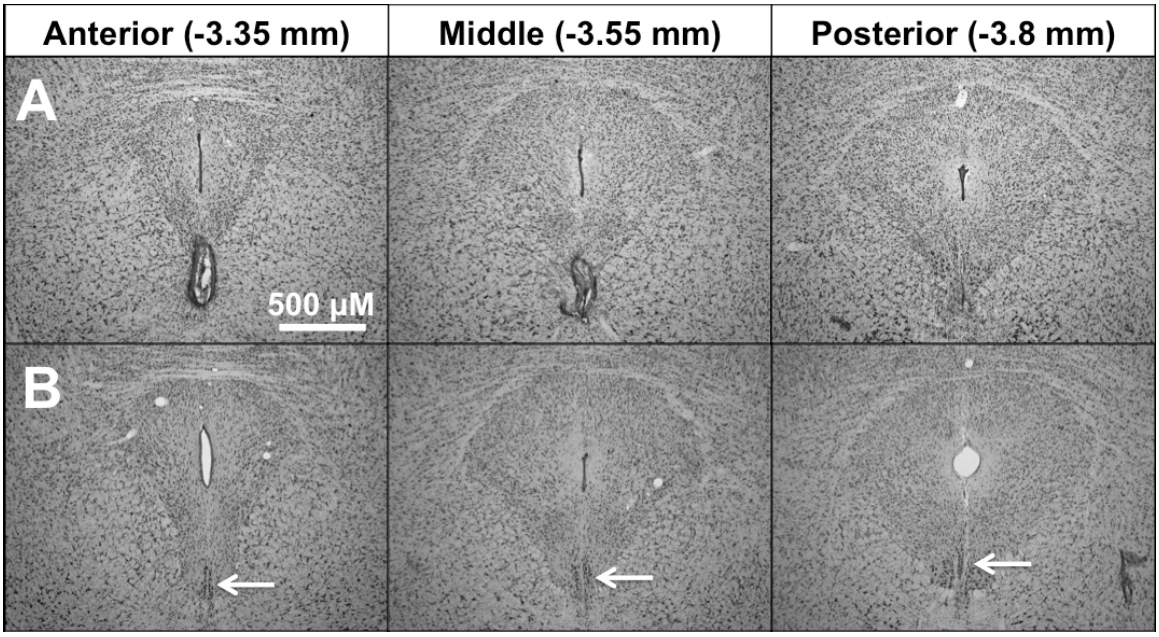
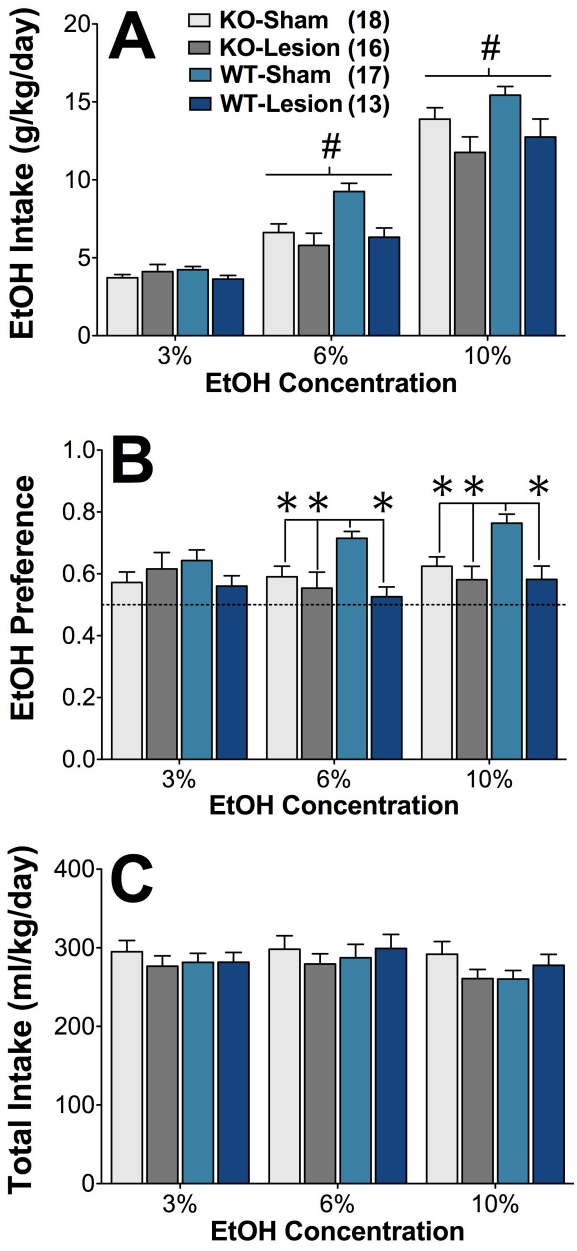


Figure 9. Effects of EWcp Lesion and Ucn1 KO on EtOH Drinking

Shown are mean ± SEM values for **(A)** EtOH Intake **(B)** EtOH Preference, and **(C)** Total Fluid Consumption in male Ucn1 KO and WT mice following either sham surgery or EWcp lesion. Asterisks indicate significant post-hoc Bonferroni difference from WT-Sham group (*p < .0083) in the presence of significant or near-significant genotype x surgery interaction. Pound signs indicate main effects of surgery (#p < .01).



surgery; $F_{1,28}$ = 22.28, p = .0001; **Fig. 9B**), but EWcp lesion had no effect on preference in Ucn1 KO mice (p = .84). Further analyses revealed that Ucn1 KO decreased EtOH preference in sham mice (simple main effect of genotype; $F_{1,33}$ = 15.82, p = .0004), but not in lesion mice (p = .65). RM-ANOVA also indicated differential effects of surgery across concentrations (surgery x concentration interaction; $F_{2,120}$ = 3.70, p = .028), although this did not significantly interact with genotype. Follow-up analyses revealed no significant main or interacting effects at the concentration of 3%. However, at 6%, EtOH preference was differentially affected by EWcp lesion in Ucn1 KO vs. WT mice (surgery x genotype interaction; $F_{1,60}$ = 5.32, p = .025). Post-hoc Bonferroni comparisons at 6% confirmed that lesion significantly reduced preference in WT mice but not Ucn1 KO mice, and that Ucn1 KO significantly reduced preference in sham mice but not lesion mice (all p < .0083). 10% EtOH preference was significantly reduced by EWcp lesion (main effect of surgery; $F_{1,60}$ = 10.67, p = .0018). Although this effect of surgery only trended toward interacting significantly with genotype ($F_{1,60}$ = 3.24, p = .077), *a priori* hypotheses justified performing post-hoc Bonferroni comparisons, which revealed that lesion reduced preference in WT but not KO mice, and that KO reduced preference in sham but not lesion mice (all p < .0083).

Effects of EWcp Lesion and Ucn1 KO on Total Fluid Intake

Total fluid consumption varied significantly across the different concentrations of EtOH (main effect of concentration; $F_{2,120}$ = 4.50, p < .05; **Fig. 9C**), in the absence of significant main or interacting effects with surgery or genotype. With surgery and genotype groups collapsed, post-hoc Bonferroni analysis found no significant differences between total fluid consumed at each concentration.

Effects of Ucn1 KO on EtOH-Induced Reward

Consistent with previous studies demonstrating the unbiased nature of the tactile floor cues used in the EtOH conditioning studies (Cunningham et al., 2003), Ucn1 KO and WT mice spent approximately 50% of their time on the grid floor during the pre-test, and this did not differ by genotype or subgroup. Following conditioning, preference for the EtOH-paired floor was apparent in WT mice, but not Ucn1 KO mice (genotype x subgroup interaction; $F_{1,45} = 4.96$, $p < .05$; **Fig. 10A**). The conclusion that deletion of *Ucn* abolished EtOH-induced CPP was supported by simple main effect analyses evaluating the impact of subgroup separately across the two genotypes. While conditioning was apparent in WT mice ($F_{1,26} = 14.45$, $p < .001$), this effect was not apparent in Ucn1 KO mice ($p = .99$).

Effects of CRF2 KO on EtOH-Induced Reward

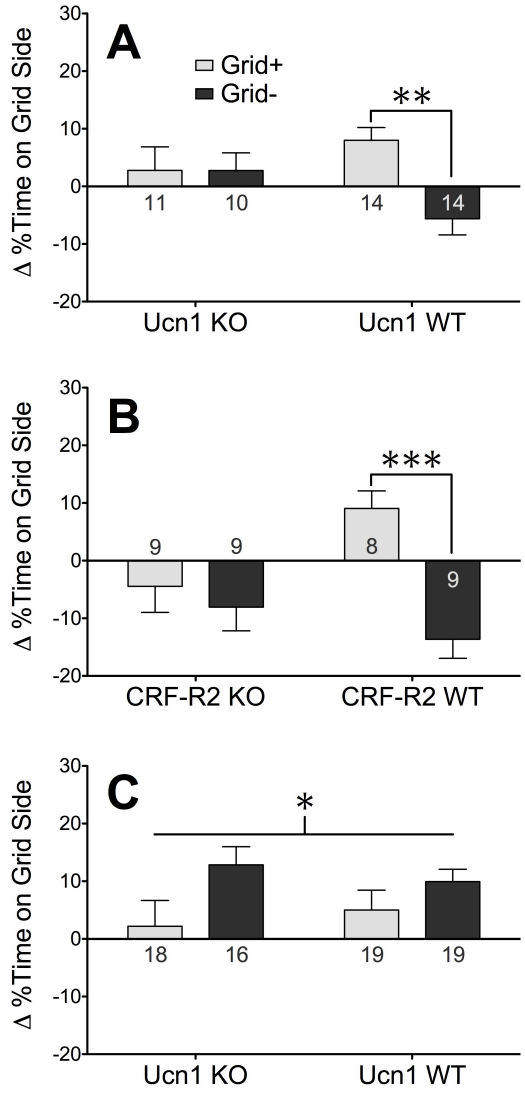
Similar to Ucn1 KO and WT mice, CRF2 KO and WT mice spent approximately half of their time on the grid floor during the pre-test, and this did not differ across genotypes or subgroups. Following conditioning, preference for the EtOH-paired floor was apparent in WT mice, but not CRF2 KO mice (genotype x subgroup interaction; $F_{1,31} = 6.22$, $p < .05$; **Fig. 10B**). The conclusion that deletion of *Crhr2* abolished EtOH-induced CPP was supported by simple main effect analyses, in which conditioning was apparent in WT mice ($F_{1,15} = 25.24$, $p < .0005$), but not CRF2 KO mice ($p = .56$).

Effects of Ucn1 KO on EtOH-Induced Aversion

In a separate experiment using an EtOH conditioning protocol that produces CPA rather than CPP, Ucn1 KO and WT mice again spent approximately 50% of their time on the grid floor during the Pre-Test, and this did not differ by genotype or subgroup. While EtOH conditioning resulted in a significant CPA (main effect of subgroup; $F_{1,68} = 5.25$, p

Figure 10. Effects of Ucn1 and CRF2 KO on EtOH Reward and Aversion

Graphs show mean ± SEM percent change in time spent on grid floor between the pre-test and the test following **(A)** EtOH-CPP in Ucn1 KO and WT mice, **(B)** EtOH-CPP in CRF2 KO and WT mice, and **(C)** EtOH-CPA in Ucn1 KO and WT mice. Multiple asterisks indicate significant difference between WT subgroups in the presence of a significant genotype x subgroup interaction (**p < .001, ***p < .0005), while single asterisk indicates significant main effect of subgroup (*p < .05; no significant interaction with genotype).



< .05; **Fig. 10C**), this effect did not interact significantly with genotype (p = .40), indicating that Ucn1 KO and WT mice were equally sensitive to the conditioned aversive effects of EtOH.

DISCUSSION

The principal findings of this study were that 2-BC CA EtOH preference (6% and 10%) depended on an interaction between whether mice expressed Ucn1, and whether mice received surgical ablation of the EWcp. In addition, I demonstrated that Ucn1 signaling (most likely via CRF2) is necessary for the conditioned rewarding effects of EtOH, and that this cannot be attributable to a generalized learning deficit in Ucn1 KO mice, as both Ucn1 genotypes demonstrated equivalent sensitivity to EtOH-induced aversion. Together, these results indicate that EWcp-Ucn1 neurons influence the magnitude of EtOH preference, likely due to Ucn1's role in sensitivity to the rewarding, but not aversive, effects of EtOH.

Although EWcp lesion and/or deletion of *Ucn* were both capable of attenuating measures of EtOH consumption, these manipulations differentially affected the outcomes of EtOH intake vs. EtOH preference. When examining EtOH intake, analyses revealed effects of EWcp lesion in both Ucn1 KO and WT mice. Follow-up examinations of the 6% and 10% concentrations indicated that EWcp lesion was equally effective at reducing EtOH-drinking in both Ucn1 KO and WT mice. The fact that EWcp lesion was effective at decreasing EtOH intake in mice lacking Ucn1 suggests that other neural systems in the EWcp besides Ucn1 may also contribute to 2-BC CA EtOH intake.

Indeed, the receptor for the orexigenic peptide ghrelin, Ghsr, is densely expressed in the mouse EWcp (Zigman et al., 2006), and systemic administration of a Ghsr antagonist not only prevented EtOH-induced neural activity within the EWcp, but also reduced intake of 20% EtOH in the standard DID and 2-BC/DID models of binge-

like drinking (Kaur and Ryabinin, 2010). Furthermore, the receptor for the anorexigenic peptide leptin (Lepr) is also expressed in the mouse EWcp, and Lepr signaling increases the expression of Ucn1 peptide by directly activating EWcp neurons (Xu et al., 2011). In addition, mutant mice that are either leptin-deficient (ob/ob) or leptin-resistant (db/db) showed decreased EtOH preference relative to their WT littermates in a 2-BC CA procedure (Blednov et al., 2004). These studies suggest that signaling via EWcp-Ghsr and/or EWcp-Lepr may be important for Ucn1's effects on EtOH intake and reward. Finally, Ucn1 is also highly co-localized in the EWcp with the anorexigenic neuropeptide CART (Koylu et al., 1998; Kozicz, 2003). A role for CART in EtOH-related behaviors has been supported by several studies (Dandekar et al., 2008; Dayas et al., 2008; King et al., 2010). Salinas et al. recently reported that genetic deletion of CART reduced 2-BC CA EtOH intake and preference, highlighting the potential contribution of EWcp-CART transmission to EtOH drinking (Salinas et al., 2012).

The EWcp also expresses high levels of the peptides CCK, nesfatin-1, and neuropeptide B (Dun et al., 2005; Foo et al., 2008; Maciewicz et al., 1984; Tanaka et al., 2003a). Since these peptides have anorexic properties, they could also contribute to EWcp's involvement in consummatory behaviors. However, reductions in EtOH intake observed here were not simply due to a non-specific decrease in consumption, because the total volume of fluid consumption was not affected, and the effect on EtOH preference was dependent on both Ucn1 genotype and the type of surgery, as discussed below.

In contrast to effects on EtOH *intake*, analysis of EtOH *preference* revealed a significant interaction between surgery and genotype. Post-hoc comparisons at concentrations of both 6% and 10% confirmed that deletion of *Ucn* reduced preference only in mice with an intact EWcp, and that lesion of EWcp reduced preference only in mice expressing Ucn1. These findings provide strong evidence that EWcp-Ucn1 neurons

are necessary for driving high EtOH preference, and suggest that dampened EtOH preference in EWcp-lesioned B6 mice can be attributed primarily to the reduction of Ucn1-positive terminals within EWcp target regions, including the LS and DRN (Bachtell et al., 2004). The potential dissociation between Ucn1's involvement in regulation of EtOH *preference* and the contribution of other EWcp peptide systems to regulation of EtOH *intake* is intriguing, and requires further investigation.

One potential caveat of implementing electrolytic lesions is that they may cause tissue damage independent of cell type, thereby potentially disrupting function of adjacent cell bodies. Indeed, the adjacent population of RLi neurons could provide DA input onto EWcp neurons, and if these neurons were also ablated, this could potentially influence the motivational effects for EtOH in the 2-BC CA paradigm. Furthermore, lesions may destroy fibers passing through the EWcp, or fibers directly innervating the EWcp, thereby complicating the interpretation of the relative importance of the EWcp cell bodies vs. their inputs vs. inputs to adjacent regions. However, the lesions produced in this study were relatively minor (0.4 mA for 5 seconds), and mice with lesions located outside of the EWcp were removed from the analysis. Furthermore, the effects of EWcp lesion were specific to Ucn1 WT mice, suggesting that the impact of this surgery on EtOH preference was localized to the EWcp-Ucn1 neuron population.

One potential caveat of examining genetically-engineered KO mice is that observed effects can sometimes be attributed to developmental compensations within systems related to the deleted gene, rather than to the absence of the gene itself. However, because Ucn1 is the only component of the CRF system that is expressed in the EWcp, and because the EWcp is the primary site of Ucn1 expression in the mammalian brain (Bittencourt et al., 1999; Kozicz et al., 1998; Vasconcelos et al., 2003), the observation that EWcp lesion differentially affected EtOH preference in Ucn1 KO and WT mice suggests that the effects of Ucn1 deletion on EtOH-related behaviors can be

primarily ascribed to the actions of EWcp-Ucn1 neurons. Furthermore, Ucn1 is only expressed postnatally in the EWcp (Cservenka et al., 2010), limiting the potential impact of compensations on development. Indeed, converging lines of evidence provide additional support for the involvement of EWcp-Ucn1 neurons in EtOH sensitivity (Bachtell et al., 2003; Ryabinin and Weitemier, 2006).

Figure 10C shows the CPA data in Ucn1 KO and WT mice. Statistical analysis identified a main effect of subgroup but no significant main or interacting effects with genotype, indicating that subgroups differed equivalently across genotypes. While Grid- mice showed an increase in percent time spent on the grid floor between pre-test and test (indicative of EtOH-induced aversion), Grid+ mice showed no change (or a slight increase) on the grid floor between pre-test and test. This apparent difference in the magnitude of CPA between subgroups suggests that, in this genetic background, the ability of EtOH to condition a place aversion may differ depending on the tactile cues used. Nevertheless, numbers of subjects in each subgroup were balanced between genotypes, and any apparent differences in the abilities of grid vs. hole to condition an aversion did not differ between Ucn1 KO and WT mice, reinforcing the conclusion that Ucn1 is not required for EtOH-induced aversion or EtOH-induced learning *per se*.

The present data complement existing literature on the contribution of specific components of the CRF system to EtOH-related behaviors. Importantly, EWcp-Ucn1 neurons may work in concert with CRF-containing neurons of the CeA, which are thought to be integral for the negative reinforcement processes that prevail during dependence and withdrawal (Heilig and Koob, 2007; Koob, 2010). CeA-CRF release is elevated during EtOH withdrawal (Merlo Pich et al., 1995; Zorrilla et al., 2001), CeA-CRF mRNA is upregulated following EtOH dependence (Roberto et al., 2010; Sommer et al., 2008), and CRF's ability to release GABA from CeA interneurons is potentiated in EtOH-dependent rats (Roberto et al., 2010). Although we have not yet fully investigated a role

for Ucn1 in EtOH dependence, the current results support a general framework in which CRF and CRF-related peptides display unique (yet partially redundant) relationships with distinct aspects of the addiction process. For example, while CRF is required for EtOH-induced psychomotor sensitization and 20% EtOH intake in the standard DID paradigm, Ucn1 is not critical for these behaviors (Kaur et al., 2012; Pastor et al., 2008).

Although Ucn1 binds with high affinity to both CRF receptors (Lewis et al., 2001; Vaughan et al., 1995), it remains unclear specifically which EtOH-related behaviors involve EWcp-Ucn1 actions at CRF1 vs. CRF2. Numerous reports described above demonstrate that genetic deletion or pharmacological blockade of CRF1 decreases EtOH consumption, and that these effects can be more pronounced in animals with a history of EtOH dependence. In contrast, several studies have concluded that CRF2 signaling acts to *decrease* EtOH consumption (Lowery et al., 2010; Sharpe et al., 2005a; Sharpe and Phillips, 2009). However, CRF2 regulation of behavior is often reported as bi-directional (Bale et al., 2000; Henry et al., 2006; Zhao et al., 2007), and intra-CeA CRF2 activation has opposing effects on EtOH self-administration in dependent vs. non-dependent rats (Funk and Koob, 2007). Indeed, the observations that deletion of *Crhr2* blocked EtOH-CPP (**Fig. 10B**) and protected against prolonged increases in EtOH preference following stress (Pastor et al., 2011a) indicate that the precise role of CRF2 signaling in EtOH drinking and reward may rely on a complex interaction between several environmental variables.

Because EWcp lesion decreased the number of Ucn1-positive terminals in the LS and DRN (Bachtell et al., 2004), and because CRF2 expression is enriched in those areas relative to CRF1 (Chalmers et al., 1995; Van Pett et al., 2000), I hypothesized that EWcp-Ucn1 mediates its effects on EtOH-related behaviors primarily via CRF2 rather than CRF1. Although this idea is supported by our observation that Ucn1 KO mice and CRF2 KO mice are both resistant to EtOH-CPP (suggesting that Ucn1 acts via CRF2 to

mediate the conditioned rewarding effects of EtOH), this hypothesis awaits further testing.

In summary, the results implicate the EWcp in EtOH intake, EWcp-Ucn1 neurons in EtOH preference, and Ucn1/CRF2 in EtOH-induced reward. Future studies examining different drinking paradigms, different concentrations of EtOH, potential effects of dependence, and additional EtOH-related behaviors will assist in delineating the specific components of the CRF system (and the specific neural substrates) that work in concert to drive the progression of EtOH addiction.

CHAPTER 3: Defining the Contribution of Ucn1 to Voluntary EtOH Drinking

INTRODUCTION

Genetic deletion of *Ucn* did not alter EtOH intake in the standard DID paradigm (Kaur et al., 2012), yet EWcp lesion and Ucn1 KO were effective at decreasing EtOH intake and preference in the standard CA 2-BC procedure described in Chapter 2. These two drinking paradigms differ in several ways, and the patterns of consumption that occur in each are differentially influenced by sensitivity to physiological effects of EtOH. Therefore, Chapter 3 of my dissertation describes research that aimed to define the experimental variables and phenotypic traits underlying Ucn1's contribution to EtOH intake.

The first identifiable difference between the standard DID and CA 2-BC procedures is the use of a single EtOH bottle in DID, as opposed to the choice between EtOH and H2O offered in 2-BC. To address this, Experiment 3.1 tested Ucn1 KO and WT mice in the modified 2-BC/DID hybrid procedure. Another primary difference between standard DID and the 2-BC CA procedure is the concentration of EtOH (20% vs. 3%, 6%, and 10%). To address this, Experiment 3.2 tested Ucn1 KO and WT mice for CA 2-BC drinking at concentrations of 10, 20, and 40% to determine whether Ucn1's influence is specific to certain concentrations of EtOH.

Additional distinctions between standard DID and CA 2-BC are the schedule of access (intermittent vs. continuous) and the length of exposure (four vs. twelve days). Therefore, Experiment 3.3 implemented a long-term IA procedure in which EtOH was offered every other day for five weeks. This design produces persistent high levels of EtOH intake (~20 g/kg/day) in B6 mice (Hwa et al., 2011), therefore testing the hypothesis that neuroadaptations in the Ucn1 system may be relevant to long-term excessive drinking. At this point, all EtOH drinking procedures longer than four days also involved escalating concentrations of EtOH, thereby confounding any conclusions made about the relative importance of each variable. For these reasons, Experiment 3.4 used

a CA 2-BC procedure in which the concentration of EtOH (10%) remained constant over the course of two weeks.

Experiment 3.5 set out to determine the specificity of Ucn1's effects on EtOH drinking. Some have argued that B6 mice primarily consume EtOH for reasons related to its caloric content or taste (Dole et al., 1985; McMillen and Williams, 1998). To address the potential contribution of Ucn1 to caloric need and taste reactivity, Ucn1 KO and WT mice were compared for food intake, as well as intake of and preference for sweet and bitter tastant solutions.

In Experiment 3.6, Ucn1 KO and WT mice were tested for sensitivity to EtOH-induced sedation with the hypothesis that low-drinking Ucn1 KO mice would be more sensitive to EtOH. Prior human studies suggested that EtOH sensitivity is an important trait in the development of excessive drinking, because subjects with a positive family history for alcoholism displayed a "low level of response" compared to family history-negative controls (i.e., they self-reported and behaviorally displayed lower levels of intoxication), despite achieving similar BECs (Schuckit, 1980).

Experiment 3.7 tested Ucn1 KO and WT mice for sensitivity to EtOH-induced locomotor activity, to determine whether Ucn1 KO mice drank less EtOH simply because they were insensitive to EtOH's behavioral effects. Chapter 2 studies already demonstrated that deletion of *Ucn* had no impact on EtOH-induced avoidance learning, arguing against the possibility that Ucn1 KO mice were generally insensitive to EtOH. However, selective breeding for acute locomotor sensitivity to EtOH's stimulant effects rendered mice more sensitive to the reward threshold-lowering effects of EtOH, hinting at a relationship between EtOH locomotor sensitivity and EtOH reward that might be relevant to the role of Ucn1 in EtOH drinking (Fish et al., 2012). Furthermore, components of the CRF system are critical for EtOH-induced locomotor sensitization,

and despite evidence against a role for Ucn1 in these studies (Pastor et al., 2008), the influence of Ucn1 on acute locomotor sensitivity to EtOH had not yet been studied.

Finally, Experiment 3.8 assessed anxiety-like behavior in Ucn1 KO and WT mice, to determine whether differences in EtOH consumption might be related to differences in basal anxiety levels between the two genotypes. EtOH-dependent animals display CRF system-mediated increases in anxiety-like behavior during withdrawal that persist throughout several weeks of abstinence (Valdez et al., 2002; Valdez et al., 2004; Valdez et al., 2003), indicating that excessive EtOH intake is related to adaptations in neural stress systems (potentially including Ucn1). Therefore, I hypothesized that Ucn1 KO mice would demonstrate lower levels of anxiety-like behavior, relative to WT control mice.

MATERIALS AND METHODS

Animals and Husbandry

For all experiments in Chapter 3, I studied groups of adult male and female Ucn1 KO and WT littermate mice generated by heterozygous breeding and weaned as described in Chapter 2. Mice were housed in a temperature- and humidity-controlled environment with *ad libitum* access to food (LabDiet 5001; LabDiet, Richmond, IN, USA) and H2O. All mice were initially housed on a 12hr/12hr light/dark cycle with lights ON/ZT-0 = 0600h, but mice in drinking studies (Experiments 3.1-3.5) were transferred to the procedure room (lights OFF/ZT-12 = 0700h or 0800h), and allowed to habituate to single housing and the reverse 12hr/12hr light-dark cycle for 1-2 weeks prior to EtOH access. During the acclimation period, mice received 24hr access to two 25 ml glass cylinder bottles with metal sipper tubes (both containing H2O) on either side of the cage, with food evenly distributed along the cage top (except for Experiment 3.3, where larger plastic bottles were used instead, and food was deposited on the cage floor).

General Procedures

For drinking studies, mice from different sexes and genotypes were pseudo-randomly distributed across the rows of the housing rack, and the side of the EtOH or tastant bottle was fully counterbalanced across groups. Furthermore, the side of the EtOH or tastant bottle was switched weekly to avoid the potential confound of a side bias (except for Experiment 3.1, which lasted less than one week, and Experiment 3.3, which aimed to achieve more stable drinking patterns for microstructure analysis). For all Chapter 3 studies, 95% EtOH was diluted either in tap H2O (drinking studies) or 0.9% saline (injection studies) to the desired concentrations, which are expressed in v/v units. Weekly body weight measurements were obtained to calculate accurate food and fluid intake variables throughout the studies. All protocols were approved by the OHSU IACUC and were performed with adherence to the NIH Guidelines for the Care and Use of Laboratory Animals.

Experiment 3.1: 2-BC/DID

Male and female Ucn1 KO and WT mice (n= 5-12 per sex, per genotype) were used. For the first three days following habituation (Days 1-3), at ZT-14, the two 25 ml H2O bottles on each cage top were replaced with two 10 ml plastic bottles (for more precise measurement of small volumes), one containing H2O and one containing 15% EtOH in H2O. At ZT-16, the 10 ml bottles were removed and replaced with the original 25 ml H2O bottles. With each exchange of 25 ml and 10 ml bottles, volumes were recorded to the nearest 0.1 ml. On Day 4, the EtOH drinking session was extended from 2hr to 4hr (ZT-14 to ZT-18), and food was collected and weighed from the cage tops at the beginning and end of the session. During food weighing, I checked the cage floor for smaller pieces of food pellets that may have fallen through the cage top grating, and if

so, included them in food weight measurements. Following the end of the 4hr session on Day 4, mice were euthanized by CO2 inhalation, and trunk blood was collected for later analysis of BECs by the Analox method, as previously reported (Kaur and Ryabinin, 2010). EtOH intake (g/kg), EtOH preference, food intake (g/kg), total caloric intake from EtOH and food combined (kilocalories [kcal] per kg), and percent calories consumed from EtOH were the dependent variables. Days 1-3 data were analyzed by three-way RM-ANOVA (between-subjects factors of sex and genotype, with day as the repeated measure). Day 4 data were analyzed by two-way ANOVA (between-subjects factors of sex and genotype). *A priori*, I hypothesized that deletion of *Ucn* would significantly reduce EtOH intake and preference. Therefore, significant interactions with genotype were followed up with simple main effect comparisons individually at each level of sex.

Experiment 3.2: Escalating 2-BC CA

Male and female Ucn1 KO and WT mice (n=7-14 per sex, per genotype) received 24hr CA 2-BC to increasing concentrations of EtOH. For the first four days following habituation (Days 1-4), at ZT-14, H2O in one of the two 25 ml bottles was replaced with 10% EtOH. On Days 5-8, the 10% EtOH was replaced with 20% EtOH, and on Days 9-12, the 20% EtOH was replaced with 40% EtOH. Bottles were read daily at ZT-14. Sex x genotype interactions for EtOH intake, EtOH preference, and total fluid intake failed to reach significance, thus sexes were combined for analyses ($F_{1,40}$ = 3.82, 0.12, 2.75; p = .058, .731, .105). EtOH intake, EtOH preference, and total fluid intake were analyzed by two-way RM-ANOVA (genotype as the between-subjects factor, EtOH concentration as the repeated measure). *A priori*, I hypothesized that deletion of *Ucn* would significantly reduce EtOH intake and preference. Therefore, post-hoc Bonferroni comparisons were made following identification of any interactions with genotype that were either significant ($p < .050$) or trending toward significance ($.050 < p < .075$).

Experiment 3.3: Escalating Long-Term IA

Ucn1 KO and WT mice (n=5-9 per sex, per genotype) were used. Every other day, one of the two H2O bottles was switched with an otherwise identical bottle containing EtOH, and switched back to H2O the next day. Bottles were weighed daily at ZT-14 (bottles-on) and at ZT-12 (bottles-off), allowing calculation of 22hr daily drinking variables. Between ZT-12 and ZT-14, mice had no access to any fluids, as this 2hr period was used for bottle weighing, recording, re-filling, exchanging, and computer analysis.

Mice received every-other-day access to EtOH (increasing concentrations of 3%, 6%, and 10% on Days 1, 3, and 5, respectively, followed by 20% on odd days from Day 7 to 37), as described previously in B6 mice (Hwa et al., 2011). Mice had 22 hrs of access to EtOH every other day (regardless of the day of the week) for 5 weeks, with each session separated by 26 hrs of no access (2 hrs fluid deprivation during bottle weighing, re-filling, exchanging, and computer analysis + 22 hrs access to two H2O bottles + 2 hrs fluid deprivation during bottle weighing, re-filling, exchanging, and computer analysis prior to the next EtOH session). On Day 37 (D37), EtOH access was cut short after a 4hr drinking session, mice were euthanized by CO2 inhalation at ZT-18, and trunk blood was collected for later analysis of BECs by two methods: gas chromatography (GC) (Finn et al., 2007) and Analox (Kaur and Ryabinin, 2010).

Experiment 3.3 was performed using a lickometer apparatus (MedPC) described previously in detail (Ford et al., 2005b). Mice were housed in small plastic cages on top of a metal grid floor, with alligator clips attached to wires connecting the grid floor to the metal spouts of the bottles. Each lick (with 10 millisecond resolution) was recorded by completion of an electrical circuit, and data were stored on an interfaced PC computer.

An EtOH bout (E-Bout) was defined as twenty or more consecutive EtOH licks (E-Licks), with each E-Lick separated by less than one min, as described previously (Ford et al., 2005a). Several E-Bout variables were generated from the E-Lick data and

used to analyze the EtOH drinking microstructure (bout frequency, bout size, interbout interval, bout length, bout rate, and latency to first bout).

Sex x genotype interactions for EtOH intake and E-Licks failed to reach statistical significance, thus sexes were combined for all analyses ($F_{1,25}$ = 0.12, 0.003; p = .736, .955). In order to validate the lickometer apparatus, mean EtOH intake and E-Lick data were averaged across all 20% EtOH sessions and analyzed by Pearson's correlation. E-Lick, EtOH intake, and total fluid intake data from across *all* EtOH drinking sessions were analyzed by RM-ANOVA (between-subjects factor of genotype, repeated measure of day). To investigate differential *patterns* of EtOH drinking between the genotypes once the concentration of EtOH had stabilized at 20%, EtOH intake and E-Lick data across 20% days only were also analyzed by linear regression models.

To maximize the likelihood of identifying genotypic differences during a specific time of day, hourly E-Lick data from the final 10 daily 20% EtOH sessions (Day 17 to Day 35) were averaged and plotted across the circadian timecourse. Following visualization of the circadian timecourse of E-Licks, I predicted that genotype differences would be greatest during the 4hr period between ZT-14 and ZT-18. Furthermore, the ZT-14 to ZT-18 interval corresponds with the timeperiod of drinking in the DID model, thereby facilitating comparison of results between studies. 4hr E-Bout data were analyzed across all EtOH drinking sessions by RM-ANOVA, and analyzed across 20% EtOH drinking sessions by linear regression. Intake, E-Lick, and E-Bout variables collected during the final 4hr drinking session on D37 were compared between genotypes by t-test. D37 BECs from GC and Analox were correlated using Pearson's correlation and then subjected to RM-ANOVA (between-subjects factor of genotype, repeated measure of method [GC vs. Analox]).

Experiment 3.4: Non-Escalating 2-BC CA

Male and female Ucn1 KO and WT mice were used (n=4-9 per sex, per genotype). For fourteen days following habituation (Days 1-14), at ZT-14, H2O in one of the two 25 ml bottles was replaced with 10% EtOH. Bottles were read daily at ZT-14 and ZT-18 in order to calculate 4hr and 24hr consumption timepoints corresponding to those analyzed in Experiment 3.3. Sex x genotype interactions for 24hr EtOH intake, EtOH preference, and total fluid intake failed to reach statistical significance, thus sexes were combined for all analyses ($F_{1,22}$ = 0.01, 0.32, 0.20; p = .92, .58, .66). RM-ANOVA was used to analyze EtOH intake and EtOH preference for 4hr and 24hr data (between-subjects factor of genotype, repeated measure of day).

Experiment 3.5: 2-BC CA Tastant Control

Male and female Ucn1 KO and WT mice were used (n=7-11 per sex, per genotype). For the first four days following habituation (Days 1-4), at ZT-14, H2O in one of the two 25 ml bottles was replaced with 3% sucrose. On Days 5-8, the concentration of sucrose was increased to 10%. On Days 9-12 and 13-16, sucrose was replaced with .003% and .015% saccharin, respectively. On Days 17-20 and 21-24, saccharin was replaced with .03% and .06% quinine, respectively. Bottles were read daily at ZT-14, and food was weighed on the final two days of each tastant concentration phase. Initial statistical analyses did not identify any interactions with concentrations, thus concentrations were combined for analysis. Sex x genotype interactions for tastant intake, tastant preference, and total fluid intake failed to reach statistical significance, thus sexes were combined for all analyses ($F_{1,22}$ = 0.53, 0.26, 0.46; p = .47, .61, .50). Tastant intake and preference, food intake, and total caloric intake were analyzed by two-way RM-ANOVA (genotype as the between-subjects factor, tastant as the repeated measure).

Experiment 3.6: EtOH-Induced Sedation

Following tastant drinking, mice from Experiment 3.5 resumed normal H2O drinking and were undisturbed for four days before being tested for their sensitivity to EtOH-induced sedation using the loss of righting reflex (LORR) procedure on three consecutive days. At ZT-16, mice were injected i.p. with a 4.0 g/kg dose of 20% EtOH (v/v) dissolved in saline (injection volume of 25.35 ml/kg), and placed in a holding cage until they appeared to be intoxicated. Mice were then placed on their backs in a V-shaped trough, and time of LORR began when mice were no longer able to right themselves within 30 seconds of being placed on their backs. Mice were observed for the following 2-3 hours. When mice righted themselves, they were repeatedly placed on their backs again until they were able to right themselves twice within 30 seconds.

The time was recorded at the moment of injection, the moment of LORR, and the moment of re-gain of righting reflex on each of the three days. On the third day, immediately following the re-gain of righting reflex, mice were euthanized by CO2 inhalation, and trunk blood samples were obtained for later BEC analysis. Sex x genotype interactions for LORR latency and LORR duration failed to reach statistical significance, thus sexes were combined for all analyses ($F_{1,27}$ = 0.36, 0.11; p = .56, .75). LORR latency and duration were analyzed separately by two-way RM-ANOVA (between-subjects factor of genotype, with day as the repeated measure). BECs on Day 3 were compared between genotypes by t-test.

Experiment 3.7: EtOH-Induced Locomotor Sensitivity

Male and female Ucn1 KO and WT mice (n=7-9 per sex, per genotype) were tested for sensitivity to EtOH-induced locomotor activity. Group-housed mice were moved to the experimental room and given 1hr to habituate before receiving an i.p. injection and undergoing a 15-min locomotor activity test in one of four sound-attenuated behavioral

chambers. Horizontal locomotor activity was detected by interruption of a 10 x 12 array of photocell beams equally spaced at a height of 1 cm along the walls of a 21 x 25 x 18 cm enclosure with a steel bar grid floor (San Diego Instruments; San Diego, CA). This chamber resided within a larger sound-attenuating box containing a fan and houselight. Horizontal activity was defined as the total number of photocell beam breaks during the 15-minute tests. Testing occurred between ZT-4 and ZT-8. Mice received 17 ml/kg saline (i.p.) and activity levels were measured immediately afterward on Days 1 and 2 to allow habituation to the apparatus, and to measure baseline activity levels, respectively. On Days 3, 5, 7, 9, and 11, mice received doses of 0.00, 0.75, 1.50, 2.00, and 2.75 g/kg EtOH dissolved in saline, administered in concentrations of 0.0%, 5.6%, 11.2%, 14.9%, and 20.5% (v/v), always with an injection volume of 17 ml/kg. The injection volume rather than the concentration of EtOH remained constant in order to match the volume injected on the first two saline habituation days. The order of EtOH doses was fully counterbalanced, and all mice received a day of rest between each EtOH testing session to minimize potential impact of pharmacological tolerance on the locomotor effects of EtOH.

The number of horizontal beam breaks during each 15 min session was used as the dependent variable, and data were analyzed by RM-ANOVA (between-subjects factors of genotype and sex). To assess potential differences in habituation and baseline activity, Days 1 and 2 were analyzed with day as the repeated measure. To assess effects of EtOH on locomotor activity, Days 3, 5, 7, 9, and 11 data were analyzed with dose as the repeated measure (0.00, 0.75, 1.50, 2.00, 2.75). Significant interactions were followed up with Bonferroni-corrected post-hoc comparisons.

Experiment 3.8: Anxiety-Like Behavior

Male and female Ucn1 KO and WT mice (n=10-15 per sex, per genotype) were tested in two widely-used rodent models of anxiety-like behavior: the elevated plus maze (EPM) and the light-dark box (LDB). Group-housed mice were moved to a dark experimental room and allowed to habituate for 1hr prior to testing on each day. Testing occurred between ZT-2 and ZT-8 on several consecutive days in which each mouse underwent both the EPM and the LDB in a fully counterbalanced order, with one day of rest between each test, in order to minimize the potential effects of repeated testing on subsequent behavior.

The EPM apparatus (Med Associates, Inc., St. Albans, VT, USA) consisted of two black opaque high-walled arms and two white open arms (51-cm long x 8-cm wide) elevated 60 cm off the ground. Small lamps were placed over the open arms, and the closed arms remained un-lit, resulting in respective lux values of 95 and 2. Mice were placed in the center platform facing a closed arm, and the following variables were scored live during a 5 min test: latency to enter open arm, entries and time spent in open and closed arms, number of head dips over the sides of open arms, rearing behavior, grooming, urination, and fecal boli. Between each session, the EPM was cleaned with H2O and a sponge, and thoroughly dried with paper towels.

The LDB (black/white box, John Crabbe Laboratory) consisted of a two-chambered apparatus comprised of one white acrylic box (28 x 28 x 30 cm) connected to a smaller black acrylic box (28 x 17 x 30 cm). A small doorway in the shared wall of the boxes allows movement between the two compartments. A small lamp was placed over the light side, and the dark side remained unlit, resulting in respective lux values of 195 and 2. Mice were placed in the dark side of the apparatus facing a dark corner, and the following variables were scored live during a 5 min test: latency to enter light side, number of dark-light and light-dark transitions, time spent in light side, rearing behavior,

grooming, urination, and fecal boli. Between each session, the LDB was cleaned with H2O and a sponge, and thoroughly dried with paper towels.

Initial analyses uncovered no significant interactions with factors of order (EPM/LDB vs. LDB/EPM), thus order groups were combined for final analyses. Sex x genotype interactions for all EPM and LDB variables failed to reach statistical significance, thus sexes were combined for all analyses ($F_{1,42}$ = 0.01-3.92; p = .938-.054). Genotypes were compared on the scored measures by t-test.

RESULTS

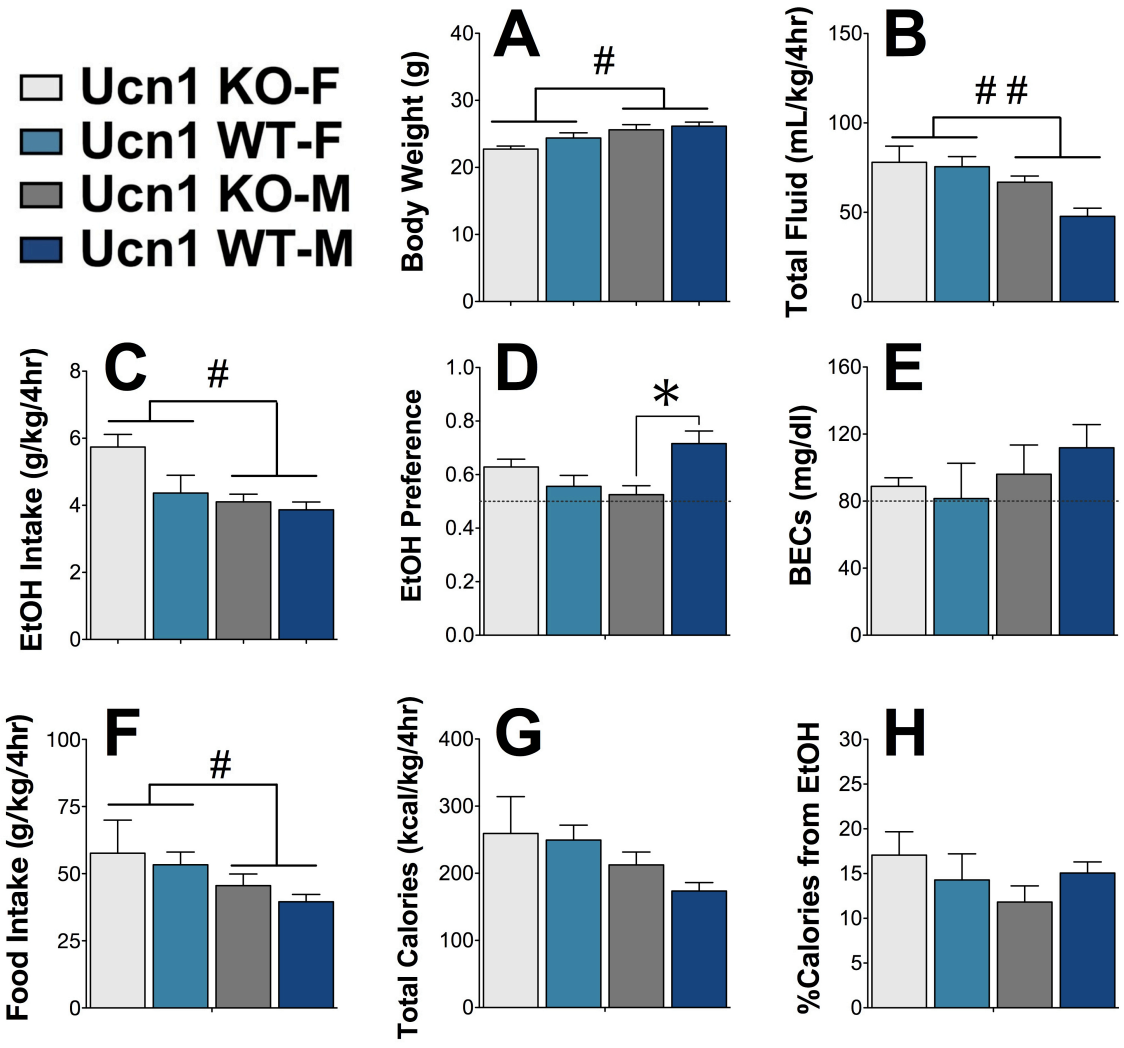
Experiment 3.1: 2-BC/DID

Female mice weighed significantly less than male mice, regardless of genotype (main effect of sex; $F_{1,30}$ = 6.55; p = .016; **Fig. 11A**). Analysis of EtOH intake, EtOH preference, and total fluid intake across Days 1-3 revealed no significant main or interacting effects of genotype, although female mice consumed more total fluid than male mice overall (mean ± SEM ml/kg/2hr: 39.2 ± 3.1 [females], 29.7 ± 1.5 [males]; main effect of sex; $F_{1,38}$ = 4.88; p = .033; data not shown).

On Day 4, total fluid intake was greater in female mice overall (main effect of sex; $F_{1,30}$ = 9.48; p = .004; **Fig. 11B**), as was EtOH intake (main effect of sex; $F_{1,30}$ = 4.63; p = .037; **Fig. 11C**), but neither of these effects significantly interacted with genotype. Analysis of Day 4 EtOH preference revealed a significant sex x genotype interaction ($F_{1,30}$ = 7.09; p = .012). Follow-up analyses revealed that Ucn1 KO male mice displayed significantly decreased EtOH preference relative to WT males (simple main effect of genotype; p < .05), whereas female genotypes did not differ (**Fig. 11D**). Mean BEC was 92.85 ± 10.91 mg/dl (range: 8.75-282.83 mg/dl), confirming that mice achieved binge-like BECs even in this modified 2-BC/DID paradigm. Analysis of BECs revealed no significant effects of sex or genotype (**Fig. 11E**). Relative to male mice, female mice

Figure 11. Experiment 3.1: 2-BC/DID

Bar graphs illustrate mean ± SEM values from data collected on D4 of the 2-BC DID study. **(A)** Female mice weighed less than male mice, **(B)** consumed more total fluid than male mice, and **(C)** consumed more EtOH than male mice overall. **(D)** Genetic deletion of *Ucn* decreased EtOH preference only in male, but not in female mice. **(E)** BECs did not differ between sexes and genotypes. **(F)** Female mice consumed more food than male mice overall. **(G)** Sexes and genotypes did not differ in total calories consumed or **(H)** percent calories consumed from EtOH. Pound signs indicate significant main effects of sex (#p < .05, ##p < .005). Asterisk indicates significant simple main effects of genotype in presence of a significant genotype by sex interaction (*p < .05).



consumed more food overall ($F_{1,30}$ = 5.50; p = .026; **Fig. 11F**), although this did not significantly interact with genotype. Total calories consumed from food and EtOH combined did not differ significantly across genotypes or sexes, nor did the percent calories consumed from EtOH (**Fig. 11G-H**).

Experiment 3.2: Escalating 2-BC CA

Genetic deletion of *Ucn* altered 24hr 2-BC CA EtOH intake differentially across the three concentrations of EtOH (genotype x concentration interaction; $F_{2,84}$ = 10.12; p = .0001). Follow-up comparisons at each concentration revealed significantly lower intake in KO vs. WT mice at concentrations of 20% (p<.01) and 40% (p<.001) (**Fig. 12A**). Similarly, analysis of EtOH preference identified a near-significant interaction between genotype and concentration ($F_{2,84}$ = 2.88; p = .062). Post-hocs confirmed lower preference in KO vs. WT mice at concentrations of 20% (p<.01) and 40% (p<.05) (**Fig. 12B**). No genotype effects on total fluid intake were identified (**Fig. 12C**).

Experiment 3.3: Escalating Long-Term 2-BC IA

Averaged from Day 7 to Day 35, daily 20% E-Licks were significantly positively correlated with daily 20% EtOH intakes in both WT mice (df = 13; r = .754; p = .0012) and Ucn1 KO mice (df = 12; r = .796; p = .0007; **Fig. 13A**). In the RM-ANOVA for all EtOH days, E-Licks were significantly lower overall in Ucn1 KO vs. WT mice (main effect of genotype; $F_{1,27}$ = 5.46; p < .05; **Fig. 13B**). In fact, the escalating pattern of E-Licks across 20% EtOH days observed in WT mice was significantly blunted in Ucn1 KO mice (linear regression; $F_{1,26}$ = 7.63; p = .01). RM-ANOVA and linear regression found no significant genotypic differences in EtOH intake and total fluid intake (**Fig. 13C-D**).

Hour-by-hour plotting of the circadian timecourse of 20% E-Licks (averaged from Day 17 to Day 35) allowed visualization of the largest differences between genotypes,

Figure 12. Experiment 3.2: Escalating 2-BC CA

Deletion of *Ucn* decreased EtOH intake and preference in the escalating concentration 2-BC CA paradigm. Bar graphs illustrate mean ± SEM values for **(A)** EtOH intake, **(B)** EtOH preference, and **(C)** total fluid intake. Asterisks indicate significant post-hoc Bonferroni comparisons between genotypes (**p*< .05, ***p*<.001, ****p*<.0001) in the presence of significant or near-significant genotype x concentration interactions. Data are shown collapsed on sex.

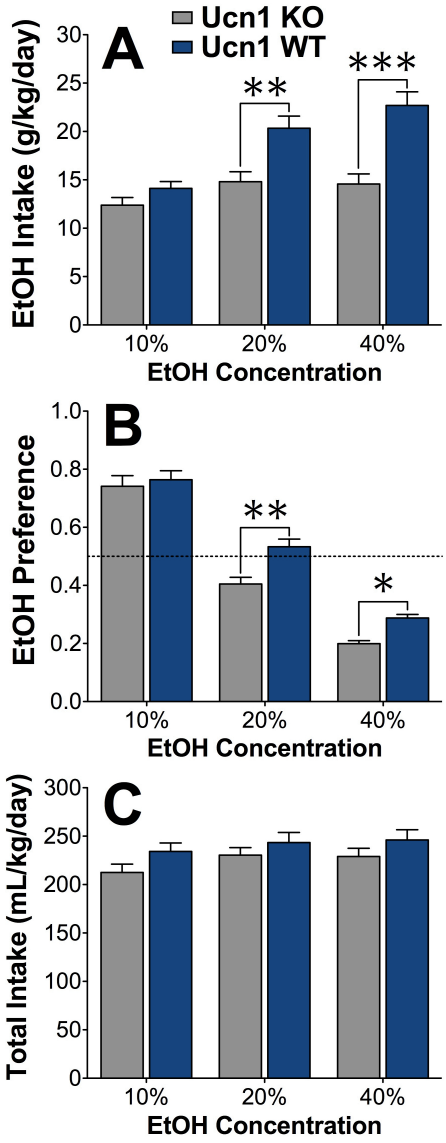
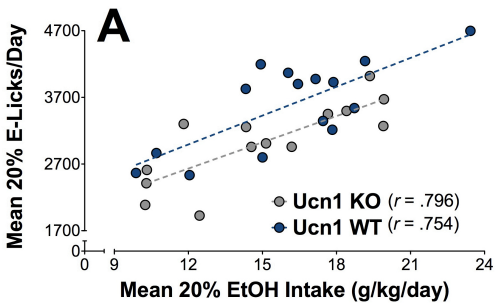
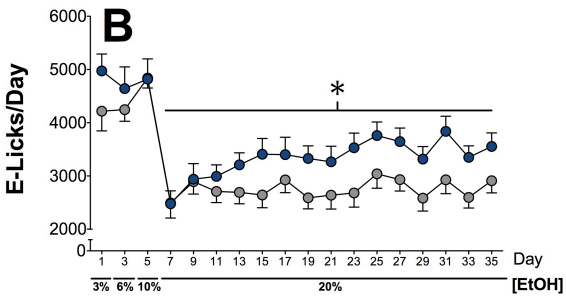
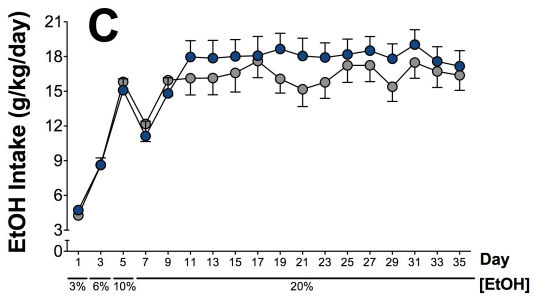


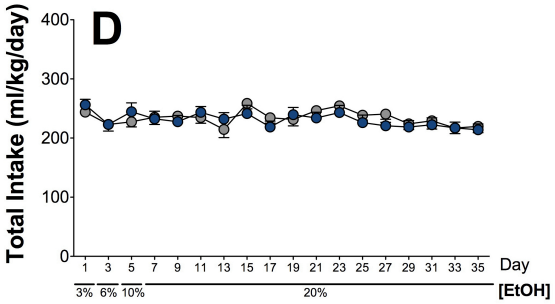
Figure 13. Experiment 3.3: Escalating Long-Term 2-BC IA

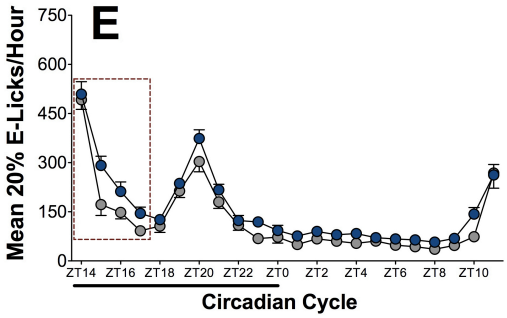
Deletion of *Ucn* decreased E-Licks and E-Bouts in the long-term 2-BC IA study. Graphs illustrate mean ± SEM values. **(A)** In both genotypes, mean daily 20% EtOH intake was significantly positively correlated with mean daily 20% E-Licks. **(B)** Overall, daily E-Licks were significantly reduced in Ucn1 KO vs. WT mice, and deletion of *Ucn* dampened the escalation of 20% E-Licks. **(C)** Daily EtOH intake and **(D)** total fluid intake were not significantly different between genotypes. **(E)** Timecourse plotting of 20% E-Licks averaged across Day 17 to Day 35 revealed the largest genotypic differences during the first 4 hrs of EtOH access (dotted red box). **(F)** Ucn1 KO significantly blunted the escalation of E-Bouts occurring within the first 4 hrs of 20% EtOH drinking sessions. Asterisks indicate significant differences between genotypes by linear regression (*p < .05, **p <.01). Data are shown collapsed on sex.

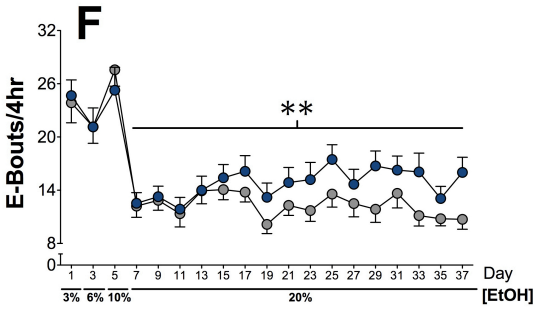

A
Mean 20% E-Licks/Day
4700
3700
2700
1700
0
0
9
12
15
18
21
24
Mean 20% EtOH Intake (g/kg/day)
Ucn1 KO (r = .796)
Ucn1 WT (r = .754)


B
E-Licks/Day
6000
5000
4000
3000
2000
0
*
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35
Day
3% 6% 10%
20%
[EtOH]


C
EtOH Intake (g/kg/day)
21
18
15
12
9
6
3
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35
Day
3% 6% 10%
20%
[EtOH]


D
Total Intake (ml/kg/day)
400
300
200
100
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35
Day
3% 6% 10%
20%
[EtOH]


E
Mean 20% E-Licks/Hour
750
600
450
300
150
0
ZT14 ZT16 ZT18 ZT20 ZT22 ZT0 ZT2 ZT4 ZT6 ZT8 ZT10
Circadian Cycle


F
E-Bouts/4hr
32
26
20
14
8
0
**
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37
Day
3% 6% 10%
20%
[EtOH]

which occurred within the first 4 hrs of the drinking session (ZT-14 to ZT-18; **Fig. 13E**). RM-ANOVA analysis of 4hr E-Bouts across all EtOH sessions revealed no significant main or interacting genotype effects. However, linear regression analysis of 4hr E-Bouts across 20% EtOH sessions indicated that deletion of *Ucn* blunted the escalating pattern of E-Bout frequency observed in WT mice ($F_{1,26}$ = 8.25; p = .008; **Fig. 13F**). Genotype did not significantly impact any of the other E-Bout variables assessed.

On the final day (D37), deletion of *Ucn* significantly reduced the number of 4hr E-Licks and E-Bouts (both t_{27} > 2.5; both p < .05; **Fig. 14A-B**), but EtOH intake values did not differ significantly between genotypes (**Fig. 14C**). D37 BEC values obtained by GC were significantly positively correlated with D37 BEC values obtained by Analox in both genotypes (*df* = 12-13; both r > .944; both p < .0001; **Fig. 14D**). Verifying the E-Lick and E-Bout measures, BECs were significantly lower in Ucn1 KO vs. WT mice (main effect of genotype; $F_{1,27}$ = 5.40; p = .028; **Fig. 14E**), and there were no significant main or interacting effects of BEC analysis method, despite BECs being slightly higher in the Analox analysis overall (main effect of method; p = .10).

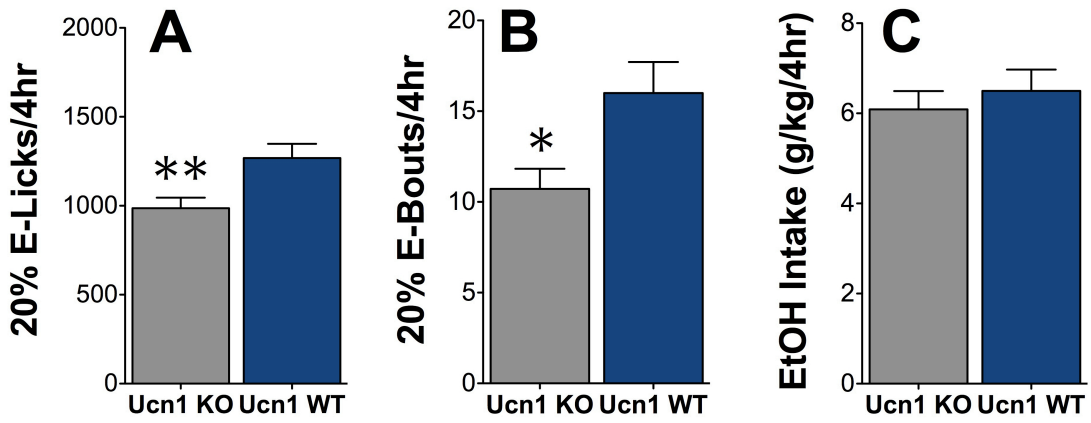
Experiment 3.4: Non-Escalating 2-BC CA

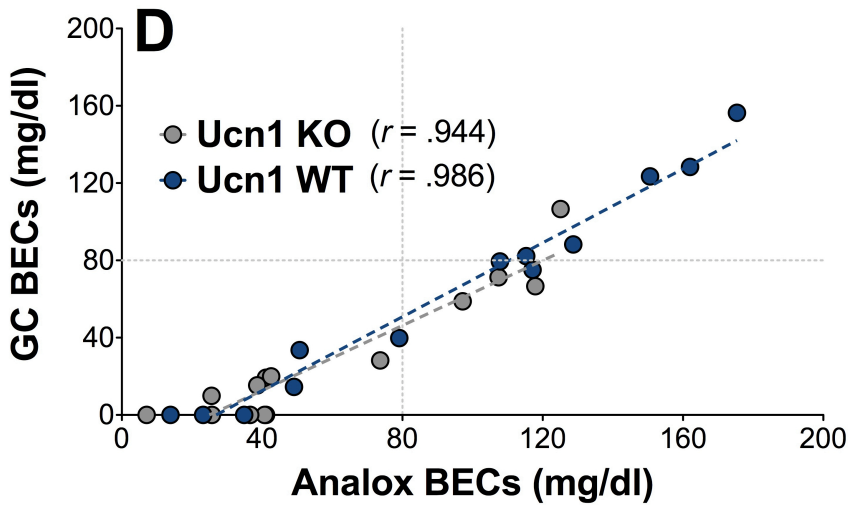
From Day 1 to 14 of the 10% CA 2-BC procedure, Ucn1 KO mice displayed little change in daily EtOH intake (13.3 to 11.5 g/kg). Similarly, WT mice changed their mean daily EtOH intake only slightly from Day 1 to 14 (12.9 to 14.2 g/kg). RM-ANOVA failed to identify a significant main effect of genotype across the two-week period, confirming that deletion of *Ucn* had no effect on EtOH intake in this non-escalating procedure (**Fig. 15A**). For EtOH preference and total fluid intake, there were also no escalations over the course of the two weeks, and no significant genotype effects (**Fig. 15B-C**).

To evaluate the possibility that Ucn1 KO and WT mice differed in the *pattern* of EtOH intake across the circadian cycle in this procedure, I also analyzed consumption

Figure 14. Experiment 3.3: Escalating Long-Term 2-BC IA (D37)

Deletion of *Ucn* blunted binge drinking on D37 of the long-term IA paradigm. Bar graphs illustrate mean ± SEM values. During the 4hr session on Day 37, Ucn1 KO mice displayed **(A)** decreased 20% E-Licks and (**B)** decreased 20% E-Bouts, relative to WT mice. **(C)** Genotypes did not significantly differ in EtOH intake**. (D)** For both genotypes, BECs analyzed by GC were significantly positively correlated with BECs analyzed by Analox. **(E)** BECs were significantly lower in Ucn1 KO vs. WT mice. Asterisks in A and B indicate significant genotypic differences by t-test (**p* <.05; ***p* <.01). Asterisk in E indicates significant main effect of genotype (**p* <.05). Data are shown collapsed on sex.


A
20% E-Licks/4hr
2000
1500
1000
500
0
**
Ucn1 KO
Ucn1 WT
B
20% E-Bouts/4hr
20
15
10
5
0
*
Ucn1 KO
Ucn1 WT
C
EtOH Intake (g/kg/4hr)
8
6
4
2
0
Ucn1 KO
Ucn1 WT


D
GC BECs (mg/dl)
200
160
120
80
40
0
Ucn1 KO (r = .944)
Ucn1 WT (r = .986)
0
40
80
120
160
200
Analox BECs (mg/dl)

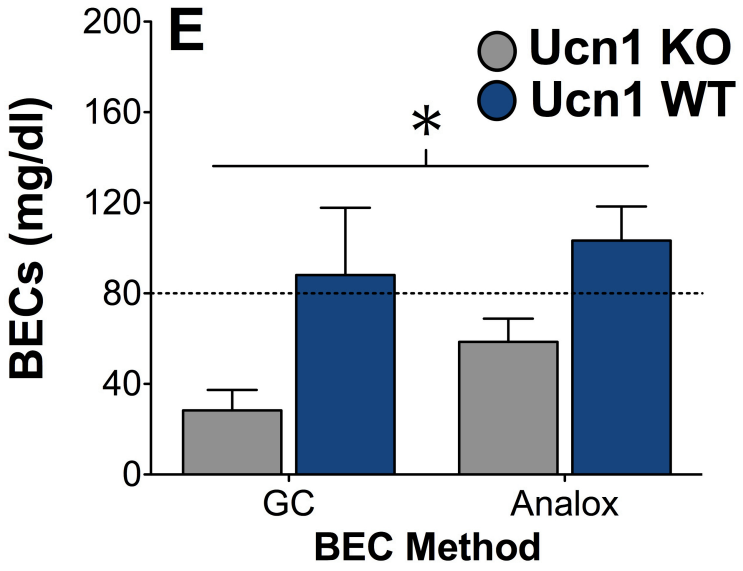
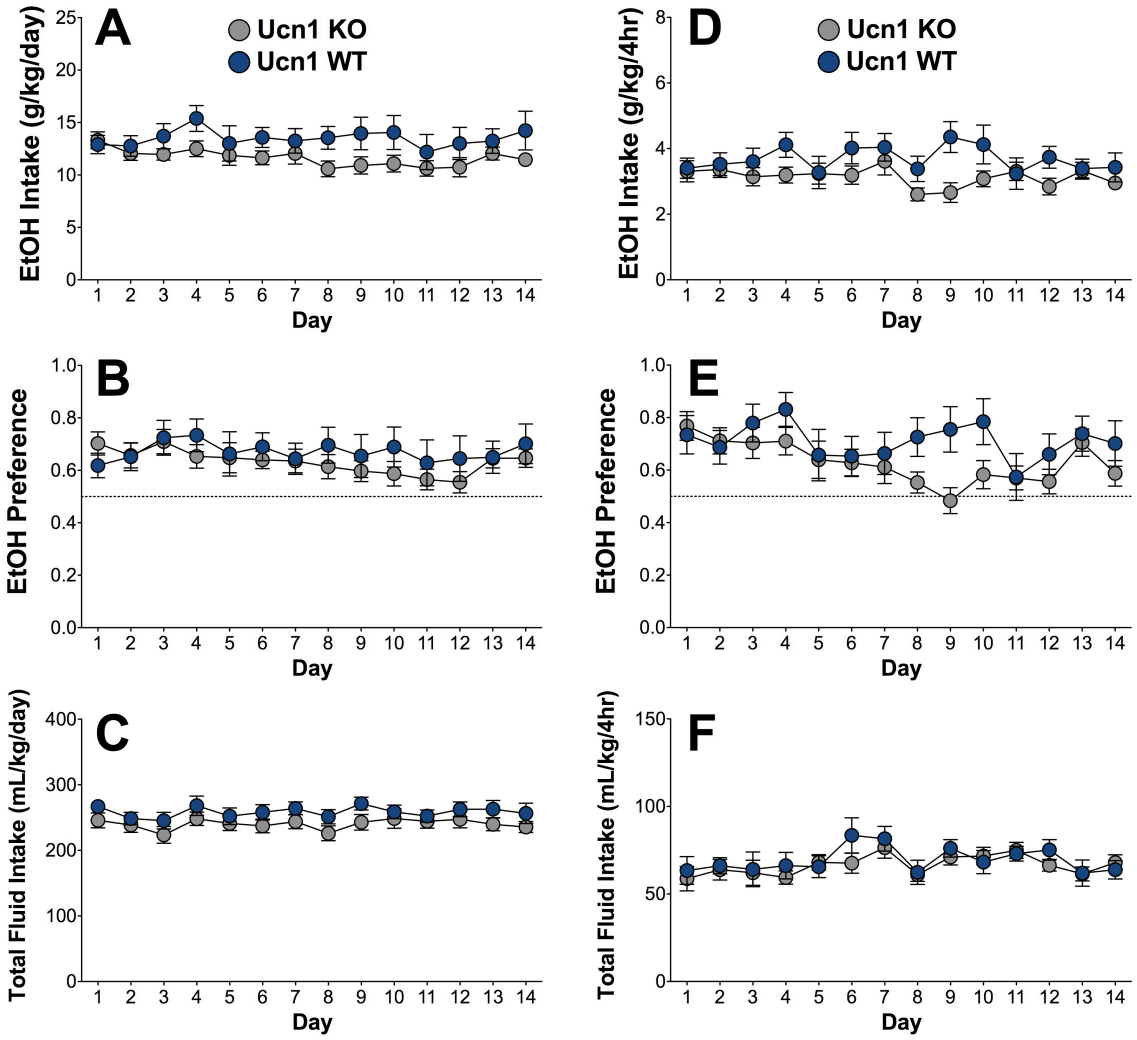

E
BECs (mg/dl)
200
160
120
80
40
0
Ucn1 KO
Ucn1 WT
*
GC
Analox
BEC Method

Figure 15. Experiment 3.4: Non-Escalating 2-BC CA

Graphs illustrate mean ± SEM values. Ucn1 KO and WT mice did not differ in **(A)** EtOH intake, **(B)** EtOH preference, or **(C)** total fluid intake over the course of two weeks of CA 10% EtOH drinking. **(D-F)** Measurements during the previously-identified 4hr period of the circadian dark cycle (ZT-14 to ZT-18) also failed to identify any significant genotype differences. Data are shown collapsed on sex.



values during the 4hr time window in the circadian dark cycle during which genotypes differed in Experiment 3.3 (ZT-14 to ZT-18). This additional analysis also failed to identify any significant genotype effects, indicating that the lack of differences in *daily* intake parameters are also reflective of the lack of differences during the "binge" period of the dark cycle (**Fig. 15D-F**).

Experiment 3.5: 2-BC CA Tastant Control

Analysis uncovered no significant genotypic differences in tastant intake, tastant preference, or total fluid intake (**Fig. 16A-C**). Furthermore, Ucn1 KO and WT mice did not differ in food consumption or total caloric intake (**Fig. 16D-E**).

Experiment 3.6: EtOH-Induced Sedation

Across the three days of LORR testing, RM-ANOVA failed to identify significant main or interacting effects of genotype on either LORR onset (**Fig. 17A**) or LORR duration (**Fig. 17B**), although both analyses uncovered highly significant main effects of day (both $F_{2,58} > 5.07$; $p < .01$). Follow-up comparisons between days with genotypes collapsed revealed evidence for tolerance, as LORR onset was significantly decreased on Day 2 relative to Day 1, and LORR duration was significantly decreased on Day 3 relative to Day 1 (both $p < .01$). Furthermore, Ucn1 KO and WT mice did not differ in BECs at the time of re-gain of righting reflex on Day 3 (**Fig. 17C**).

Experiment 3.7: EtOH-Induced Locomotor Sensitivity

Initial analyses of habituation and baseline locomotor activity levels on Days 1 and 2 did not identify any main or interacting effects with genotype, consistent with previous data showing equivalent basal activity levels between Ucn1 KO and WT mice (Giardino et al., 2011b). Analysis of the EtOH locomotor dose-response curve uncovered a significant

Figure 16. Experiment 3.5: 2-BC CA Tastant Control

Graphs illustrate mean ± SEM values. Ucn1 KO and WT mice did not differ in **(A)** tastant intake, **(B)** tastant preference, nor **(C)** total fluid intake during 2-BC CA tastant drinking. Furthermore, genotypes did not significantly differ in consumption of **(D)** food or **(E)** total calories during tastant access. Data are shown collapsed on sex and concentration.

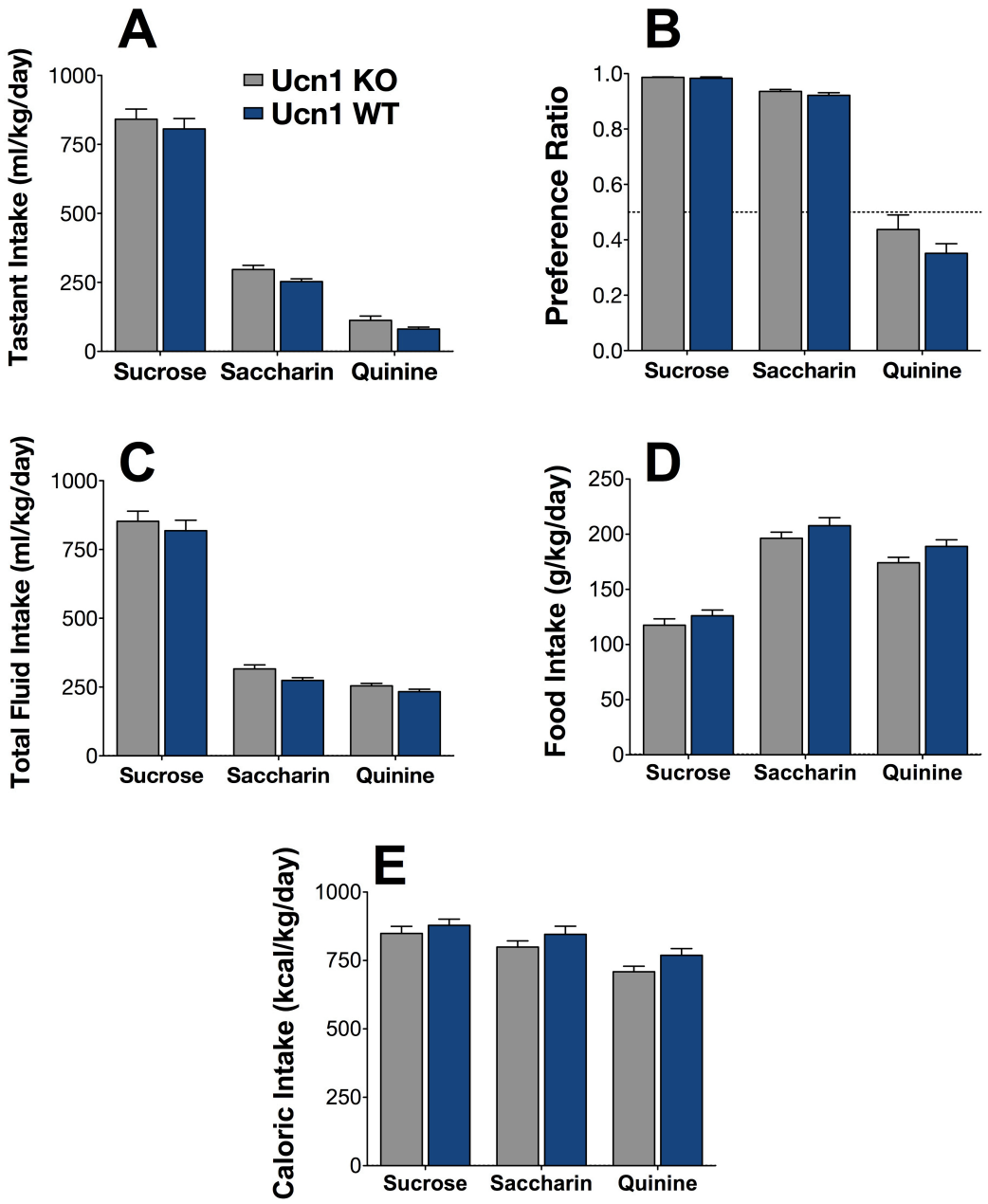


Figure 17. Experiment 3.6: EtOH-Induced Sedation

Graphs illustrate mean ± SEM values. Across three consecutive days of repeated testing with 4.0 g/kg EtOH, deletion of *Ucn* had no effect on **(A)** the latency to LORR onset, nor **(B)** the total LORR duration. **(C)** BECs at time of regaining LORR on Day 3 were also similar between genotypes. Pound signs indicate significant main effects of day (both $p < .01$) followed by significant differences from Day 1 when genotypes were collapsed (##$p < .01$). Data are shown collapsed on sex.

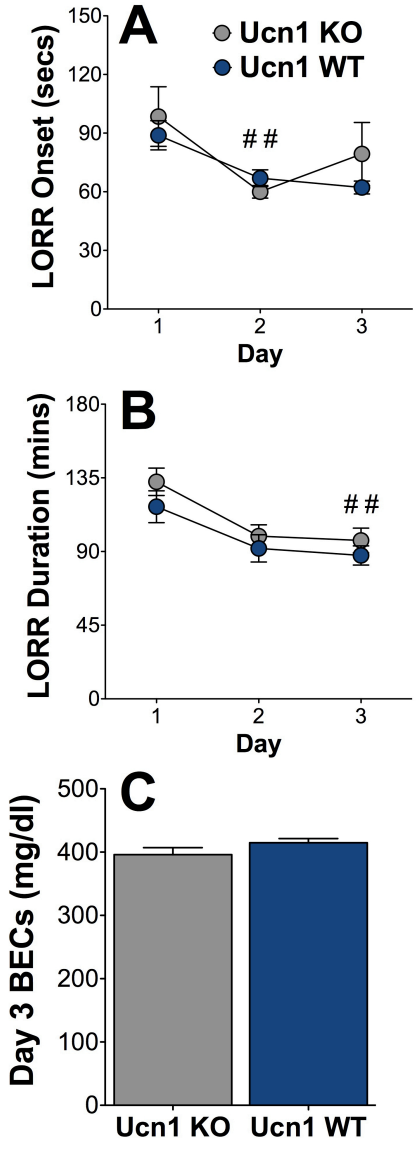
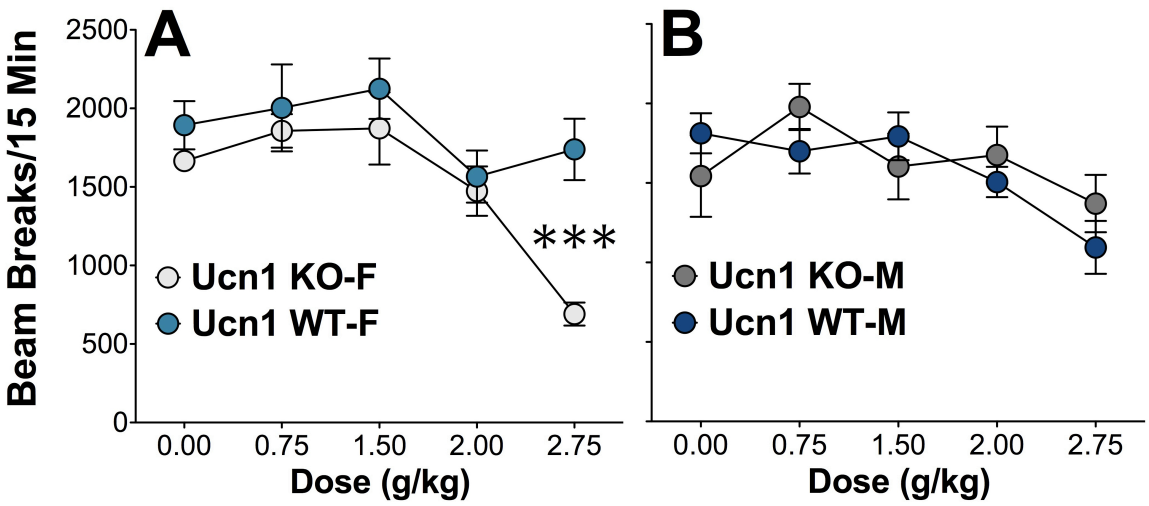


Figure 18. Experiment 3.7: EtOH-induced locomotor activity.

Graphs illustrate mean ± SEM values. Deletion of *Ucn* accentuated the locomotor depressant effects of 2.75 g/kg EtOH in **(A)** female mice, but not in **(B)** male mice. Asterisks indicate significant sex x genotype x dose interaction and significant Bonferroni post-hoc comparison between female genotypes at 2.75 g/kg (****p* <.001).



sex x genotype x dose interaction ($F_{4,224}$ = 2.76; p = .031), justifying separate analyses of genotype and dose within each sex. **Figure 18A** shows that among female mice, deletion of *Ucn* enhanced sensitivity to the locomotor depressant effects of 2.75 g/kg EtOH, despite the genotype x dose interaction not quite reaching statistical significance ($F_{4,112}$ = 2.50; p = .053; Bonferroni p < .001). **Figure 18B** shows that male Ucn1 KO and WT mice were equally sensitive to EtOH's effects on locomotor activity (main effect of dose; $F_{4,112}$ = 4.61; p = .003; dose x genotype interaction p = .203).

Experiment 3.8 – Baseline anxiety measures

Across the EPM (**Fig. 19**) and the LDB (**Fig. 20**), Ucn1 KO and WT mice displayed similar profiles of anxiety-like behavior. However, deletion of *Ucn* increased rearing behavior in both paradigms (**Fig. 19H, 20F**), suggesting that Ucn1 KO mice display greater exploratory behavior, relative to Ucn1 WT mice (both t_{48} > 2.34; both p < .05).

DISCUSSION

The first four studies in Chapter 3 altered several variables (EtOH choice, EtOH concentration, schedule of EtOH access, and length of EtOH exposure) in order to better define the drinking phenotype influenced by Ucn1. Relative to WT controls, mice lacking Ucn1 displayed decreased EtOH drinking only in experiments that a) involved long-term EtOH exposure (i.e. $\geq$4 days), and b) produced escalating EtOH intakes. These data suggest that Ucn1 undergoes time- and/or dose-dependent neuroadaptations contributing to the excessive intake phenotype. Thus, Ucn1's contribution to voluntary oral EtOH consumption may be especially relevant to the forms of pathological drinking observed in human alcoholics.

Experiment 3.3 used a lickometer system to provide a high-resolution view of time-dependent changes in EtOH consumption. The long-term IA procedure produced

Figure 19. Experiment 3.8: Anxiety-Like Behavior (EPM)

Graphs illustrate mean ± SEM values. Ucn1 KO and WT mice did not differ in **(A)** latency to enter an open arm, **(B)** time spent in the open arms, **(C)** open arm entries, **(D)** percent of arm entries into the open arm, **(E)** closed arm entries, **(F)** number of head dips over the side of open arms, or **(G)** number of grooming bouts. Panel **(H)** shows that relative to WT mice, Ucn1 KO mice displayed greater rearing behavior (*p <.05). Data are shown collapsed on sex and testing order.

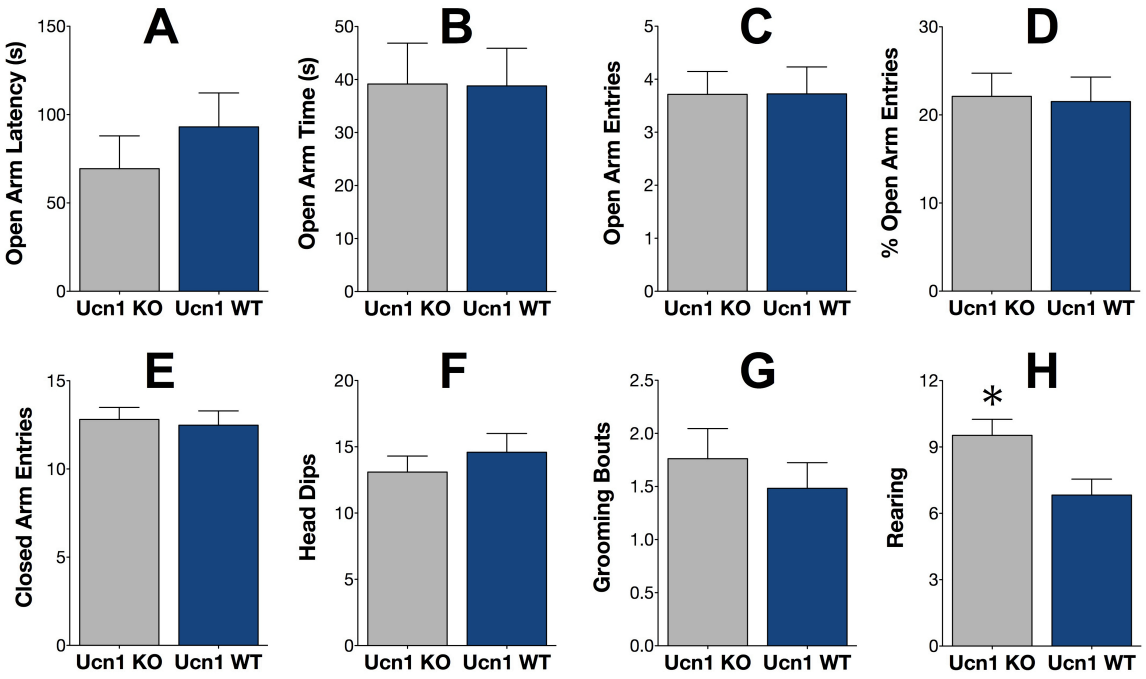
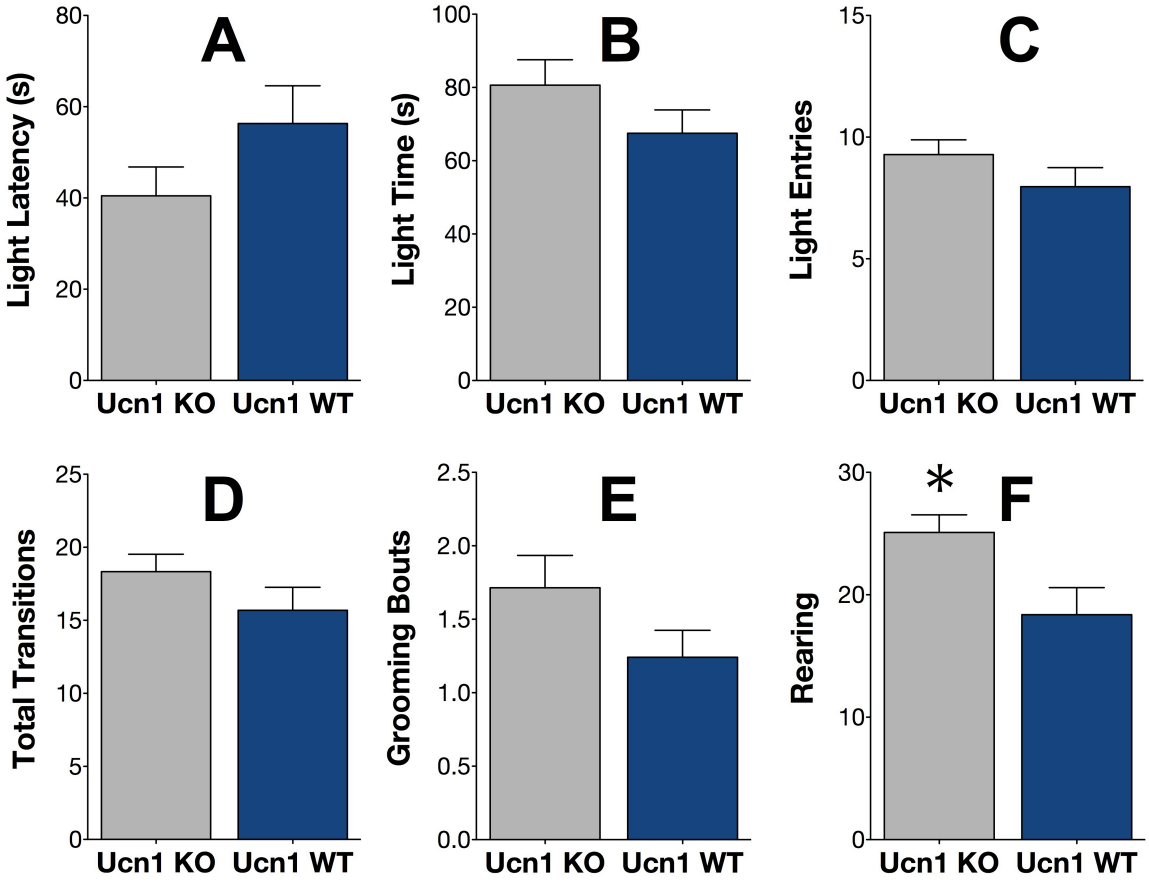


Figure 20. Experiment 3.8: Anxiety-Like Behavior (LDB)

Graphs illustrate mean ± SEM values. Ucn1 KO and WT mice did not differ in **(A)** latency to enter the light side of the box, **(B)** time spent in the light side of the box, **(C)** number of entries into the light side of the box, **(D)** total light/dark transitions, or **(E)** number of grooming bouts. Panel **(F)** shows that relative to WT mice, Ucn1 KO mice displayed greater rearing behavior (**p* <.05). Data are shown collapsed on sex and testing order.



progressive escalations of E-Licks and E-Bouts in WT mice that were significantly blunted in Ucn1 KO mice, although EtOH intake did not differ significantly between genotypes. Amidst the data collection phase, I plotted the circadian patterns of drinking, and used this rhythm to identify a time window during which the genotypes markedly differed in E-Licks. Not surprisingly, this time window coincided with that used in the DID paradigm, suggesting that mice were achieving binge-like BECs during their first 4 hrs of EtOH access. On D37, during the identified 4hr time window, Ucn1 WT mice achieved mean BECs of 103.3 mg/dl, while Ucn1 KO mice failed to meet the binge criteria, reaching mean BECs of 58.6 mg/dl. This delineation may hold particular relevance to human populations, where the extent of Ucn1 signaling could determine the difference between moderate vs. problem drinking.

However, it is worth noting the imperfect relationship between the effects of genotype on EtOH intake vs. alternative measures of EtOH consumption (E-Licks, E-Bouts). Because excessive motor activity around the sipper spout (i.e., bumping the spout) may inadvertently result in the loss of fluid without a recorded “lick” (or vice-versa), the dichotomy between g/kg EtOH intake and E-Licks may represent a different pattern of home-cage behavioral patterns between the genotypes. On the other hand, the E-Lick and E-Bout results accurately reflected the observed differences in BECs, suggesting that the lickometer afforded precise measures of consumption. With this in mind, the lack of genotype differences in g/kg intake could be attributable to the fact that weighing the large plastic bottles was a less precise method than reading the smaller graduated cylinders (an important procedural distinction between Experiment 3.3 and all of the other drinking studies performed in Chapters 2, 3, 4, and 5).

Chapter 2 identified a role for Ucn1 in the conditioned rewarding effects of EtOH, yet it remained unclear how Ucn1 might contribute to other traits that influence voluntary EtOH consumption. For example, some have suggested that B6 mice consume EtOH

primarily for its caloric contents (McMillen and Williams, 1998), which could be a confounding factor influencing Ucn1's effects on EtOH-seeking. Ucn1 KO and WT mice did not differ in food or total caloric intake in Experiments 3.1 and 3.5, nor did they differ in the percent of calories consumed from EtOH vs. food in Experiment 3.1. Because male Ucn1 KO mice displayed lower EtOH preference ratios relative to male WT mice in the absence of differences in food intake (Experiment 3.1), this indicates that caloric need is not a primary factor explaining genotypic differences in preference for EtOH. However, it remains to be determined whether Ucn1 KO and WT mice differ in food intake in the paradigms where they differ in EtOH intake (i.e. escalating 2-BC CA or IA).

Orosensory cues are also known to influence EtOH acceptance (Cunningham and Niehus, 1997). To assess taste reactivity, I offered Ucn1 KO and WT mice increasing concentrations of sucrose, saccharin, and quinine, each with a length of exposure that had been adequate to observe differences in EtOH intake (8 days). These control experiments showed that deletion of *Ucn* did not influence preference for sweet or bitter tastes. However, the interpretation of these findings could be limited by the lack of a washout period between exposure to different tastants, and by the absence of a counterbalanced order of tastant presentation.

Mice from the tastant experiment were also tested for LORR. Past studies identified an inverse relationship between sensitivity to EtOH-induced body sway and problem drinking (Schuckit, 1994). This measure of motor incoordination may be related to the sedative effects of EtOH observed in LORR. Due to the time-dependent genotypic differences in EtOH intake observed in Experiments 3.2 and 3.3, I also hypothesized that Ucn1 might contribute to EtOH *tolerance*. Thus, Ucn1 KO and WT mice were repeatedly tested for LORR on three consecutive days, during which tolerance to sedation developed (i.e., LORR onset decreased from Day 1 to Day 2, and LORR duration decreased from Day 1 to Day 3). No significant genotype differences were identified in

the latency or duration of LORR, suggesting that Ucn1 may not contribute to EtOH-induced sedation or tolerance. Furthermore, I observed similar BECs between the genotypes at the time of re-gain of the righting reflex, agreeing with previous studies that loss of Ucn1 expression does not lead to differences in the rate of EtOH elimination (Kaur et al., 2012; Pastor et al., 2008).

Experiment 3.7 showed that Ucn1 KO may lead to a sex-specific increase in sensitivity to the locomotor depressant effects of 2.75 g/kg EtOH. However, another interpretation could be that the lack of locomotor depression in female Ucn1 WT mice is the more striking result. Nevertheless, the different behaviors of male and female mice suggest that this could be an estrous-related effect that differentially impacts locomotor activity in the two genotypes. This finding awaits replication in a separate cohort of mice not subjected to multiple doses of EtOH. The multiple dosing schedule (although fully counterbalanced) could have contributed to sensitization and/or tolerance to the locomotor effects of EtOH. These effects might have still been apparent despite the use of a one-day “washout period” between EtOH dose testing.

Finally, Experiment 3.8 attempted to identify differences in anxiety-like behavior between Ucn1 KO and WT mice, as several experiments found associations between CRF system activity, anxiety-like behavior, and excessive EtOH intake (Valdez et al., 2002; Valdez et al., 2004; Valdez et al., 2003). The precise role of Ucn1 in stress- and addiction-related behaviors has remained convoluted, as the methods implemented thus far focused primarily on exogenous administration of Ucn1, rather than direct manipulation of endogenous Ucn1 levels within the brain (Ryabinin et al., 2008; Sajdyk et al., 1999; Spiga et al., 2006; Spina et al., 1996; Spina et al., 2002; Weitemier and Ryabinin, 2006). As the literature currently stands, Ucn1 may possess anxiogenic or anxiolytic actions, depending on the site of action and the prior stress experience of the individual.

In my hands, Ucn1 KO and WT mice did not differ in traditional measures of anxiety-like behavior across two well-validated tasks. I discovered that KO of Ucn1 increased exploratory rearing behavior in both the EPM and the LDB. Despite these reliable differences that could be reflective of differences in habituation to novelty, Ucn1 KO and WT mice did not differ in locomotor activity levels during habituation to a novel environment (i.e., exposure to the activity chamber following saline injections on Days 1 and 2 of the locomotor study). Unfortunately, I could not measure rearing behavior and vertical activity in the locomotor chambers, which may have been useful in providing additional measures of exploratory behavior. Although intriguing, it remains unclear how differences in rearing behavior could be related to differences in voluntary EtOH intake.

All studies in Chapter 3 used both male and female KO and WT mice, but it is unclear whether sample sizes were always sufficient to detect differential effects of genotype across sexes. Experiments 3.1 and 3.7 identified significant sex x genotype interactions, but these studies could also be underpowered, and would benefit from replication in larger cohorts of male and female KO and WT animals. Unfortunately, there are prohibitive costs associated with breeding, weaning, genotyping, and testing multiple lines of CRF system mutant mice. Therefore, I took special care to limit unnecessary testing of KO and WT animals, especially if levels of variability were already reasonable within each group. The conclusion that Ucn1 KO produced largely sex-independent effects on EtOH-related behavior should be interpreted cautiously.

In summary, Ucn1 KO produced a selective decrease in long-term, escalating EtOH consumption that could not be accounted for by differences in caloric need, taste reactivity, sensitivity to EtOH sedation and tolerance, EtOH metabolism, EtOH insensitivity, or baseline anxiety levels. The results from Experiment 3.3 showed that the loss of Ucn1 may protect against the high levels of drinking that are observed following repeated intermittent abstinence. Progressive escalations in binge-like consumption

could reflect adaptations similar to those that occur during the transition to EtOH dependence. For example, a recent study reported the appearance of physical withdrawal symptoms in B6 mice that underwent the long-term IA paradigm for 16 weeks (Hwa et al., 2011). To directly test the hypothesis that Ucn1 contributes to the development of EtOH dependence, the Ryabinin Lab is currently assessing Ucn1 KO and WT mice using the chronic intermittent EtOH vapor model.

CHAPTER 4: Long-Term EtOH Drinking: Effects on Gene Expression in the Centrally Projecting Edinger-Westphal Nucleus

INTRODUCTION

Because reduction of Ucn1 levels by genetic KO in Chapter 3 selectively decreased EtOH intake and preference, I predicted that EtOH drinking produced adaptations in neural *Ucn* gene expression. Based on the EWcp lesion studies in Chapter 2, I reasoned that long-term EtOH consumption altered levels of *Ucn* mRNA specifically within the EWcp. In addition, Chapter 1 studies provided several additional candidate genes to further explore with regard to EtOH drinking. Therefore, I designed a series of experiments in which expression levels of several EWcp-enriched genes of interest (including *Ucn*) were assessed following voluntary EtOH consumption.

For selection of mRNA transcripts for Chapter 4 analyses, I focused on neuropeptide-related genes in general, and candidate genes that differed between strains in Chapter 1. After the Chapter 1 studies were complete, a report on the effects of leptin receptor (Lepr) expression and signaling in EWcp-Ucn1 neurons indicated that Lepr was another EWcp candidate gene that should be added to the qPCR arrays (Xu et al., 2011). To conserve space on the qPCR plates so as to run samples from two mice on each plate (n=48 wells per mouse), several genes that were included in Chapter 1 were removed from the list used in Chapter 4. Reasons for removal from the list included either the lack of identifiable expression differences between B6 and D2 mice, or the lack of a clear relationship to the stress response or the neural effects of EtOH.

Custom qPCR arrays were used to compare EWcp gene expression levels between H2O control mice and mice that underwent long-term EtOH drinking. In two separate experiments each including time-matched H2O control groups, EtOH-experienced mice were euthanized during a period of forced deprivation (No Access), or during a period of binge-like drinking (Access), and EWcp samples were isolated.

EWcp gene expression changes that occur during forced EtOH abstinence might reflect long-term adaptations underlying the persistent high EtOH intakes observed in

the IA paradigm (i.e., a stress-like state of negative affect, or the emergence of negative reinforcement process that perpetuate further drinking). Gene expression changes occurring following a binge-like drinking session might reflect either genes underlying the acute rewarding effects of intoxicating doses of EtOH, or genes induced by acute EtOH that later contribute to adaptations in other genes observed during forced abstinence.

The long-term IA paradigm was selected for these studies because it resembles the pattern of consumption observed in human alcoholics (high levels of EtOH intake interspersed with repeated periods of abstinence from EtOH), and because it will facilitate comparison of results to other recent investigations of neural CRF system contributions to long-term IA drinking in both mice and rats (Cippitelli et al., 2011; Hwa et al., 2013; Simms et al., 2013). Furthermore, Chapter 3 studies generally found stronger effects of Ucn1 KO on EtOH drinking at higher concentrations of EtOH, and identified a binge-like time window in the long-term IA paradigm during which genotypes differed, suggesting that adaptations in gene expression might be observable at a similar timepoint before or after intake of high-concentration (20%) EtOH.

Because reduction of Ucn1 levels by genetic KO decreased EtOH drinking in Chapters 2 and 3, I hypothesized that *Ucn* mRNA would be elevated in EtOH-exposed vs. H2O-control mice. In addition, because the EWcp may represent a substrate for the effects of stress on EtOH drinking, I also hypothesized that other anxiety-promoting neuropeptide genes in the EWcp (*Adcyap1, Cck, Cart, Nucb2*) would be upregulated in EtOH-experienced mice. Originally, I predicted that these genes would be upregulated immediately following an EtOH drinking session (i.e., under Access conditions). After observing modest effects on gene expression in that experiment, and reflecting on the effects of EtOH on the extrahypothalamic CRF system (reviewed above), I next hypothesized that neuropeptide-related EWcp genes might instead be upregulated in the absence of EtOH (i.e., under No Access conditions).

MATERIALS AND METHODS

General Procedures

Adult male B6 mice arrived from JAX-West at 8 weeks of age and were initially group-housed for 1 week in a temperature- and humidity-controlled environment with *ad libitum* access to food (LabDiet 5001; LabDiet, Richmond, IN, USA) and H2O. Only male mice were used because experiments in Chapter 3 did not identify any systematic sex differences between genotypes. Although Experiments 3.1 and 3.7 did identify significant interactions of genotype with sex, it is unclear how these studies of short-term DID and locomotor sensitivity would relate to the effects of long-term IA drinking on gene EWcp expression. Furthermore, fluctuations in hormones within female mice could contribute to increased variability in gene expression data, and would further complicate statistical analysis. Mice were moved to the procedure room and allowed to habituate to single housing and a reverse light-dark cycle (ZT-0/lights OFF at 7 or 8 am) for 1-2 weeks prior to EtOH access. During the acclimation period, mice received 24hr access to two 25 ml glass cylinder bottles with metal sipper tubes (both containing H2O) on either side of the cage, and food evenly distributed along the cage top.

Mice from different groups were pseudo-randomly distributed across the rows of the housing rack, and the side of the EtOH bottle was fully counterbalanced across groups. Furthermore, the side of the EtOH bottle was switched weekly to avoid the potential confound of a side bias. H2O control mice had identical bottle conditions with the exception that they only received H2O. Weekly body weight measurements were obtained to calculate accurate food and fluid intake variables throughout the studies. All protocols were approved by the OHSU IACUC and were performed with adherence to the NIH Guidelines for the Care and Use of Laboratory Animals.

Experiment 4.1: No Access Conditions

Following habituation, mice were randomly split into EtOH (n=24) and H2O-Control (n=12) groups. Mice underwent the long-term IA procedure described in Experiment 3.3, receiving every-other-day access to EtOH (increasing concentrations of 3, 6, and 10% on Days 1, 3, and 5, respectively, followed by 20% on odd days from Day 7 to 37). However, unlike Experiment 3.3, which was performed using a lickometer apparatus, grid-floor housing in smaller cages, and plastic 50 ml bottles, Experiment 4.1 was performed with the standard shoebox cages and 25 ml glass bottles used for all other experiments. Furthermore, because there was no long time requirement for setting up the lickometer apparatus in between sessions, EtOH and H2O drinking days were each exactly 24 hrs in Chapter 4 studies. Food intake and caloric variables were calculated for Day 31, as previously described in Chapter 3.

At the beginning of Day 37 (D37), mice were euthanized by CO2 at ZT-14, which is immediately prior to the time at which they would have normally received access to EtOH. Thus, EtOH mice in the No Access experiment had experienced 24 hours of EtOH abstinence at the time of euthanasia. Following euthanasia, brains were dissected, and tissue samples containing the EWcp from each animal were dissected prior to undergoing RNA extraction, isolation, and quantification, as described in Chapter 1. Only mice with EWcp samples that met high-quality RNA criteria (260/280 values between 1.8 and 2.2, concentrations $\geq$ 5.4 nanograms per μl) were used for the gene expression analyses.

Although a full 96-well qPCR array was used for each individual animal in Chapter 1, I was more selective in the choice of candidate genes to be included in Chapter 4. Thus, EWcp samples from two different mice were analyzed on a single 96-well plate. The 48 wells analyzed for each animal included: five wells for RT and genomic DNA controls, six wells for housekeeping genes (**Table 7**), 23 wells for EWcp-

Table 7. Chapter 4: List of Housekeeping Genes

Asterisks indicate genes that were included in the average of housekeeping genes used to normalize the mRNA levels of the genes of interest.

Gene Symbol	Gene Name
18S	18S ribosomal RNA
*Actb**	Beta-actin
*Gapdh**	Glyceraldehyde 3-phosphate dehydrogenase
Gusb	Beta-glucuronidase
*Hprt**	Hypoxanthine-guanine phosphoribosyltransferase
*Hsp90ab1**	Heat shock protein 90-beta
*Reep5**	Receptor expression-enhancing protein 5

enriched genes of interest, three for ITFs, eight for DA-related genes, and three for CRF-related genes other than *Ucn* (**Table 8**). Mean CTs for the six housekeeping genes included on the qPCR array were first normalized to *18S* mRNA levels (diluted 1:100,000), and then compared between EtOH and H2O groups by t-test. Normalized levels of housekeeping transcripts that did not show significant group differences (5 out of 6) were then averaged and used to normalize the quantitative expression of all genes of interest included on the array.

For each individual gene of interest, CT values were normalized by the equation $2^{-\Delta CT}$, where ΔCT = the CT for the gene of interest subtracted from the mean CT value of the housekeeping genes. Additionally, data were analyzed by the $2^{-\Delta\Delta CT}$ method, in order to determine fold change levels of mRNA in EtOH mice expressed relative to H2O mice ("calibrator" group). The mean $2^{-\Delta\Delta CT}$ values were compared by t-test between groups (significance threshold at $p < .05$), and data are presented as mean $2^{-\Delta\Delta CT}$ values ± SEM. Bonferroni correction for multiple comparisons was not applied, as I aimed to identify as many differentially-expressed genes as possible. Correlational analyses performed on the EtOH group aimed to identify relationships between mRNA levels and measures of EtOH consumption, using an adjusted significance threshold of $p < .025$ to account for multiple comparisons. For genes that were significantly correlated with EtOH intake, I performed control correlations in the H2O control group, correlating the mean H2O intake from 20% EtOH days to the mRNA levels of the gene being assessed.

Experiment 4.2: Access Conditions

Following habituation, mice were randomly split into EtOH (n=24) and H2O (n=24) groups. EtOH mice underwent the long-term IA procedure described in Experiment 4.1. On D37, EtOH access was cut short after a 4hr drinking session (ZT-14 to ZT-18),

Table 8. Chapter 4: List of Genes of Interest

Including the six housekeeping genes and five controls, each half of the 96-well array included 23 EWcp-enriched, three ITFs, eight DA-related, and three CRF-related genes.

Gene Symbol	Category	Gene Name
Adcyap1	EW-Enriched	Pituitary adenylate cyclase-activating polypeptide
Cart	EW-Enriched	Cocaine- and amphetamine-regulated transcript
Cck	EW-Enriched	Cholecystokinin
Cds2	EW-Enriched	Phosphatidate cytidylyltransferase 2
Dlk1	EW-Enriched	Delta-like homolog 1
Gabre	EW-Enriched	GABA-A receptor subunit epsilon
Gabrq	EW-Enriched	GABA-A receptor subunit theta
Ghsr	EW-Enriched	Growth hormone secretagogue (ghrelin) receptor
Gpx3	EW-Enriched	Glutathione peroxidase 3
Lepr	EW-Enriched	Leptin receptor
Ndn	EW-Enriched	Necdin
Nenf	EW-Enriched	Neuron-derived neurotrophic factor
Neto1	EW-Enriched	Neuropilin and tolloid-like 1
Nucb2	EW-Enriched	Nucleobindin-2 (Nesfatin-1)
Pcsk1	EW-Enriched	Proprotein convertase subtilisin/kexin type 1
Peg3	EW-Enriched	Paternally-expressed gene 10
Peg10	EW-Enriched	Paternally-expressed gene 3
Postn	EW-Enriched	Periostin, osteoblast specific factor
Prmt2	EW-Enriched	Protein arginine methyltransferase 2
Ptprn	EW-Enriched	Protein tyrosine phosphatase, receptor type N
Scg2	EW-Enriched	Secretogranin-2, secretoneurin
Sncg	EW-Enriched	Gamma-synuclein
Ucn	EW-Enriched	Urocortin-1
Egr1	ITFs	Early growth response 1
Fos	ITFs	FBJ osteosarcoma oncogene
Fosb	ITFs	FBJ osteosarcoma oncogene B
Th	DA-Related	Tyrosine hydroxylase
Ddc	DA-Related	Dopamine decarboxylase
Slc6a3	DA-Related	Dopamine reuptake transporter
Drd1a	DA-Related	Dopamine receptor 1
Drd2	DA-Related	Dopamine receptor 2
Drd3	DA-Related	Dopamine receptor 3
Drd4	DA-Related	Dopamine receptor 4
Drd5	DA-Related	Dopamine receptor 5
Crhr1	CRF-Related	CRF receptor 1
Crhr2	CRF-Related	CRF receptor 2
Crhbp	CRF-Related	CRF binding protein

during which food intake was also calculated. Mice were euthanized by CO2 inhalation at ZT-18, and trunk blood was collected for analysis of BECs. As in Experiment 4.1, EWcp tissue samples were collected from all mice immediately after euthanasia, and analyzed as described above. All genes of interest were normalized to the same set of housekeeping genes used for analysis of Experiment 4.1.

RESULTS

Experiment 4.1 (No Access): Long-Term IA Drinking

EtOH and H2O groups did not differ in body weight or total fluid intake (**Fig. 21A-B**). For mice in the EtOH group, daily 20% EtOH intake stabilized at ~20 g/kg (**Fig. 21C**). Preference for 20% EtOH increased progressively (main effect of day; $F_{14,168} = 8.21$; $p < .0001$), reaching significantly higher levels on D35 relative to D7 (Bonferroni $p < .0001$; **Fig. 21D**). Consummatory variables collected on Day 31 revealed that EtOH mice consumed less food than H2O controls ($t_{20} = 3.99$; $p = .0007$; **Fig. 21E**), yet there was no difference in total calories consumed between the groups (**Fig. 21F**), as mice in the EtOH group consumed ~20% of their total calories from EtOH (**Fig. 21G**).

Experiment 4.1 (No Access): Gene Expression Findings

Following normalization to *18S*, mRNA levels of housekeeping genes were similar between groups, excluding *Gusb* ($t_{20} = 3.15$; $p = .0059$). All genes of interest were normalized to the remaining housekeeping genes: *Actb*, *Gapdh*, *Hprt*, *Hsp90ab1*, and *Reep5*. Normalized mRNA levels of 17/37 genes of interest (including *Ucn* and other neuropeptide-related transcripts) were elevated in EtOH mice, relative to H2O mice (**Table 9**). Mean daily 20% EtOH intake was significantly positively correlated with *Fos* mRNA levels ($r = .633$; $df = 11$; $p = .020$; **Fig. 21H**), and significantly negatively correlated with *Drd2* mRNA levels ($r = -.649$; $df = 11$ $p = .016$) (**Fig. 21I**), despite neither

Figure 21. Experiment 4.1 (No Access): Long-Term IA Drinking

Graphs illustrate mean ± SEM values, and in come cases display individual data points for each group. **(A)** Body weights and **(B)** total fluid intake did not differ significantly between groups. **(C)** EtOH intake reached high stable levels, and **(D)** EtOH preference progressively increased over the course of 20% EtOH days. **(E)** On Day 31, mice in the EtOH group consumed less food than H2O controls. **(F)** Day 31 total caloric intake values were equivalent between groups. **(G)** Mice in the EtOH group consumed 19.7% of their total calories from EtOH on Day 31. **(H)** Mean 20% EtOH intake was significantly positively correlated with *Fos* mRNA levels and **(I)** significantly negatively correlated with *Drd2* mRNA levels. Asterisks indicate significant difference by t-test (***p = .0007).

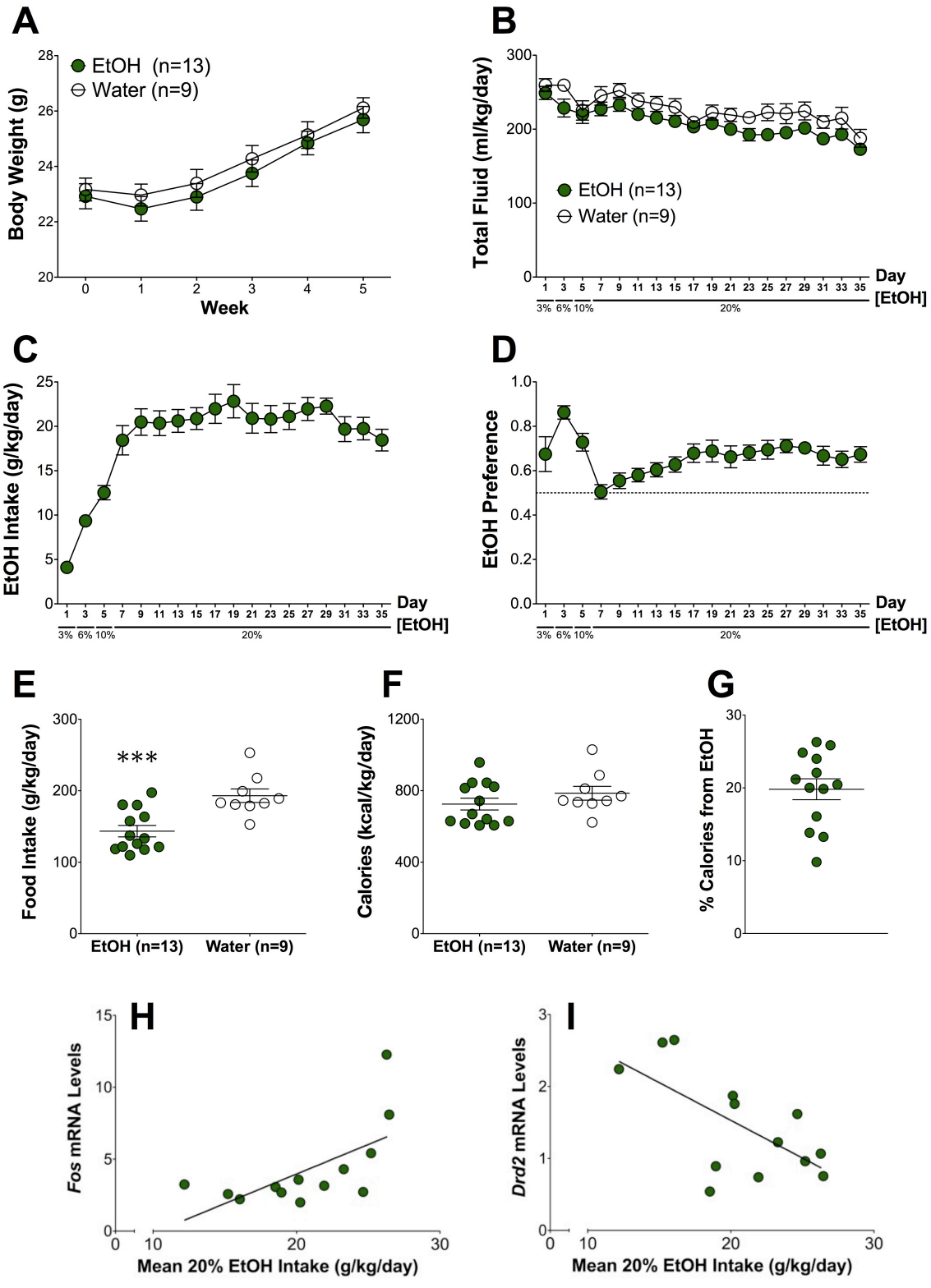

A
EtOH (n=13)
Water (n=9)
Body Weight (g)
Week
B
Total Fluid (ml/kg/day)
EtOH (n=13)
Water (n=9)
Day
[EtOH]
3% 6% 10% 20%
C
EtOH Intake (g/kg/day)
Day
[EtOH]
3% 6% 10% 20%
D
EtOH Preference
Day
[EtOH]
3% 6% 10% 20%
E
Food Intake (g/kg/day)
***
EtOH (n=13)
Water (n=9)
F
Calories (kcal/kg/day)
EtOH (n=13)
Water (n=9)
G
% Calories from EtOH
H
Fos mRNA Levels
Mean 20% EtOH Intake (g/kg/day)
I
Drd2 mRNA Levels
Mean 20% EtOH Intake (g/kg/day)

Table 9. Experiment 4.1 (No Access): Gene Expression Findings

Seventeen of 37 genes of interest were significantly upregulated in EtOH vs. H2O mice (1.4-fold to 2.9-fold). No genes were significantly downregulated in EtOH vs. H2O mice.

Gene	*p*	Fold	Gene Name and Information
Adcyap1	.0052	1.69	Pituitary AC-activating peptide (PACAP; stress neuropeptide)
Cds2	.0028	1.79	Phosphatidate cytidylyltransferase 2
Gabrq	.0236	1.56	GABA-A receptor subunit theta
Gpx3	.0134	1.84	Glutathione peroxidase 3
Ndn	.0040	1.75	Necdin (Deleted in Prader-Willi syndrome; imprinted)
Nenf	.0393	1.45	Neuron-derived neurotrophic factor
Nucb2	.0166	1.63	Nucleobindin-2 (Nesfatin-1; stress neuropeptide)
Pcsk1	.0005	1.90	Proprotein convertase 1 (Cleaves long-form peptides)
Peg3	.0055	1.88	Paternally-expressed gene 3 (Imprinted)
Peg10	.0165	1.82	Paternally-expressed gene 10 (Imprinted)
Postn	.0258	2.24	Periostin (Ligand for integrins that support cell migration)
Prmt2	.0131	1.57	Protein arginine methyltransferase 2 (Methylation; ER-α)
Scg2	.0001	2.01	Secretogranin-2 (Packages neuropeptide vesicles)
Ucn	.0183	2.02	Urocortin-1 (Stress neuropeptide)
Drd5	.0072	2.92	Dopamine D5 receptor (D1-like, Gs-coupled)
Crhbp	.0022	1.74	CRF binding protein (Interacts with Ucn1)
Crhr1	.0476	1.86	CRF type-1 receptor (Interacts with Ucn1)

of these genes being differentially expressed between groups. Mean H2O intakes from 20% EtOH days in H2O control mice were not significantly correlated with either *Fos* or *Drd2* mRNA levels (both $r < .28$; $df = 7$; $p > .15$).

Experiment 4.2 (Access): Long-Term IA Drinking

Similar to Experiment 4.1, EtOH and H2O mice did not differ in body weights or total fluid intakes (**Fig. 22A-B**). EtOH intake reached high, stable levels, and EtOH preference showed a progressive increase (**Fig. 22C-D**). During the final 4hr drinking session on D37, EtOH mice averaged EtOH intakes of 4.68 g/kg, an EtOH preference ratio of 52.54%, and BECs of 78.22 mg/dl, falling just below the criterion for binge drinking (**Fig. 23A-C**). EtOH mice consumed significantly less food relative to H2O controls on D37 ($t_{23} = 2.26$; $p < .05$; **Fig. 23D**). Total caloric intake tended to differ between groups, but did not reach significance ($p = .09$), as EtOH mice received ~20% of their total calories from EtOH (**Fig. 23E-F**).

Experiment 4.2 (Access): Gene Expression Findings

Cck mRNA levels were elevated in EtOH vs. H2O mice ($t_{23} = 2.13$; $p < .05$; **Fig. 24A**), and *Peg3* mRNA levels were downregulated in EtOH vs. H2O mice ($t_{21} = 2.56$; $p < .05$; **Fig. 24B**). No other genes were significantly differentially expressed between groups. Mean 20% EtOH intake was significantly positively correlated with *Cck* mRNA levels ($r = .664$; $df = 12$; $p = .010$; **Fig. 24C**), and D37 BECs were significantly positively correlated with *Fos* mRNA levels ($r = .615$; $df = 12$; $p = .019$; **Fig. 24D**), despite *Fos* levels not differing significantly between groups. Mean H2O intake from 20% EtOH days in H2O control mice was not significantly correlated with *Cck* mRNA ($r = -.35$; $df = 9$ $p = .29$).

Figure 22. Experiment 4.2 (Access): Long-Term IA Drinking

Graphs illustrate mean ± SEM values. **(A)** Body weights and **(B)** total fluid intakes did not differ between EtOH and H2O groups. **(C)** EtOH intake reached high, stable levels, and **(D)** EtOH preference showed a progressive increase over 20% IA EtOH days.

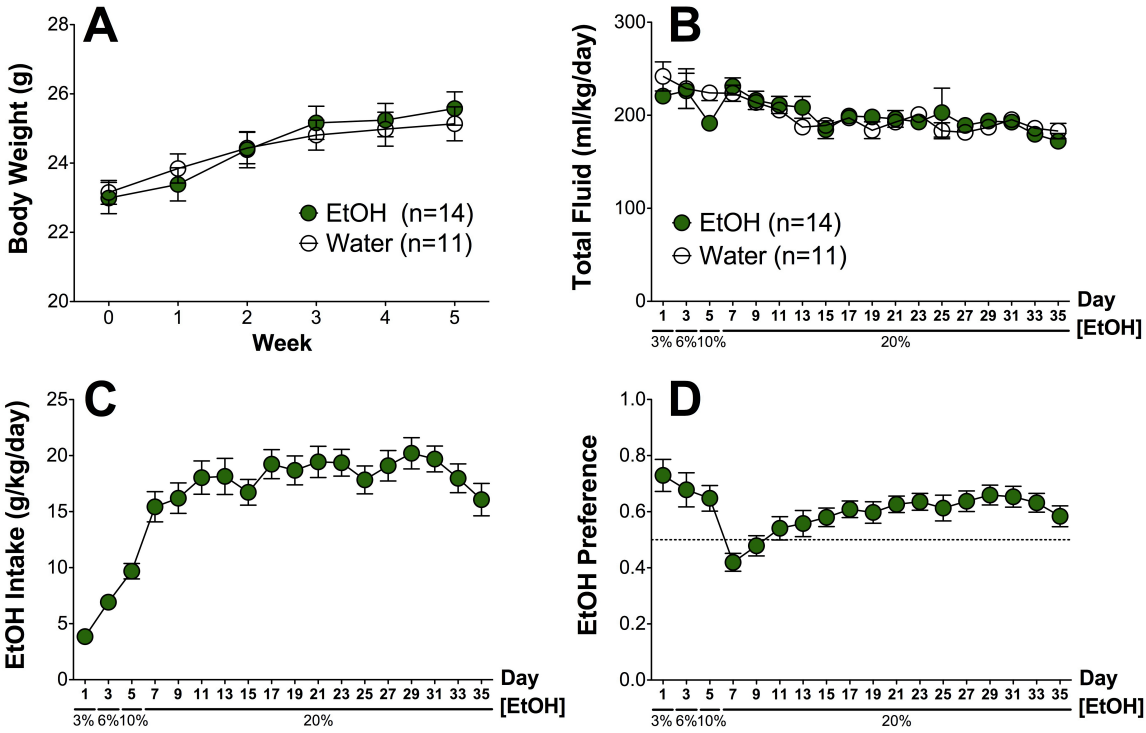


Figure 23. Experiment 4.2 (Access): Long-Term IA Drinking (D37)

Graphs illustrate mean ± SEM values and display individual data points for each group. During the final 4hr drinking session on D37, mice in the EtOH group averaged **(A)** EtOH intakes of 4.68 g/kg, **(B)** a 52.54% EtOH preference ratio, and **(C)** BECs of 78.22 mg/dl. **(D)** EtOH mice consumed significantly less food relative to H2O controls (t-test, **p* < .05). **(E)** Total caloric intake did not differ between groups (*p* = .09). **(F)** EtOH mice received 18.4% of their estimated total caloric intake from EtOH**.**

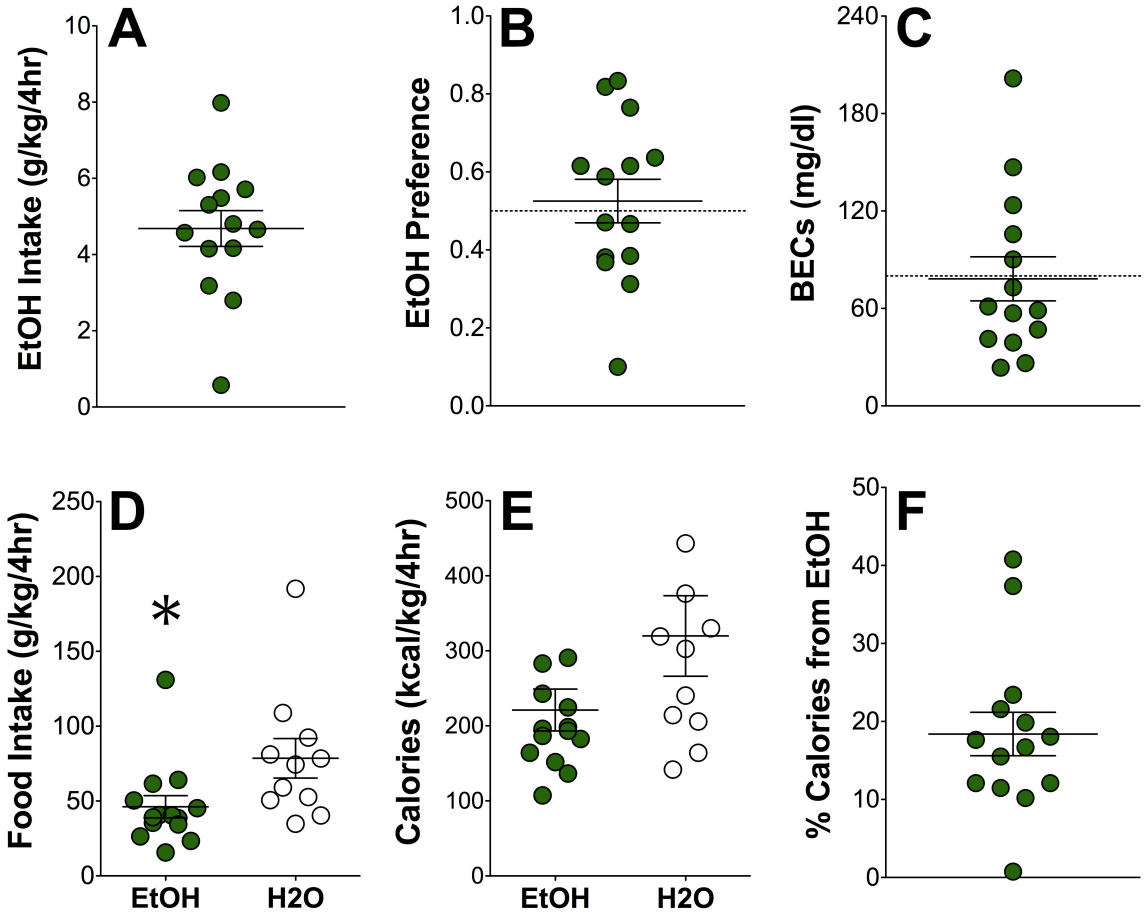
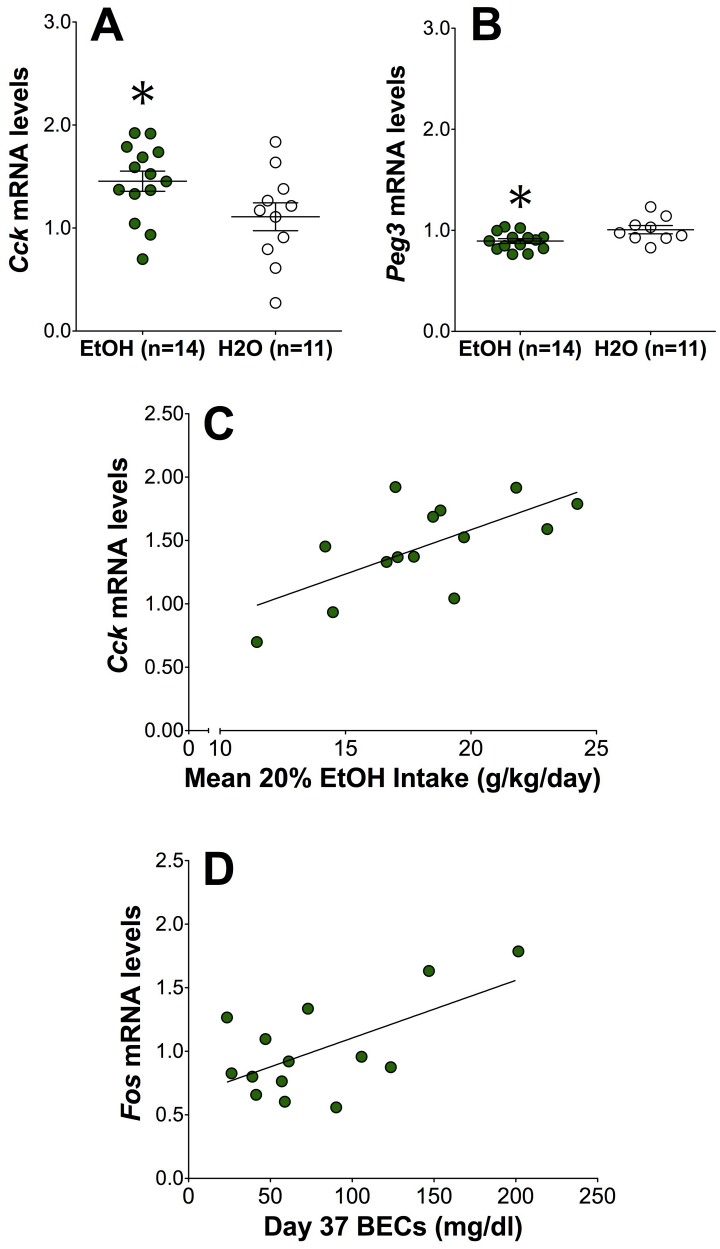


Figure 24. Experiment 4.2 (Access): Gene Expression Findings

Graphs illustrate mean ± SEM values and/or display individual data points for each group. **(A)** *Cck* was upregulated, and **(B)** *Peg3* was downregulated in EtOH vs. H2O mice. **(C)** *Cck* levels were positively correlated with mean daily 20% EtOH intake (r = .664; df = 12; p = .010), and **(D)** *Fos* levels were significantly positively correlated with D37 BECs (r = .615; df = 12; p = .019).



DISCUSSION

Chapter 4 assessed the transcriptional profile of the EWcp following long-term EtOH drinking, finding that several genes were differentially regulated during forced EtOH abstinence (No Access) vs. acute binge-like intoxication (Access). Contrary to my original hypothesis, mRNA levels of *Ucn* were selectively upregulated in the absence of EtOH, but not immediately following an EtOH drinking session. One interpretation of these results is that *Ucn* expression is increased by abstinence from excessive EtOH intake, and then undergoes homeostatic readjustment back to baseline levels following an acute EtOH drinking session. In other words, the persistent high EtOH intakes displayed by B6 mice in the long-term IA procedure could be partially explained by an effort to maintain EWcp-Ucn1 homeostasis. This mechanism could also explain why Ucn1 KO mice were less susceptible to the progressively increased EtOH drinking observed in Chapters 2 and 3.

No Access conditions also increased the expression of several other genes encoding neuropeptides (PACAP [*Adcyap1*], Nesfatin-1 [*Nucb2*]), as well as several genes encoding proteins important for neuropeptide processing (proprotein convertase subtilisin/kexin type 1 [*Pcsk1*]), release (secretogranin-II [*Scg2*]), and signaling (CRFBP, CRF1). These findings allow me to speculate that rates of neuropeptide synthesis and release in the EWcp are particularly enhanced in the absence of EtOH following long-term binge-like drinking. Because EWcp-neuropeptides possess anxiogenic properties, the No Access gene expression profile likely reflects a stress-like condition similar to (yet distinct from) the negative affective state that characterizes withdrawal following EtOH dependence. Although B6 mice rarely show physical withdrawal symptoms during EtOH abstinence, these adaptations may reflect changes in the affective, rather than physical, aspects of acute abstinence. However, in order to establish a relationship between

affective state and EWcp candidate gene expression, comparisons of stress-related behaviors between B6 and D2 mice during EtOH abstinence would be required.

The elevated levels of *Ndn*, *Peg3*, and *Peg10* during No Access are notable, as each transcript is genetically imprinted and expressed only from the male-inherited allele, hinting at sex-specific effects of EtOH on the EWcp. Indeed, estrogen receptor beta is co-expressed with Ucn1 in the EWcp (Derks et al., 2007), and *Ucn* levels fluctuate over the estrous cycle in female rats (Derks et al., 2010). However, analysis of sex differences in EtOH effects on EWcp gene expression was not possible here, as Chapter 4 studies used male mice only.

Experiment 4.1 also revealed that levels of *Drd2* mRNA were inversely correlated with mean daily 20% EtOH intake. *Drd2* (encoding the dopamine receptor 2 subtype; Drd2) is expressed in the midbrain primarily on DA neurons of the VTA and RLi, where it acts as an inhibitory autoreceptor. Thus, the low levels of *Drd2* mRNA found in high-drinking mice during forced abstinence may serve to alleviate a deficit in RLi DAergic transmission caused by long-term EtOH consumption. Interestingly, a recent report from the Ryabinin Lab showed that inhibition of the VTA using a Drd2 agonist induced c-Fos expression in the EWcp (Ryabinin et al., 2013). By raising the possibility that VTA DA neurons exert tonic inhibitory control directly on the EWcp, these data provide evidence for a novel stress-reward link. In this model, EWcp-Ucn1 neurons are inhibited at baseline, but become excited during periods of DAergic dysfunction (for example, following exposure to stress or long-term IA drinking).

Although previous c-Fos studies found that the EWcp was not particularly sensitive to EtOH withdrawal (Kozell et al., 2005), it is notable that *Fos* mRNA during No Access was positively correlated with mean 20% daily EtOH intake. This suggests that EWcp ITF expression can be induced by the absence of EtOH in a dose-dependent

manner, analogous to the effects of acute EtOH (Bachtell et al., 2002b; Sharpe et al., 2005b).

Experiment 4.2 revealed elevated levels of *Cck* mRNA in EtOH vs. H2O mice following Access, and identified a significant positive correlation between *Cck* mRNA levels and mean daily 20% EtOH intake. These findings coincide with higher levels of *Cck* in the EWcp of B6 vs. D2 mice, thereby suggesting that EWcp-CCK has the potential to both regulate and be regulated by EtOH drinking. In such a model, enhanced basal levels of EWcp-*Cck* could predispose B6 mice to high levels of EtOH intake, thereby producing further EtOH-induced elevations in EWcp-*Cck* and perpetuating EtOH intake in a feed-forward manner. Despite these results from the Access experiment, *Cck* levels were not significantly elevated in EtOH mice following No Access, identifying an important distinction between *Cck* and *Ucn*.

Interestingly, *Peg3* was the only transcript significantly altered in both the No Access and Access studies. The opposite direction of *Peg3* adaptations in the two experiments suggests that this gene may be particularly sensitive to acute EtOH exposure and subsequent abstinence. The significant positive correlation between *Fos* mRNA levels and BECs in the Access experiment is consistent with several previous studies that documented a dose-dependent induction of c-Fos in the EWcp following EtOH injection or voluntary drinking (Bachtell et al., 2002b; Sharpe et al., 2005b). Furthermore, this result provides evidence that, like the EWcp response to repeated cocaine exposure, EtOH-induced c-Fos expression in the EWcp does not habituate over the long-term (Spangler et al., 2009).

One minor limitation of the current study is that EtOH mice in the Access experiment did not achieve mean BECs that surpassed the binge threshold (78.2 mg/dl). However, these 14 mice included in the analysis are only a subset of the original 24 that completed the behavioral portion of the study. In this larger group of EtOH mice, mean

BECs were 88.1 mg/dl, indicating that this procedure is capable of producing verifiable binge BECs at this D37 ZT-18 timepoint. This suggests that the inability of EtOH mice to surpass binge threshold in the Access study was simply due to chance exclusion of mice with binge BECs whose EWcp RNA samples did not meet criterion for gene expression analysis. In any case, behavioral data from the complete set of n=24 EtOH mice are nearly identical to the subset of n=14 EtOH mice presented here.

An additional limitation is that these experiments were only capable of assessing the EWcp gene expression profile at two timepoints. Future studies may offer a more dynamic view of the long-term effects of EtOH drinking by investigating changes in EWcp gene expression that persist weeks or months following cessation of EtOH access. Although the No Access findings at the 24hr timepoint are relevant because they reflect the state of the EWcp at the exact point at which mice would normally begin drinking during their next EtOH session, the relatively short-term adaptations occurring at 24hr of abstinence may contrast substantially with long-term changes.

In addition, these long-term IA experiments would benefit from concomitant measures of stress reactivity throughout (i.e. HPA-axis hormone profiles, anxiety-like behavior during No Access, etc.). In terms of trying to compare the effects of this procedure on the EWcp to the effects of EtOH dependence on the extended amygdala CRF system, additional measures of the negative affective state may inform the degree to which long-term IA adaptations correspond to the changes observed following long-term EtOH vapor.

Intriguingly, five of the genes that were upregulated in EtOH vs. H2O mice (*Cck, Postn*, *Ucn*, *Fos,* and *Drd5*) were also upregulated in B6 vs. D2 mice (Chapter 1), providing further justification for investigating their potential roles in mediating EtOH-related behaviors via the EWcp. *Drd5* is a particularly interesting candidate, as Drd5 signaling is critical for the mechanism of cocaine-induced long-term potentiation of

synaptic plasticity that occurs in VTA-DA neurons (Argilli et al., 2008). Therefore, Drd5 likely has similar actions in the RLi, suggesting that the long-term IA procedure produces neuroplasticity-related adaptations in adjacent DA neurons that interact directly with the EWcp.

In summary, these experiments independently assessed the effects of acute binge-like EtOH drinking and subsequent forced EtOH abstinence on EWcp gene expression. Taken with the limitations described, the data highlight a role for CCK in acute EtOH drinking, and implicate roles for PACAP, Nesfatin-1, and Ucn1 in the abstinent, stress-like state following long-term drinking. All four of these neuropeptides possess anxiogenic properties. Therefore, the EWcp is potentially a key neural substrate underlying the effects of stress on EtOH drinking, and conversely, underlying the effects of long-term EtOH drinking on stress reactivity.

CHAPTER 5: Genetic Knockdown in the Centrally Projecting Edinger-Westphal Nucleus: Effects on Long-Term Ethanol Drinking

INTRODUCTION

Findings from Chapters 2 and 3 highlighted a role for EWcp-Ucn1 in excessive EtOH intake, as EWcp lesions decreased EtOH preference in an Ucn1-dependent manner, and Ucn1 KO reduced voluntary EtOH drinking in several paradigms. However, the interpretations of these findings may be accompanied by certain caveats. Electrolytic lesions of cell body populations are also capable of destroying fibers of passage, which could lead to an incorrect conclusion about the relative contributions of the EWcp vs. afferent inputs to non-EWcp neurons. In terms of the constitutive genetic KO approach, compensatory changes in expression of other CRF system ligands (or other stress-related neuropeptides within the EWcp) may counteract the loss of Ucn1, thereby potentially confounding the interpretation of behavioral changes ascribed to Ucn1's function. Although these caveats are unlikely to explain the data produced in Chapters 2 and 3, more targeted approaches may be required to definitively confirm that a specific gene within a specific neuronal population contributes to the studied behavior in the expected manner.

For these reasons, Chapter 5 involved the use of RNA interference (RNAi). Discovered in 1998, RNAi refers to an endogenous mechanism in which short (19-21 base-pair) sequences of DNA are transcribed into non-coding RNAs capable of downregulating gene expression in a transcript-specific manner via complementary mRNA binding and targeting for degradation (Fire et al., 1998). More recently, viral-encoded RNAi was adopted as a research tool to manipulate expression levels of genes of interest (Xia et al., 2002). In combination with traditional behavioral neuroscience techniques, this method can now be applied *in vivo* in an anatomically-specific manner via targeted stereotaxic brain surgery (Hommel et al., 2003).

To pursue viral-mediated RNAi of EWcp candidate genes, I established a collaboration between the Ryabinin Laboratory and the Laboratory of Dr. Alon Chen at

the Weizmann Institute in Rehovot, Israel. The Chen group recently created and validated several lentiviruses that employ RNAi to downregulate specific components of the CRF system. Specifically, the Chen Lab reported substantial stress-related behavioral effects following site-specific central KD of CRF, CRF1, or CRF2 (Elliott et al., 2010; Lebow et al., 2012; Regev et al., 2012; Sztainberg et al., 2011). Here, I describe the effects of EWcp-specific KD of Ucn1 on baseline consummatory variables and excessive EtOH intake in the long-term IA paradigm. The long-term IA procedure was chosen for these studies because Experiment 3.3 specifically identified a 4hr time window in the circadian dark cycle of the long-term IA procedure in which KO of Ucn1 substantially impacted escalation of drinking and binge-level consumption. Furthermore, I chose the long-term IA procedure for these studies in order to complement the gene expression experiments performed in Chapter 4, and to allow comparison with other investigations of the brain CRF system that used this drinking model (Cippitelli et al., 2011; Hwa et al., 2013; Simms et al., 2013).

Based on results from Chapters 2, 3, and 4, I hypothesized that EWcp-specific KD of Ucn1 by lentiviral-mediated RNAi would attenuate the high levels of EtOH drinking observed in the long-term IA procedure. Furthermore, I predicted that these effects would occur independently of any alterations of baseline consummatory behavior (food and H2O intake). I also tested the effects of EWcp-Ucn1 KD on anxiety-like behavior, although mixed results from previous investigations of anxiety-like behavior in Ucn1 KO mice (including those from Chapter 3) limited my ability to form a directional hypothesis about the potential effects.

MATERIALS AND METHODS

Lentiviral Targeting Strategy

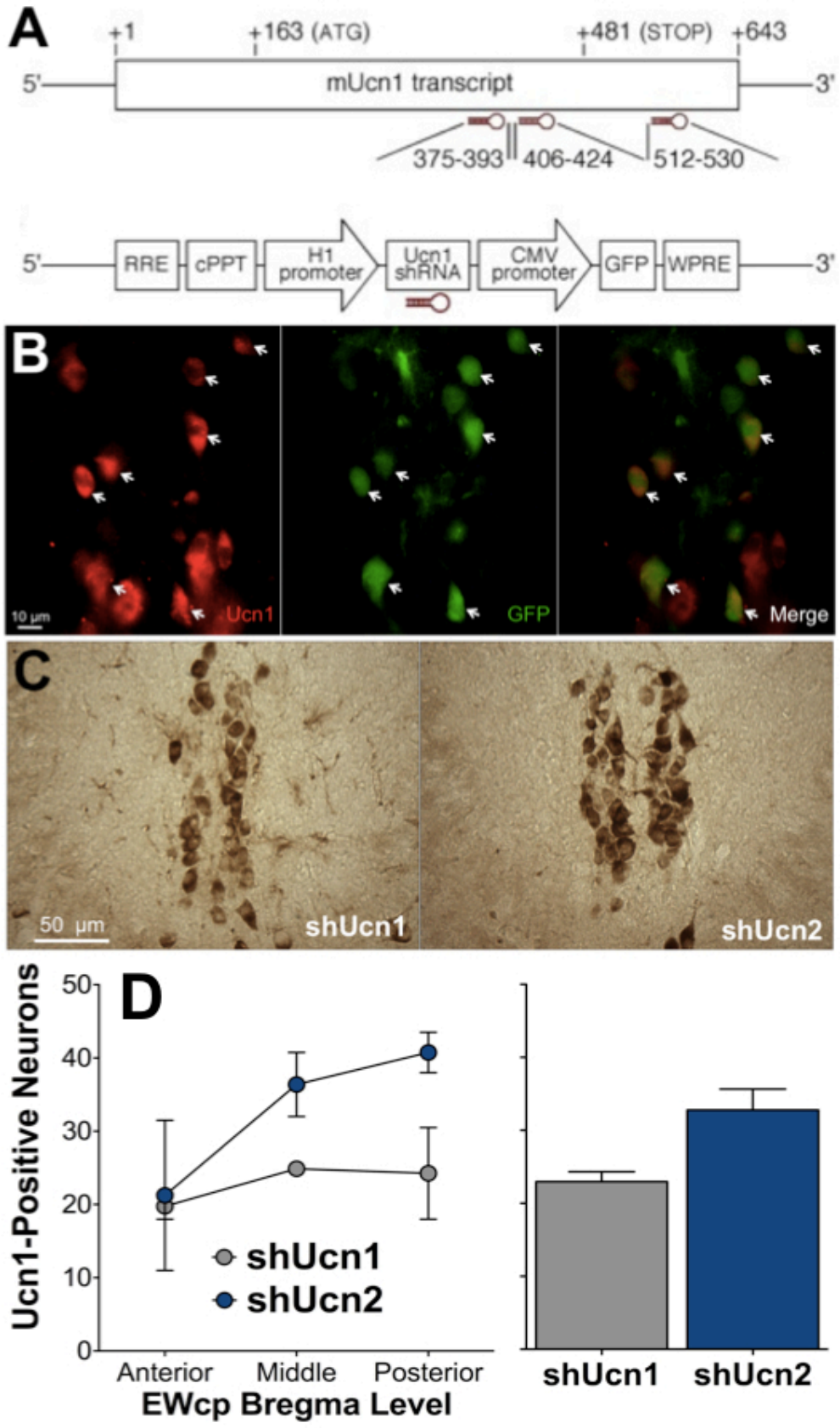
Lentiviruses contained shRNAs targeted against either the mouse *Ucn* or *Ucn2* genes. shRNAs were tagged with GFP and expressed under the constitutive H1 promoter (**Fig. 25A**), similar to previous CRF system KD approaches published by the Chen Laboratory (Elliott et al., 2010; Lebow et al., 2012; Regev et al., 2012; Sztainberg et al., 2011). The *Ucn2*-shRNA was chosen as a control sequence because it is distinct from any sequence that has complementarity to the *Ucn* gene, yet the remainder of the vector is identical between the two viruses. Using the *Ucn* and *Ucn2* viruses in the EWcp is a favorable approach because the EWcp contains only *Ucn* and not *Ucn2*. This approach would also facilitate comparison to studies of *Ucn2* KD in brain regions where the *Ucn* shRNA would serve as a control (for example, in the LC, where *Ucn* is not expressed, but *Ucn2* is abundant).

Animals and Surgical Procedures

Adult male B6 mice arrived from JAX-West and were initially group housed in a temperature- and humidity-controlled environment with *ad libitum* access to food (LabDiet 5001; LabDiet, Richmond, IN, USA) and H2O. Only male mice were used because studies in Chapter 3 failed to find consistent sex differences in EtOH drinking between genotypes (although it is unclear whether group numbers per sex per genotype were sufficient to consistently identify sex differences, were they present). Mice were anesthetized by isofluorane, received 0.3 ml s.c. Carprofen, and underwent stereotactic lentiviral infusion surgery. A small hole was drilled in the left skull at -3.5 mm (A/P) and +1.0 mm (M/L) from bregma. A borosillicate glass micropipette injector was lowered 4.3 mm into the brain at a 15° angle, terminating in the middle EW at a depth of 3.9 mm beneath the skull (D/V), along the midline. One μl of the Ucn1 shRNA virus (shUcn1) or

Figure 25. Lentiviral-Mediated EWcp-Ucn1 KD by RNA Interference

Graphs illustrate mean ± SEM values. **(A)** Lentiviral targeting strategy, in which a 19 base-pair shRNA with a sequence complementary to the mouse *Ucn* gene was tagged with GFP and expressed under control of the constitutive H1 promoter. Control lentivirus is identical, except the 19 base-pair shRNA is complementary to the mouse *Ucn2* gene. **(B)** Three weeks following lentiviral surgery, Ucn1-positive neurons within the EWcp were infected with the shUcn1 virus, indicated by co-localization of GFP with Ucn1 (white arrows). **(C)** IHC photomicrographs demonstrated lower Ucn1-IR in the EWcp of shUcn1- vs. shUcn2-injected mice. **(D)** Quantification of Ucn1-positive neurons revealed lower IR in shUcn1 vs. shUcn2 mice at the protein level in the EWcp. (Bar graph represents average across all three bregma levels of the EWcp).


A
+1
+163 (ATG)
+481 (STOP)
+643
5'
mUcn1 transcript
3'
375-393
406-424
512-530
5'
RRE
cPPT
H1 promoter
Ucn1 shRNA
CMV promoter
GFP
WPRE
3'
B
10 μm
Ucn1
GFP
Merge
C
50 μm
shUcn1
shUcn2
D
Ucn1-Positive Neurons
50
40
30
20
10
0
shUcn1
shUcn2
Anterior
Middle
Posterior
EWcp Bregma Level
shUcn1
shUcn2

the Ucn2 shRNA virus (shUcn2) was infused via a 5 μl Hamilton syringe connected to the injector via plastic tubing over the course of 5 mins, and the injector remained in place for an additional 2 mins following infusion. Mice initially recovered for 1 hr in a fresh warm cage placed on a heating pad prior to being returned to the colony (single-housed). All protocols were approved by the OHSU IACUC and were performed with adherence to the NIH Guidelines for the Care and Use of Laboratory Animals.

General Behavioral Procedures

For both EtOH drinking and anxiety-like behavior studies, mice were moved to the experimental room (reverse 12hr/12hr dark-light cycle; ZT-12 = 0800h) 5-10 days following surgical procedures, and acclimated to these conditions for two additional weeks prior to the experiment start. During acclimation, weekly measures of body weight, H2O, and food allowed calculation of baseline consummatory variables. Preliminary RM-ANOVA identified no significant interactions with the factor of week, thus data were collapsed across weeks and compared between viral groups by t-test.

Long-Term IA EtOH Drinking

For this study, 35 mice received lentiviral surgery (n=20 shUcn1, n=15 shUcn2). Following the EtOH-free baseline, a small cohort of mice was euthanized at 3 weeks post-surgery for pilot analyses of EWcp-Ucn1 KD (n=2-3 per viral group). Remaining mice (n=13-17 per group) underwent the long-term IA procedure described in Experiment 4.2, with n=2 per viral group serving as EtOH-naïve H2O control animals, leaving n=11-15 per group in the EtOH-exposed cohort. On Day 33 (D33) of the IA protocol, mice were euthanized by CO2 at ZT-18, trunk blood was collected for later analysis of BECs by Analox, and brains were dissected and placed in 2% PFA in PBS for later IHC processing as described below.

ZT-18 was chosen as the timepoint of euthanasia in this study because Experiment 4.2 showed that ZT-18 is a timepoint at which Ucn1 levels are not affected by recent EtOH exposure. By using this timepoint, I avoided a potential confound in attempting to verify EWcp-Ucn1 KD in groups that could be consuming different amounts of EtOH, since 24hr abstinence from long-term IA drinking alters *Ucn* mRNA expression (Experiment 4.1). For analysis of IA behavioral data, I performed RM-ANOVA across all EtOH days (between-subjects factor of virus, with day as the repeated measure). For analysis of data from D33, 4hr variables were compared between viral groups by t-test.

Tissue Processing

Brains were rapidly dissected and stored in 2% PFA in PBS overnight. Brains were then transferred to 20% sucrose in PBS for 24 hours prior to being stored in 30% sucrose in PBS for 24-96 hours prior to being sliced in 30 μm sections on a Leica cryostat. Sections from the EWcp were collected in 0.1% NaN3 in PBS for later IHC staining and verification of adequate lentiviral infection. Midbrain slices were stained for Ucn1 using the Phoenix antibody (1:5000) and underwent secondary labeling with either the standard DAB method (mice from the pilot study at 3 weeks post-surgery), or with the Alexa-555 fluorescent antibody (mice from the IA study at 8 weeks post-surgery), as previously described (Spangler et al., 2009).

KD Analyses at the Protein Level

Only subjects with extensive GFP labeling within the EWcp were included in KD analyses (4/5 for the pilot subjects, 25/30 for the IA study subjects). For pilot KD analysis at 3 weeks post-surgery in mice with extensive GFP labeling in the EWcp (n=2 per group), I counted the number of DAB-stained Ucn1-positive cells in the EWcp (4-6 EWcp sections per subject). For analysis of KD at 8 weeks post-surgery in mice with extensive

GFP labeling in the EWcp (EtOH mice, n=9-12/group; H2O control mice, n=2/group), I obtained 20x photomicrographs of EWcp-Ucn1 immunofluorescence (4-6 EWcp sections per subject), and used ImageJ to determine the density of Ucn1-IR.

Brightness and exposure settings were matched for all slices across all subjects, and data were analyzed under blinded conditions. Measures of EWcp-Ucn1 KD were analyzed by RM-ANOVA (between-subjects factor of virus, with EWcp bregma level as the repeated measure). Anterior, middle, and posterior EWcp were defined as -3.3 to -3.4, -3.5 to -3.6, and -.3.7 to -3.9 mm from bregma, respectively. Due to the RM-ANOVA design, only mice with viable sections across each of the three bregma levels were included in the analyses.

The first completion of the KD analysis in EtOH mice from the IA study yielded complete sets of tissue from 4-7 per viral group. Attrition of subjects from the original group sizes (9-12 per group) was primarily due to lack of slices chosen for the stain that represented the entire rostral-caudal axis of the EWcp (i.e., two anterior and three middle slices, but no posterior slices). In a few isolated cases, slices were of imperfect quality due to difficulties during cryostat slicing, slice mounting, or slide coverslipping. Importantly, slices were always of sufficient quality to clearly detect whether extensive GFP labeling reached the EWcp, but this did not necessarily mean the slice was also suitable for IR density analysis. Due to these difficulties in the first experiment, I replicated the IHC experiment in the EtOH mice from the IA study using a more thorough method for collecting slices across the entire length of the EWcp, and performed an additional analysis on tissue from a larger subset of animals (n = 7-10 per group).

Baseline Anxiety-Like Behavior

This study used 16 male B6 mice that received surgery as described above (n=8 per viral group), and were tested in the two models of anxiety-like behavior described in

Experiment 3.8. Singly-housed mice were moved to a dark experimental room and allowed to habituate for 1hr prior to testing on each day. Testing occurred between ZT-14 and ZT-18, but was otherwise identical to that described in Experiment 3.8 (n=4 per order, per viral group). Post-mortem analysis revealed that all subjects displayed extensive GFP labeling within the EWcp, thus all subjects were included in the behavioral analysis. Data were analyzed by two-way ANOVA with between-subjects factors of virus and order, and Bonferroni post-hoc comparisons performed between viral groups in the presence of significant virus x order interactions.

RESULTS

EWcp-Ucn1 KD at 3 Weeks Post-Surgery

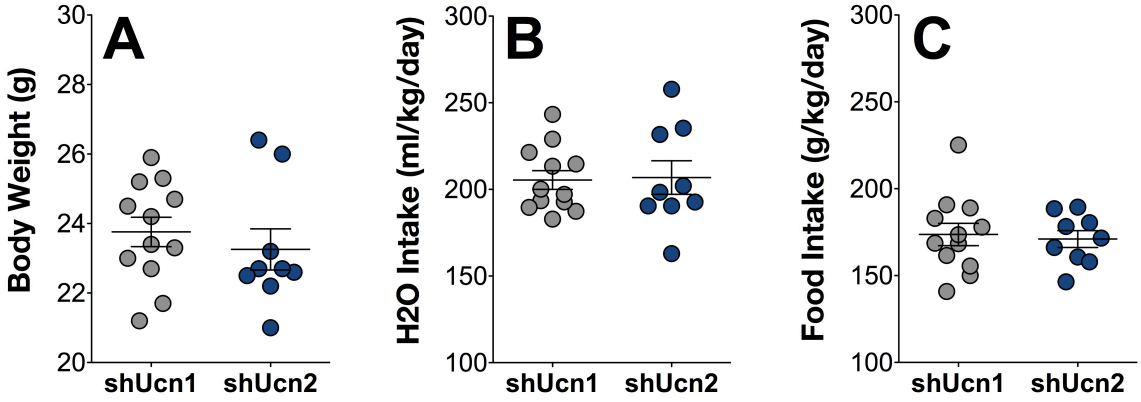
Pilot subjects with infusions into the ventromedial periaqueductal gray displayed Ucn1-IR and GFP-IR within the same multipolar cell bodies, indicating EWcp-specific lentiviral infection (**Fig. 25B**). Representative photomicrographs and quantitative cell counts showed that shUcn1-infected mice displayed a 29.9% reduction in EWcp-Ucn1 protein expression relative to viral control mice, suggesting successful KD despite my inability to perform statistical analysis due to low group numbers (n = 2/group; **Fig. 25C-D**).

Effects of EWcp-Ucn1 KD on Baseline Consummatory Behavior

During the two-week habituation period (beginning 5-10 days following lentiviral surgery), shUcn1 and shUcn2 mice did not differ in body weight, H2O intake, or food intake (**Fig. 26A-C**).

Figure 26. Effects of EWcp-Ucn1 KD on Baseline Consummatory Behavior

Graphs illustrate mean ± SEM values and individual data points. During the two-week reverse light/dark cycle habituation period, shUcn1 (n=12) and shUcn2 (n=9) mice did not differ in **(A)** body weight, **(B)** H2O intake, or **(C)** food intake.



Effects of EWcp-Ucn1 KD on Long-Term IA Drinking

In the long-term IA experiment, viral groups did not differ significantly in body weight or total fluid intake (**Fig. 27A-B**). Lentiviral KD of EWcp-Ucn1 significantly reduced EtOH intake and preference overall (n = 9-12 per group, main effects of virus; both $F_{1,285}$ > 5.35; p < .05; **Fig. 27C-D**). Virus did not significantly impact food intake, total caloric intake, or percent calories consumed from EtOH (**Fig. 27E-G**).

Effects of EWcp-Ucn1 KD on Long-Term IA Drinking (Day 33)

Analysis of the final 4hr session on D33 revealed no significant group differences in EtOH intake, preference, or BECs (**Fig. 28A-C**). Further analysis of D33 data revealed no significant group differences in total fluid intake, food intake, total caloric intake, or percent calories consumed from EtOH (**Fig. 28D-G**).

EWcp-Ucn1 KD at 8 Weeks Post-Surgery

Representative photomicrographs of anterior and posterior EWcp slices demonstrated reduced Ucn1-IR among shUcn1 vs. shUcn2 mice (**Fig. 29A**). Quantitative measures showed that the density of EWcp-Ucn1-IR was significantly decreased (44.1% reduction) in shUcn1 vs. shUcn2 mice, indicating that lentiviral KD remained effective in mice exposed to long-term IA EtOH drinking and euthanized 8 weeks later (n=4-7 per group, main effect of virus, $F_{1,18}$ = 7.19; p = .025; **Fig. 29B**). Replication of the Ucn1 IHC experiment confirmed effective KD at 8 weeks post-surgery in a larger cohort of EtOH-exposed mice (n = 7-10 per group; $F_{1,30}$ = 4.99; p = .041; **Fig. 29C**). I also compared Ucn1-IR between the groups of mice that served as EtOH-naïve H2O controls in the long-term IA study. Density of Ucn1-IR was decreased in these shUcn1 vs. shUcn2 mice, but low group numbers prevented reliable statistical analysis (**Fig. 29D**).

Figure 27. Effects of EWcp-Ucn1 KD on Long-Term IA Drinking

Graphs illustrate mean ± SEM values. **(A-B)** Lentiviral KD of Ucn1 had no significant effect on body weight or total fluid intake over the course of the long-term intermittent EtOH drinking experiment. **(C-D)** Reduction of EWcp-Ucn1 levels by lentiviral KD significantly reduced EtOH intake and EtOH preference (*main effect of virus; $p < .05$). **(E-G)** EWcp-Ucn1 KD did not alter food intake, total caloric intake, or the percent of calories consumed from EtOH.

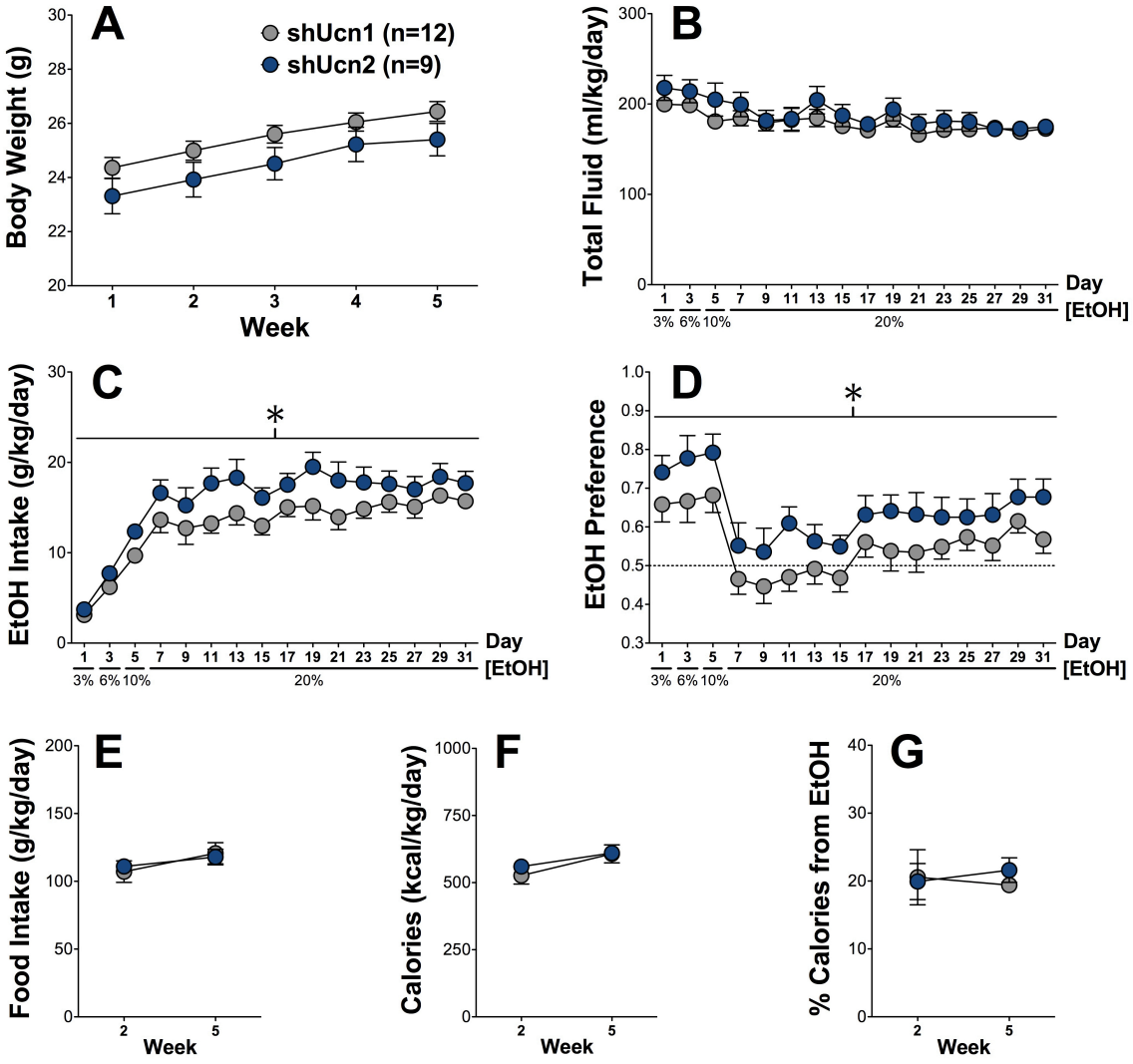


Figure 28. Effects of EWcp-Ucn1 KD on Long-Term IA Drinking (D33)

Graphs illustrate mean ± SEM values and individual data points. During the 4hr session on D33, shUcn1 and shUcn2 mice did not differ in **(A)** EtOH intake, **(B)** EtOH preference, **(C)** BECs, **(D)** total fluid intake, **(E)** food intake, **(F)** total caloric intake, or **(G)** percent calories from EtOH.

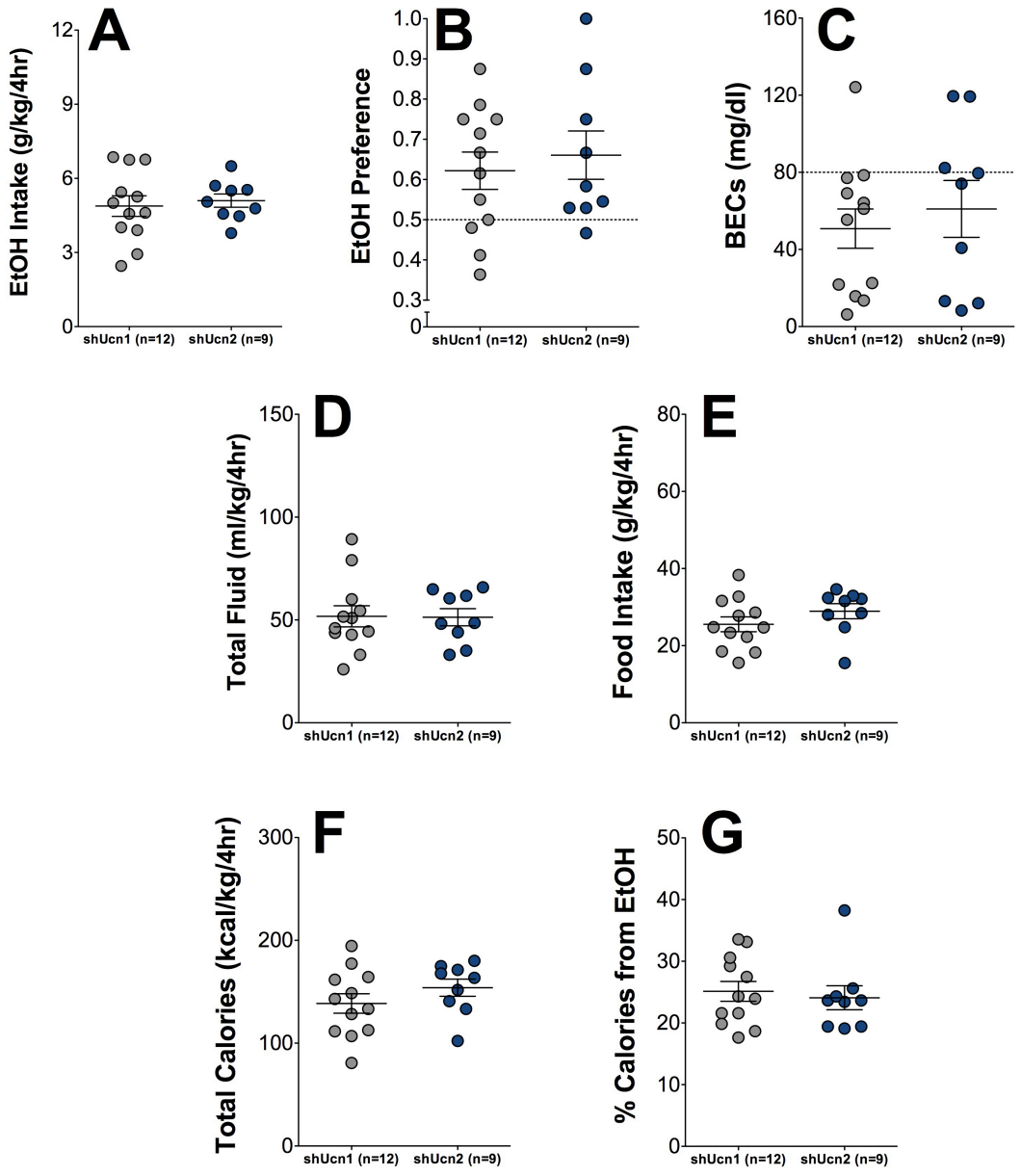
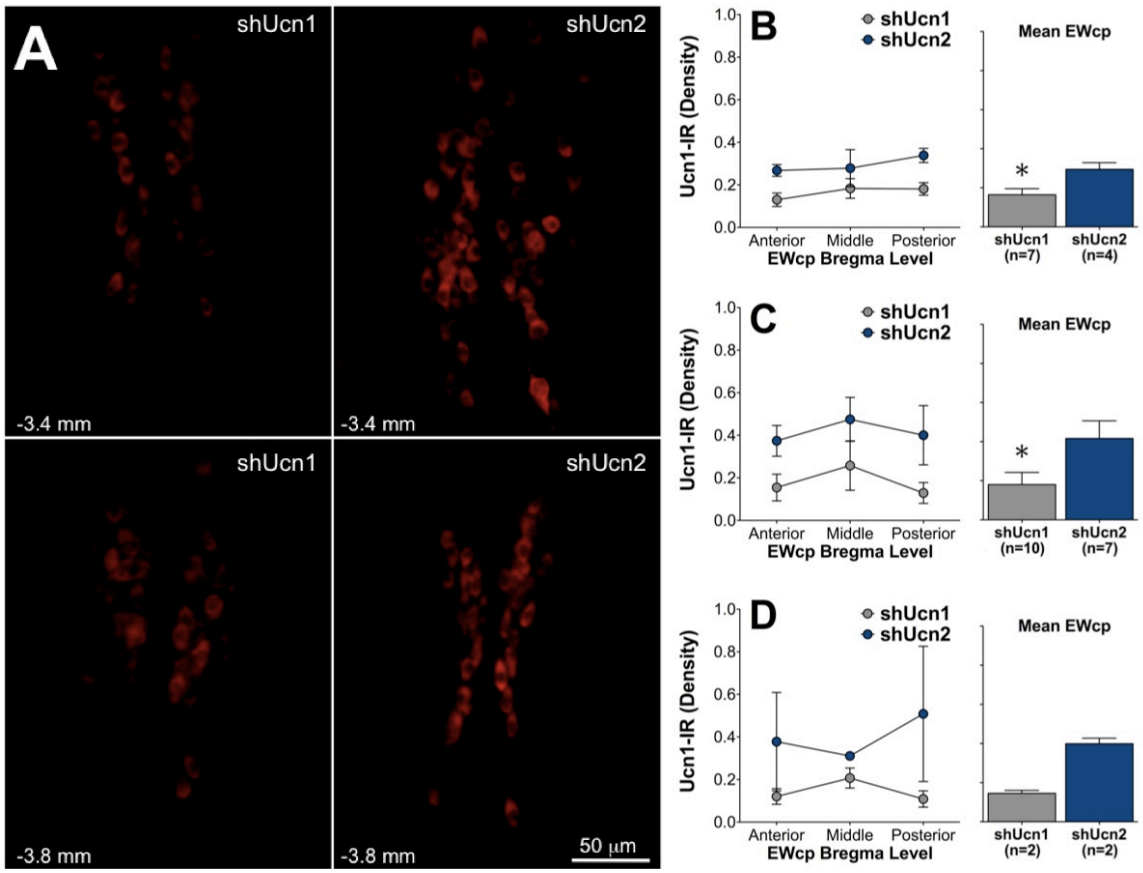


Figure 29. EWcp-Ucn1 KD at 8 Weeks Post-Surgery

(A) Representative photomicrographs of the EWcp taken at 20x resolution following long-term IA EtOH drinking demonstrate reduced Ucn1-IR at two different bregma levels in the EWcp of shUcn1 vs. shUcn2 mice. **(B)** Results of first IHC experiment showing reduced Ucn1-IR in EtOH-drinking shUcn1 vs. shUcn2 mice (n=4-7/group). **(C)** Results of second IHC experiment showing reduced Ucn1-IR in shUcn1 vs. shUcn2 mice (n=7-10/group). **(D)** EWcp-Ucn1 KD analysis in EtOH-naïve H2O control animals from the long-term IA study also revealed lower Ucn1-IR density in shUcn1 vs. shUcn2 mice (n=2/group). All graphs illustrate mean ± SEM values, bar graphs represent averages across all three bregma levels of the EWcp. *main effect of virus; $p < .05$.



Effects of EWcp-Ucn1 KD on Anxiety-Like Behavior (EPM)

Mice were tested in the EPM and LDB in a counterbalanced order (n=4 per order, per viral group). In the EPM, open arm latency and time spent in the open arm revealed no significant main or interacting effects (**Fig. 30A-B**). However, analyses of open arm entries and percent entries into the open arm revealed differences between viral groups that depended on the order of testing (virus x order interaction; both $F_{1,12} > 4.77$; $p < .05$). Post-hocs confirmed that open arm entries and percent entries into the open arm were significantly increased in shUcn1 vs. shUcn2 mice, but only in those that received the EPM following the LDB (**Fig. 30C-D**). Closed arm entries were significantly increased in mice that received the EPM after the LDB, but this did not differ by viral group (main effect of order; $F_{1,12} = 5.85$; $p = .032$; **Fig. 30E**). No significant main or interacting effects were observed in analyses of remaining EPM variables (**Fig. 30F-H**).

Effects of EWcp-Ucn1 KD on Anxiety-Like Behavior (LDB)

In the LDB, analysis of latency to enter the light revealed no significant effects (**Fig. 31A**). However, analysis of light time found that shUcn1 mice spent significantly more time in the light, relative to shUcn2 mice, regardless of order of testing (main effect of virus; $F_{1,12} = 4.96$; $p = .046$; **Fig. 31B**). Mice that received the LDB first also spent more time in the light overall, although this effect did not significantly interact with virus (main effect of order; $F_{1,12} = 19.42$; $p = .0009$). Mice who received the LDB first also made more light entries and more total transitions (**Fig. 31C-D**), although neither of these effects significantly interacted with virus (main effects of order; both $F_{1,12} > 5.12$; $p < .05$). No significant effects were observed for grooming (**Fig. 31E**). Mice that received the LDB first reared significantly more than mice that received the LDB second, regardless of viral group (main effect of order; $F_{1,12} = 80.39$; $p < .0001$; **Fig. 31F**).

Figure 30. Effects of EWcp-Ucn1 KD on Anxiety-Like Behavior (EPM)

In the EPM, **(A)** latency to enter the open arm and **(B)** open arm time did not differ between shUcn1 and shUcn2 mice. **(C)** Open arm entries and **(D)** the percent of open arm entries were greater in shUcn1 vs. shUcn2 mice, but only when they received the EPM second. **(E)** Closed arm entries were significantly greater in mice that received the EPM second, but did not differ by viral group. **(F-H)** Viral groups did not differ in head dips, grooming bouts, or rearing. Graphs illustrate mean ± SEM values, n=4 per order, per viral group. Asterisks indicate presence of significant virus x order interaction and significant Bonferroni comparison between shUcn1 and shUcn2 mice that received the EPM second (*$p < .05$). Pound sign indicates main effect of order (#$p < .05$).

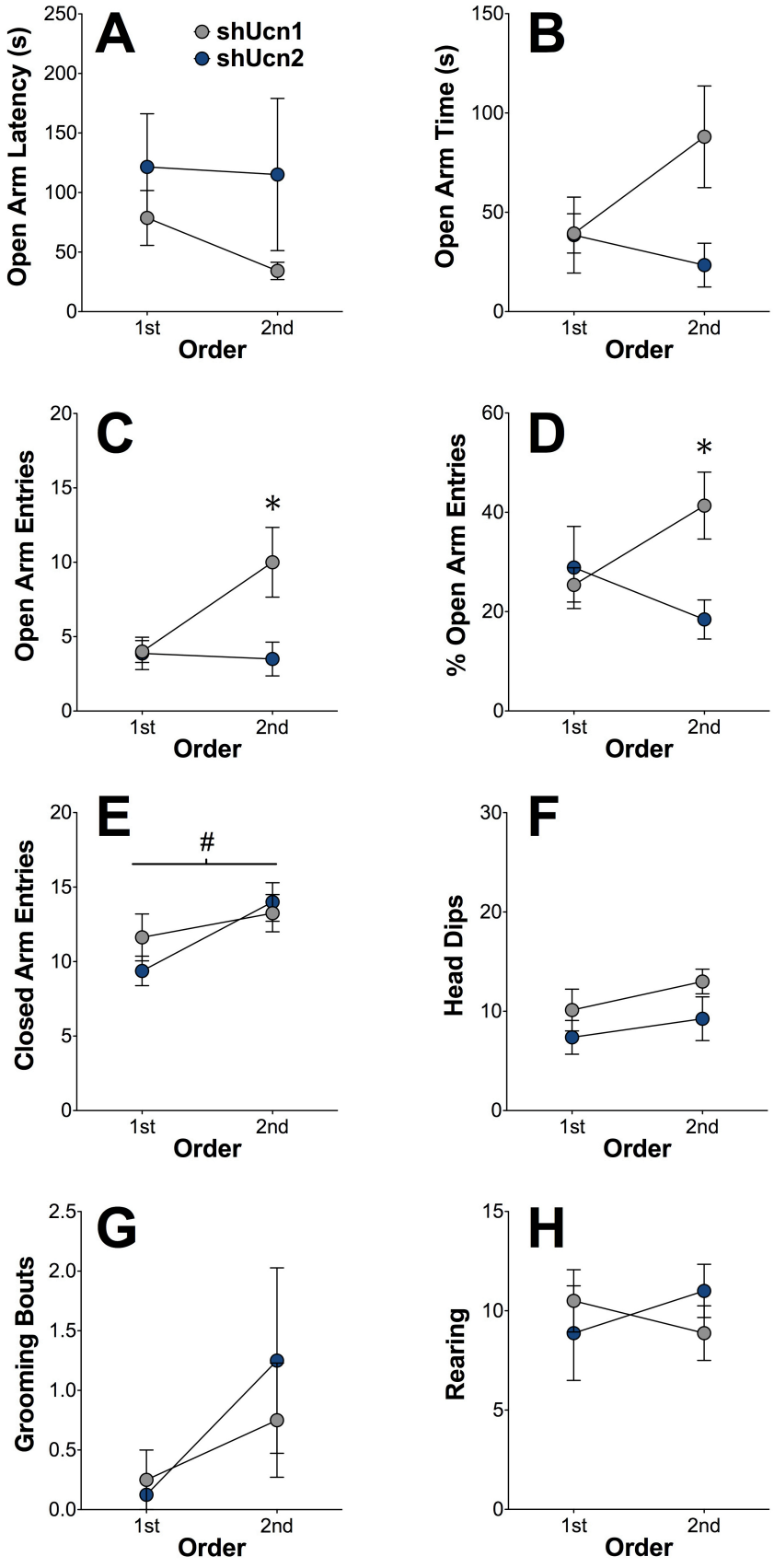
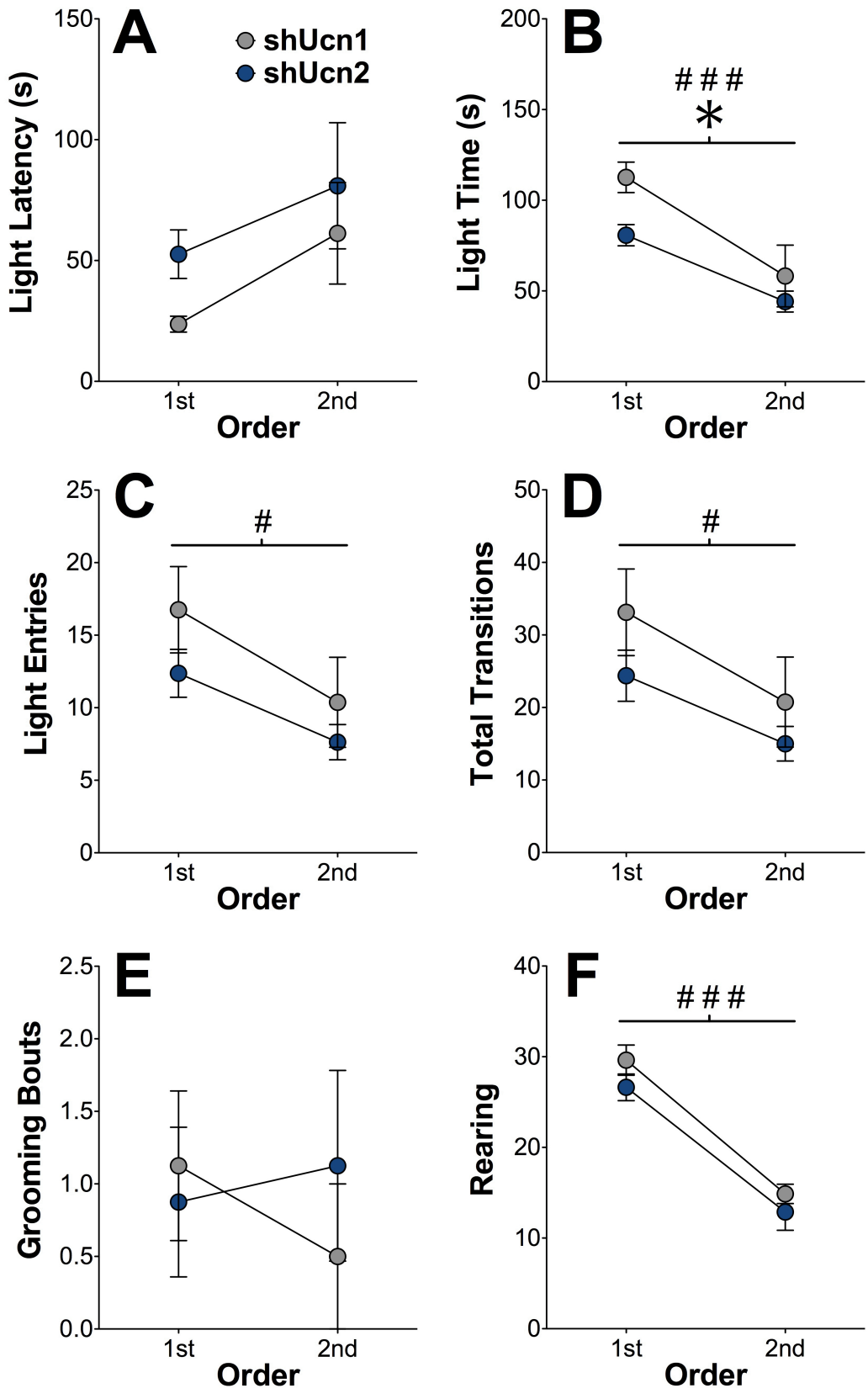

A
shUcn1
shUcn2
Open Arm Latency (s)
250
200
150
100
50
0
1st
2nd
Order
B
Open Arm Time (s)
150
100
50
0
1st
2nd
Order
C
Open Arm Entries
20
15
10
5
0
*
1st
2nd
Order
D
% Open Arm Entries
60
40
20
0
*
1st
2nd
Order
E
Closed Arm Entries
20
15
10
5
0
#
1st
2nd
Order
F
Head Dips
30
20
10
0
1st
2nd
Order
G
Grooming Bouts
2.5
2.0
1.5
1.0
0.5
0.0
1st
2nd
Order
H
Rearing
15
10
5
0
1st
2nd
Order

Figure 31. Effects of EWcp-Ucn1 KD on Anxiety-Like Behavior (LDB)

In the LDB, **(A)** latency to enter the open arm and **(B)** open arm time did not differ between shUcn1 and shUcn2 mice. **(C)** Open arm entries and **(D)** the percent of open arm entries were greater in shUcn1 vs. shUcn2 mice, but only when they received the EPM second. **(E)** Closed arm entries were significantly greater in mice that received the EPM second, but this did not differ by viral group. **(F-H)** Viral groups did not differ in head dips, grooming bouts, or rearing behavior. Graphs illustrate mean ± SEM values, n=4 per order, per viral group. Asterisk indicates significant main effect of virus (*p < .05). Pound signs indicate main effects of order (#p < .05, ###p < .0001).


A
shUcn1
shUcn2
Light Latency (s)
150
100
50
0
1st
2nd
Order
B
###
*
Light Time (s)
200
150
100
50
0
1st
2nd
Order
C
#
Light Entries
25
20
15
10
5
0
1st
2nd
Order
D
#
Total Transitions
50
40
30
20
10
0
1st
2nd
Order
E
Grooming Bouts
2.5
2.0
1.5
1.0
0.5
0.0
1st
2nd
Order
F
###
Rearing
40
30
20
10
0
1st
2nd
Order

DISCUSSION

Prior optimization of stereotaxic targeting led to successful EWcp viral infection in a high percentage of animals following surgery. The majority of mice lacking EWcp infection displayed a complete absence of GFP in the brain, likely due to improper ejection of the viral solution from the glass micropipette, caused by the presence of air bubbles in the plastic tubing connected to the Hamilton syringe.

Pilot studies of EWcp-Ucn1 KD at 3 weeks post-surgery observed reduction of protein levels by ~30% (n=2/group). Analysis of EtOH-naïve mice that went through the IA experiment as H2O controls and were euthanized at 8 weeks post-surgery also revealed reduction of EWcp-Ucn1 protein levels (**Fig. 29D**), suggesting that KD was effective long-term. EWcp-Ucn1 levels were significantly reduced by ~40% at 8 weeks post-surgery in mice that underwent the long-term IA EtOH drinking study, providing further evidence of potent and long-lasting KD. To provide a more thorough analysis of KD in a larger EtOH-naïve cohort, *Ucn* mRNA levels are currently being compared between shUcn1 and shUcn2 mice using qPCR following tissue punch microdissection of the EWcp. Importantly, EWcp-Ucn1 KD had no effects on body weight or baseline H2O and food intake in the absence of EtOH, confirming than any effects of the shUcn1 virus on EtOH drinking behavior could not be attributed to an overall effect on consummatory behavior.

Consistent with several converging lines of evidence, EWcp-specific KD of Ucn1 reduced EtOH intake and preference. RM-ANOVA identified significant main effects of virus that did not significantly interact with day, indicating lower EtOH consumption in shUcn1 mice overall. However, upon visual inspection of the data, the effects appear to be driven primarily by differences across Days 7-21, and the magnitude of the effect on intake appears to wane over the final 1-2 weeks of the study. No differences were identified between viral groups on D33, suggesting that any effects of virus had

dissipated by this timepoint. Because lentiviral vectors have the ability to integrate into the DNA, KD effects are expected to be permanent. Mice transduced with lentiviral KD vectors can pass on the fully-functioning viral DNA to their progeny (Tiscornia et al., 2003). In other words, a decrease in viral effectiveness over time is not a likely explanation for these time-dependent effects on EtOH consumption. Thus, although effects of KD were long-lasting, adaptations in other EWcp neuropeptide systems may have compensated for the loss of Ucn1, thereby obscuring any viral group differences by the fifth week of EtOH drinking.

With regard to compensations, one potential candidate is CART, which is also highly expressed in the EWcp and known to mediate voluntary EtOH intake (Salinas et al., 2012). However, Ucn1 KO mice do not display elevated EWcp-CART-IR relative to WT littermates, suggesting that this adaptation also does not occur following EWcp-Ucn1-KD. Nevertheless, Chapter 4 revealed EtOH-induced upregulation of several neuropeptide-related genes besides *Ucn*, any of which could undergo changes in a manner intimately tied to Ucn1 tone. For example, Experiment 4.2 specifically implicated *Cck* as an EWcp candidate gene regulated by binge-like EtOH intake. Although I have yet to examine whether CCK levels are upregulated in the EWcp of Ucn1 KO or KD mice, current studies in the Ryabinin Lab are addressing the role of CCK in the long-term IA procedure and anxiety-like behavior by adopting a viral-mediated RNAi approach, similar to that used to address Ucn1.

One potential limitation of the long-term IA study performed here is that mice in neither viral group reached mean BECs that surpassed the binge threshold. Despite mean 4hr EtOH intakes of ~5 g/kg on D33, only 1-3 mice in each viral group surpassed the binge criterion. Reasons for these lower-than-expected levels of intoxication could be related to the viral surgical procedures that these mice experienced, which is the main distinction between studies in Chapter 5 and those throughout the rest of the

Dissertation. Although all mice appeared healthy following surgery (normal motor activity and steady increases in body weight over time), viral challenge in the EWcp could have deleterious effects that impact the likelihood of drinking binge-like doses of EtOH.

With this in mind, the strongest pieces of evidence from the Chapter 3 studies supporting a role for Ucn1 in EtOH drinking were provided by Experiment 3.2 (in which intakes of 20% and 40% EtOH were reduced substantially by Ucn1 KO), and by Experiment 3.3 (in which Ucn1 WT but not KO mice surpassed the binge criterion following a 20% EtOH drinking session). Although intake of 40% EtOH occurred in the 2-BC CA procedure when drinking is more spread out over the course of the day, it is likely that the WT mice consuming ~23 g/kg/day of 40% EtOH were reaching the binge threshold at some point during the day. This suggests that Ucn1 may only be recruited by EtOH drinking when binge levels are reached repeatedly over time. Indeed, this could explain the lack of effect of Ucn1 KO on non-escalating 2-BC 10% CA drinking (Experiment 3.4), as well as the lack of effect of EWcp-Ucn1 KD in this Chapter, where mice failed to reach the binge threshold for unknown reasons.

A similar idea has been put forth to explain the variable involvement of the CRF1 receptor in binge EtOH consumption (i.e., effectiveness of CRF1 antagonists in drinking-in-the-dark when intakes are high, but not in drinking-in-the-light when intakes are low). Although the data to support this hypothesis are currently lacking, it is an interesting concept worth pursuing, and could explain why EWcp-Ucn1 KD did not significantly reduce BECs on D33 of this study.

The results from studies of EWcp-Ucn1-KD on anxiety-like behavior resolved a longstanding dispute on the role of Ucn1 in anxiety by showing that shUcn1 mice are relatively anxiolytic compared to controls, indicating that endogenous Ucn1 therefore facilitates anxiety-like behavior. This effect appears to differ from the previous reports in Ucn1 KO mice, and also differs from my own findings in Ucn1 KO mice, where I

observed increased exploration in mice lacking Ucn1. Although effects of KD in the EPM depended on order of testing (as significant effects were only observed in the half of mice with previous experience on the LDB), results from the LDB showed that shUcn1 mice spent more time in the light side of the box relative to controls, irrespective of the testing order. Importantly, these mice were tested in the anxiety behavior paradigms at ~3-4 weeks post-surgery. This is a timepoint where effective KD appears to be present, and also a timepoint at which shUcn1 and shUcn2 mice differ in EtOH drinking.

However, these effects should be interpreted with caution, as repeated testing can have unexpected effects on subsequent behavior. With this in mind, it is worth noting that analyses of anxiety-like behavior in Chapter 3 did not detect any significant interactions with order of testing, indicating Ucn1 KO mice displayed increased exploration in both the EPM and the LDB, regardless of their prior experience. Nevertheless, the anxiolytic-like phenotype observed in both the EPM and LDB is consistent with the broader framework implicating Ucn1 in the extrahypothalamic stress response. The similar effects of EWcp-Ucn1-KD on anxiogenesis and EtOH drinking suggest that these behavioral processes are regulated by overlapping neural systems, and that they may influence each other via this common neural substrate. As alluded to in the Discussion of Chapter 4, assessments of HPA markers, stress-related behaviors, and EWcp gene expression profiles throughout the course of the IA procedure would provide useful information on the effects of long-term IA drinking on the potential emergence of a negative affective state at protracted periods of abstinence. By combining such an approach with the EWcp-Ucn1 KD technique, one could theoretically test whether increases in HPA hormones, stress reactivity, or EWcp gene expression following abstinence from the IA procedure could be attenuated by lentiviral reduction of EWcp-Ucn1 levels.

A related question that remains from these experiments is the extent to which KD of EWcp-Ucn1 has similar or distinct effects compared to KD of other stress-related neuropeptides within the EWcp. Data collected in mice that received intra-EW viral manipulations of CCK levels have not identified a consistent relationship with anxiety-like behavior, although these studies are still in preliminary stages. Future work on the roles of individual EWcp neuropeptide components in driving the high intake phenotype observed in the long-term IA paradigm and the anxiogenic phenotype observed in the EPM and LDB will advance our knowledge of midbrain neuropeptide circuits driving the relationship between stress and excessive EtOH drinking. This information may eventually provide additional strategies for treatment of psychiatric conditions of negative affect and substance abuse.

GENERAL DISCUSSION

Summary of Findings

The study in Chapter 1 took advantage of publicly available tools in the ABA to identify several genes that were selectively expressed within the EWcp, and used tissue punch microdissection of the EWcp in combination with array expression profiling to quantify those transcripts within tissue samples of the EWcp from adult male B6 and D2 mice. The results, which expand on several previous studies that analyzed protein–level expression of ITFs and neuropeptides within the EWcp, confirmed that mRNA levels of several EWcp-enriched genes and two ITFs were greater within naive B6 mice, relative to naïve D2 mice. These findings are paralleled by differences in voluntary EtOH drinking between B6 and D2 strains, indicating that increased neural activity and neuropeptide-related gene expression within the EWcp is associated with higher levels of EtOH intake. Because EWcp gene expression data were generally reflective of protein levels, these data support a framework in which rates of neuropeptide synthesis and release are elevated in the EWcp of B6 vs. D2 mice. Within the context of previous findings on the CRF system, elevated release of Ucn1 and other stress-related neuropeptides would be expected to facilitate EtOH consumption, thereby providing a potential mechanism to explain genotypic differences in EtOH drinking.

This hypothesis was pursued in Chapter 2, where standard 2-BC CA EtOH drinking was examined following reduction of EWcp-Ucn1 expression via electrolytic lesion, genetic KO, or both. In addition, Chapter 2 used place conditioning experiments in Ucn1 and CRF2 genetic KO and WT mice. Taken together, these studies provided further support for a role for the EWcp in EtOH intake and EWcp-Ucn1 neurons in EtOH preference. Furthermore, these studies established that Ucn1 and CRF2 both underlie EtOH-induced reward. Thus, although Ucn1 binds to both CRF1 and CRF2, and although CRF2 can be bound by all three Ucns, these data suggest that the Ucn1-CRF2

ligand-receptor combination contributes to the conditioned rewarding effects of EtOH. Because there is extensive overlap between the neurotransmitter systems driving EtOH-induced reward and voluntary EtOH binge drinking (Crabbe et al., 2011a), these results suggested a role for endogenous EWcp-Ucn1 activity in facilitating EtOH intake.

Chapter 3 further characterized the EtOH-related phenotype of the Ucn1 KO mouse model by utilizing several different drinking paradigms, and by testing mice for additional EtOH-related traits and anxiety-like behaviors. Together with previous efforts from the Ryabinin Lab (Kaur et al., 2012), and with the data from Chapter 2, these studies demonstrated that constitutive, global loss of Ucn1 selectively reduced EtOH consumption only in long-term experiments that employed increasing concentrations of EtOH (leading to progressively increasing levels of EtOH intake). The differences in EtOH consumption observed between genotypes in Experiments 3.2 and 3.3 could be related to Ucn1's influence on EtOH reward processing (Chapter 2), sensitivity to locomotor effects of EtOH (Experiment 3.6), exploratory behavior (Experiment 3.8), or a combination of the three. Despite these corresponding effects of Ucn1 KO on EtOH drinking and related traits, further studies are required to clarify the way in which additional behavioral domains are related to Ucn1's influence on EtOH drinking.

Using the long-term IA model, Chapter 4 revealed that EWcp genes of interest underwent differential adaptations depending on the presence or absence of EtOH. Genes encoding neuropeptides and related proteins were particularly elevated during No Access, and the stress-related neuropeptide gene *Cck* was also upregulated following EtOH Access. These data complemented the findings from Chapter 1, suggesting that excessive EtOH intake is associated with greater EWcp-neuropeptide synthesis and release. The EWcp is known as a central stress locus, and many of the neuropeptide components altered by EtOH are related to the stress response. Therefore, the binge-like intake observed upon resumption of EtOH access in the long-term IA procedure may

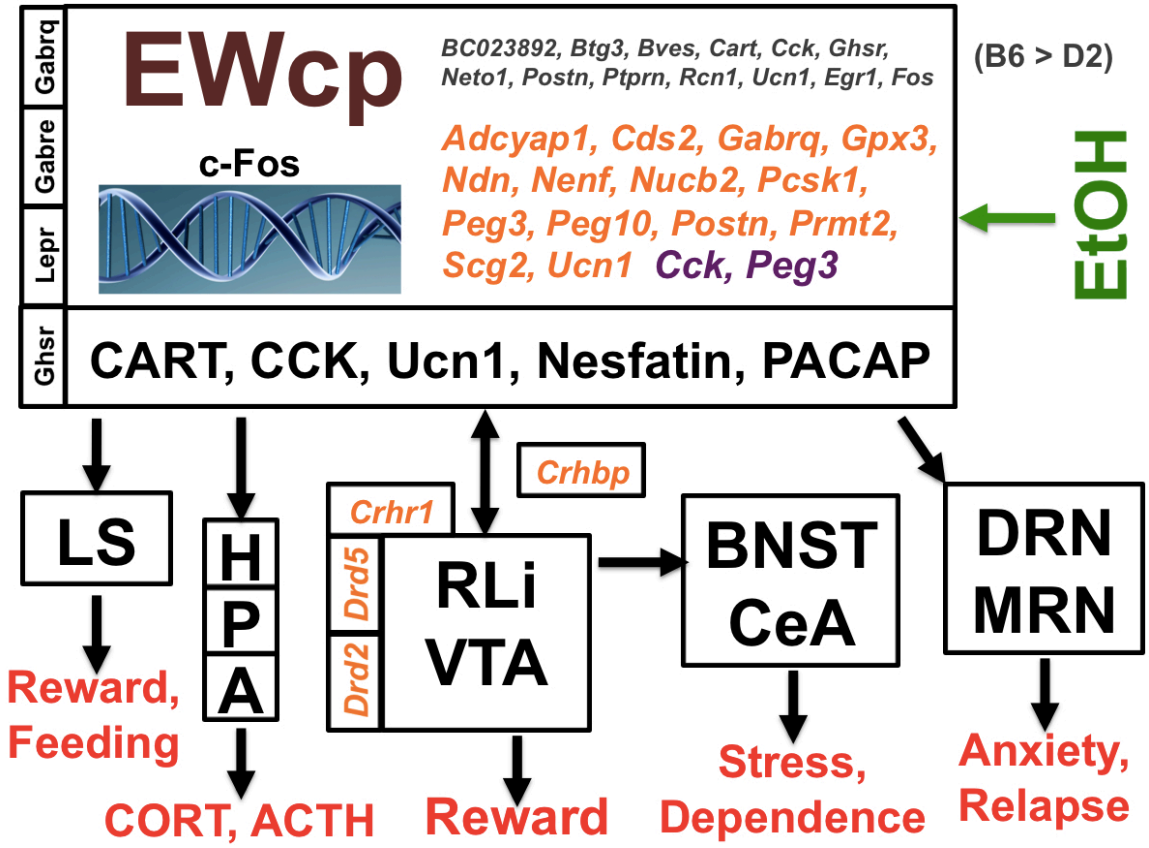
serve to counteract the stress-like state of EtOH abstinence mediated via neuroadaptations occurring in the EWcp. This concept could potentially explain the results obtained in Chapter 5, in which successful KD of EWcp-Ucn1 selectively reduced EtOH drinking, and reduced basal anxiety-like behavior under certain conditions. Further measurements of stress-related behavior throughout the long-term IA procedure (during acute and protracted abstinence, as well as during active intoxication) would be required to firmly establish a conceptual framework linking IA-induced stress-like behavior, adaptations in the genetic profile of the EWcp, and contributions of Ucn1 release to EtOH preference. The long-term IA procedure is still relatively understudied in mice, and its impact on the HPA-axis and corresponding behavior is not currently understood. The observation that EWcp-Ucn1-KD reduced both voluntary EtOH intake and anxiety-like behavior points out that the influence of EWcp neuropeptides on brain stress circuits could interact with environmental factors to drive excessive EtOH drinking.

The EWcp: Neuroanatomical and Genetic Mechanisms of Behavior

Figure 32 illustrates one speculative theory of the EWcp's role in behavior. In this view, the EWcp processes internal reward cues and external signals from the environment via direct interactions with midbrain DA neurons, raphe 5-HT neurons, and forebrain limbic systems that drive stress and consummatory functions. The model also asserts that transcriptional activity in the EWcp is enhanced by exposure to drugs of abuse (particularly EtOH) (Bachtell et al., 2002a; Spangler et al., 2009). This could occur either *directly* through EtOH's actions on GABA-A receptors containing the epsilon and theta subunits (Gabre and Gabrq, which are both EWcp-enriched), or *indirectly* via RLi DA neurons that relay reward signals to the EWcp following drug-induced changes in Drd2/Drd5 expression (as documented in Chapter 4). *Ddc* mRNA levels also differed

Figure 32. The EWcp: Neuroanatomical and Genetic Mechanisms of Behavior

The EWcp is enriched in multiple anxiogenic neuropeptides (CART, CCK, Ucn1, Nesfatin, PACAP). Ghsr and Lepr provide inputs about internal homeostatic challenges. Gabre and Gabrq could provide signals from direct interactions of EtOH with GABA-A receptors. Several genes were inherently upregulated in EtOH-preferring B6 vs. EtOH-avoiding D2 mice (GRAY). Activation of EWcp c-Fos expression by EtOH could alter transcription of mRNAs encoding peptide neurotransmitters and other proteins responsible for processing and release of neuropeptides. EtOH-induced adaptations in EWcp gene expression were observed under both No Access (ORANGE) as well as Access (PURPLE) conditions. No Access conditions also upregulated CRF- and DA-related genes, likely expressed on DA neurons of the RLi (ORANGE). When released onto various nodes in limbic circuits, EWcp stress neuropeptides are capable of driving multiple behavioral domains related to stress and drug addiction.



between B6 and D2 mice in Chapter 1 studies, suggesting that differential rates of DA synthesis between strains could explain differences in EtOH-related behaviors driven by RLi interactions with the EWcp.

The model further presumes that transcriptional activity in the EWcp is enhanced by environmental stressors (Janssen and Kozicz, 2012). Together with the findings in Chapter 5 showing that EWcp-Ucn1 can contribute toward anxiogenesis, there is evidence for anxiogenic properties of all investigated EWcp neuropeptides (including PACAP, CART, CCK, and Nesfatin-1) (Hashimoto et al., 2011; Hofmann et al., 2013; Rotzinger et al., 2010; Stanek, 2006). Therefore, activation of the EWcp could either mobilize stress peptides for release, or inhibit their activity to facilitate stress recovery (Kozicz, 2007). In any case, both stressors and drugs of abuse activate the EWcp, providing a candidate substrate for the effects of stress on consumption of addictive substances.

The EtOH dependence paradigms described previously found that subjects with a chronic history of EtOH vapor or liquid diet exposure displayed enhanced anxiety-like behavior that was associated with dysregulation of extended amygdala CRF systems. Given the enrichment of stress neuropeptides in the EWcp (and the relation of Ucn1 to CRF), it is likely that the EWcp undergoes similar adaptations contributing to the stress-like state of EtOH withdrawal. This hypothesis could be tested by assessing the genetic profile of the EWcp following EtOH dependence, or by performing KD of EWcp-Ucn1 prior to EtOH dependence, analogous to the studies of voluntary drinking that I completed in Chapters 4 and 5. Consistent with the idea that withdrawal from chronic EtOH produces a negative hedonic state, stress is a major factor for precipitating relapse in dependent alcoholics (Sinha, 2001). The potential involvement of the EWcp-Ucn1

system in driving relapse of EtOH-seeking in the stress-induced reinstatement model awaits further investigation (discussed in detailed below).

In addition to the relationship between stress and addictive behavior, many of the stress neuropeptide system components expressed in the EWcp have dual roles in the consummatory behavior. Indeed, the EWcp is not only influenced by external threats, but also receives direct information about internal challenges to homeostasis via receptors for the feeding-related hormones leptin and ghrelin (Lepr, Ghsr) (Xu et al., 2009; Xu et al., 2011). Considerable overlap exists between the neural substrates of craving for food and drugs of abuse (Volkow et al., 2012), and stress is a common risk factor for obesity and addiction (Sinha and Jastreboff, 2013). Furthermore, previous examinations by the Ryabinin Laboratory provided evidence suggestive of a role for EWcp-Ghsr signaling in DID binge drinking (Kaur and Ryabinin, 2010). Therefore, motivation for food and natural rewards may contribute to the effects of EWcp-Ucn1 on EtOH drinking. However, Chapter 5 showed that KD of EWcp-Ucn1 did not alter food intake or total caloric intake either under baseline conditions (**Fig. 26A-C**), or at a timepoint when it had considerable effects on EtOH intake and preference (**Fig. 27E-F**). Together with the absence of effects of EWcp lesion and Ucn1 KO on overall consumption of food and fluids (Chapters 2 and 3), these data suggest that consummatory influences alone are insufficient to explain the role of the EWcp-Ucn1 in EtOH drinking behavior.

In the proposed model, individuals with inherently upregulated neural activity of EWcp neurons (perhaps via increased levels of *Fos* and *Egr1* mRNA) display higher levels of the above-mentioned stress-related neuropeptides and their processing components. These conditions could permit enhanced synthesis and release of stress-related neurotransmitters onto target regions including the LS, H, RLi/VTA, and DRN/MRN (**Fig. 32**). These sites are key limbic areas known to mediate the response to homeostatic signals and emotional stimuli.

The LS drives motivated behavior related to social affiliation and drug reward (Koolhaas et al., 1998; Talishinsky and Rosen, 2012), and is also associated with stress-dependent, CRF2-mediated anxiety-like behavior (Henry et al., 2006). Previous studies found that Ucn1-CRF2 signaling in the LS undergoes plastic changes following repeated cocaine exposure (Liu et al., 2005), suggesting that EtOH could produce similar changes in regulation of LS activity by Ucn1. By acting in the LS (perhaps through CRF2 signaling), Ucn1 may facilitate consummatory behavior via the extensive network of LS projections to H nuclei (Risold and Swanson, 1997). Ucn1 could also impact the H *directly* via projections from the EWcp. Ucn1 fibers are present in the H (Bittencourt et al., 1999; Kozicz et al., 1998; Weitemier et al., 2005), and ongoing genetically-defined tracing studies in the Ryabinin Lab are poised to reveal that these Ucn1 projections arise from the EWcp. Characterization of such a pathway could provide a mechanism for EWcp-Ucn1 regulation of HPA-axis activity, either by forming synapses directly on PVN-CRF neurons, or through interactions with other stress-related peptide systems in H nuclei (including Ucn2 and Ucn3; **Fig. 2**).

Because VTA DA neurons exert Drd2-sensitive tonic inhibitory control over the EWcp (Ryabinin et al., 2013), and RLi DA neurons intermingle with neuropeptide neurons (Bachtell et al., 2002a; Gaszner and Kozicz, 2003), the model asserts that signals from the mesolimbic DA pathway are transmitted to the EWcp. Such a connection provides a substrate for regulating the stress response via relay of information about drug reward or drug-related cues. Intriguingly, firing rates of RLi DA neurons are increased by EtOH, and these cells display EtOH-induced synaptic plasticity of glutamate (but not GABA) transmission (Li et al., 2013). Glutamatergic adaptations in RLi-DA neurons were observed following acute EtOH, but not following chronic intermittent EtOH vapor. These data suggest that effects of EtOH on RLi-DA glutamate plasticity are related to EtOH-induced reward, but diminish as chronic EtOH exposure

leads to long-term reward deficits. Although direct projections from the EWcp to the extended amygdala have not been documented, RLi DA neurons provide direct inputs onto the BNST (Petit et al., 1995). Stressors and drugs of abuse both activate Ucn1 neurons of the EWcp and CRF neurons of the extended amygdala, suggesting that the RLi might serve as a key intermediary for coordinating stress and reward signals via interactions with these two anatomically-distinct domains of the CRF system.

CRFBP and CRF receptors regulate glutamate neuroplasticity and stress-induced reinstatement of cocaine-seeking through actions on VTA DA neurons (Ungless et al., 2003; Wang et al., 2007). These findings suggest that similar physiological and behavioral effects could occur via CRF receptor signaling in the RLi. Studies in Chapter 4 found that *Crhbp* and *Crhr1* were both upregulated within samples taken from EtOH mice in the No Access experiment. These findings coincided with elevations in *Ucn* mRNA, suggesting that abstinence from long-term IA drinking potentiates local release of EWcp-Ucn1 and subsequent interactions with CRFBP and CRF1 in RLi DA neurons. Alternatively, increased expression of *Crhbp* and *Crhr1* could reflect adaptations produced by release of CRF from an unknown source, rather than release of Ucn1 from the EWcp. Although the tissue punch microdissection technique does not allow me to definitively state that CRFBP and CRF1 adaptations occur within RLi DA neurons, these components are known to be expressed in the VTA (but not the EWcp or EWpg), suggesting that the RLi is the most likely candidate for the site of these effects.

The DRN/MRN network is also a major target of EWcp neuropeptides. These regions likely mediate the affective response to Ucns/CRF2 signaling via alterations in 5-HT transmission within cortical and limbic circuits. As described in the General Introduction, the MRN is a key site for mediating the effects of stressors on reinstatement of operant EtOH-seeking behavior (Le and Shaham, 2002). These effects are mediated by the CRF system, but the underlying ligand-receptor combination(s)

remain unknown. One possibility is that these effects are mediated by CRF projections from the BNST that act via MRN-CRF1 signaling. Another possibility is that these effects are mediated via Ucn1 projections from the EWcp that act via MRN-CRF1 and/or MRN-CRF2 signaling. Interestingly, the pharmacological stressor yohimbine (alpha-2 adrenoreceptor antagonist) attenuated EtOH-induced c-Fos expression in the EWcp (Bachtell et al., 2002a), suggesting that its ability to reinstate EtOH-seeking could be related to its anti-EtOH actions in the EWcp. Alternatively, stress-induced EtOH relapse could be mediated by MRN-CRF2 signaling driven by projections of LC-Ucn2 or BNST-Ucn3. In fact, these scenarios are not mutually exclusive. Given the anatomical convergence of multiple CRF/Ucns ligands in the MRN, the complex interactions of the CRFBP, and the evidence for ligand-directed signaling at CRF receptors, stress-induced reinstatement of EtOH-seeking could occur through any combination of CRF-related mechanisms occurring in the MRN.

This example serves to illustrate the difficulties in interpreting studies of the CRF system that relied solely on exogenous pharmacological manipulations. Even the interpretation of single-gene KO studies can be limited by compensations in partially-redundant components of the CRF system (**Table 2**). By performing region-specific genetic manipulations of individual CRF system components, significant progress will be made in unraveling the underlying neurobiology of the stress response.

The EWcp, Long-Term EtOH Drinking, and Allostatic Mechanisms

The findings in the above Chapters support a theory in which genetically predisposed individuals with inherently high levels of *Ucn*, *Cart, Cck,* or *Ghsr* within the EWcp display a potentiated response to acute EtOH via upregulation of transcriptional activity (*Fos* and *Egr1*). This acute EtOH-induced activation produces short-term adaptations in gene expression (*Cck*, *Peg3*), which cause additional adaptations (particularly among

neuropeptide-related genes: *Ucn, Adcyap1, Nucb2, Pcsk1, Scg2*) that predominate during abstinence from repeated exposure. Upregulation of these neuropeptide systems may lead to a negative affective state that enhances the likelihood of continuing to participate in repeated voluntary EtOH intoxication.

Such repeated intermittent adaptation produces physiological wear-and-tear, and efforts to counteract the stress-like state of "No Access" by maintaining EWcp homeostasis may lead to a persistent cycle of compulsive, binge-like drinking. This constant need for adaptation comes at an extreme cost to the organism, and addiction to drugs of abuse can be conceptualized as a disorder of allostasis (the process of maintaining homeostasis through behavioral change) (Koob, 2003; Koob and Le Moal, 2001). Indeed, the "allostatic load" that can be generated by attempting to constantly maintain homeostasis is hypothesized to be a major contributor to stress-related psychiatric disease in general (McEwen, 1998). The theoretical involvement of EWcp-Ucn1 allostasis in voluntary binge-like drinking certainly fits within the larger literature implicating extrahypothalamic CRF stress systems in allostatic contributions to excessive intake during EtOH dependence (Heilig and Koob, 2007; Koob, 2008; Koob, 2010; Valdez and Koob, 2004). Therefore, these findings hold relevance to clinical populations suffering from EtOH dependence, binge-like drinking, and stress-induced relapse, suggesting that continued understanding of the EWcp's genetic contributions to behavior may reveal further mechanisms for treatment of psychiatric disorders.

Future Directions

Future experiments to follow up on this work should focus on developing reliable, sensitive methods for detecting stress during EtOH abstinence in B6 mice. Traditional measures of HPA-axis reactivity may not accurately reflect the affective state, and anxiety-like behavior in the EPM and LDB may not capture the anhedonia associated

with EtOH withdrawal. Conditioned avoidance of EtOH abstinence-related cues may provide a behavioral index of withdrawal-induced aversion, and could be used to test whether Ucn1 contributes to binge-like drinking through potentiation of a negative affective state.

With regard to the EWcp, further study of the genes altered during Access vs. No Access conditions (as well as studies examining *acute* EtOH-induced changes in gene expression) may delineate the mechanisms contributing to progressive escalation of EtOH intake. Genes encoding Pcsk1 and Scg2 are of special interest, as inhibition of their functions within the EWcp could block the processing and release of *multiple* EWcp neuropeptides. Such interventions could produce profound effects on behavior, relative to approaches that selectively target *individual* neuropeptide genes. However, we must consider that the present data support distinct roles for *Cck* and *Ucn* in EtOH drinking. Thus, despite their similar anxiogenic properties, each EWcp neuropeptide may produce unique effects on the brain at each step throughout the addiction process.

The EWcp tissue punch technique may result in collection of cells from adjacent neuronal populations (EWpg, RLi, DRN). Therefore, future studies may analyze the EWcp with greater precision by adopting laser capture microdissection or fluorescence-assisted cell sorting methods. In addition, gains in understanding EWcp function may be accomplished through the use of additional genetically-encoded viral manipulations (overexpression as a complementary approach to knockdown, pathway tracing, optogenetic activation/inhibition, designer receptors/ligands, etc.). New technologies are increasingly being applied to the study of the brain, and future use of these tools may provide powerful insight into the EWcp's role in stress and addiction-like behavior.

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