CHARACTERIZATION OF THE ROLE OF THE CENTRALLY-PROJECTING EDINGER-WESTPHAL NUCLEUS IN ETHANOL-SEEKING BEHAVIORS

By

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List of Abbreviations

2-BC, 2-bottle choice

ANOVA, analysis of variance

AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

AUD, alcohol use disorder

BEC, blood ethanol concentration

BLA, basolateral amygdala

BNST, bed nucleus of the stria terminalis

$Ca^{2+,}$ calcium

CART, cocaine and amphetamine-regulated transcript

CB1, cannabinoid type 1 receptor

CCK, cholecystokinin

CCKA, cholecystokinin receptor, type A

CCKB, cholecystokinin receptor, type B

CeA, central nucleus of the amygdala

Cl^{-}, chloride

CG, ciliary ganglion

ChAT, choline acetyltransferase

CNO, clozapine-N-Oxide

CPA, conditioned place aversion

CPP, conditioned place preference

CRF, corticotropin-releasing factor

CRF1R, corticotropin-releasing factor type 1 receptor

CS, conditioned stimulus

CRF2R, corticotropin-releasing factor type 2 receptor

CTA, conditioned taste aversion

DA, dopamine

DAT, dopamine transporter

DID, drinking-in-the-dark

DIO, double-floxed inverse orientation

DSM, diagnostic and statistical manual of mental disorders

DREADDs, designer receptors exclusively activated by designer drugs

DRN, dorsal raphe nucleus

ERK, extracellular signal–regulated kinases

EtOH, ethanol

EW, Edinger-Westphal nucleus

EWcp, centrally-projecting Edinger Westphal nucleus

EWpg, pre-ganglionic Edinger Westphal nucleus

Exp, Experiment

Fig, Figure

GABA, gamma-amino butyric acid

GABAAR, gamma-amino butyric acid A receptor

GABABR, gamma-amino butyric acid B receptor

G+, GRID+

G-, GRID-

IEG, immediate early gene

IP, intraperitoneal

IPSCs, inhibitory postsynaptic currents

KO, knockout

LC, locus coeruleus

LepRb, leptin receptor, type b

LH, lateral hypothalamus

LS, lateral septum

LTP, long-term potentiation

M+B, muscimol + baclofen

mPFC, medial prefrontal cortex

mRNA, messenger RNA

NAcc, nucleus accumbens

NK1R, neurokinin 1 receptor

NMDA, N-methyl-D-aspartate receptor

NREM, non-rapid-eye-movement-sleep

POA, preoptic area

PBS, phosphate-buffered saline

PVN, periventricular nucleus of the hypothalamus

PVT, paraventricular nucleus of the thalamus

RM, repeated measures

SA, self-administration

SC, spinal cord

SNc, substantia nigra parts compacta

SP, substance P

SpV, trigeminal nucleus

Ucn1, urocortin 1

US, unconditioned stimulus

VTA, ventral tegmental area

WT, wild-type

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Abstract

Alcohol use disorder (AUD) is a chronic disease characterized in part by excessive intake of ethanol (EtOH) and repeated relapsing events. Research on the neurobiological underpinnings that regulate (EtOH) intake and EtOH-seeking behaviors is essential for the development of treatments in the future. Previous literature has shown that the centrally-projecting Edinger Westphal nucleus (EWcp) is sensitive to EtOH, and may also regulate EtOH intake. The aim of this dissertation was to examine the role of the centrally-projecting Edinger-Westphal (EWcp) nucleus in EtOH-seeking behaviors.

In **Chapter 2**, I used the conditioned place preference (CPP) procedure to assess the involvement of the EWcp in conditioned EtOH-seeking behaviors. Pharmacological inhibition of the EWcp during the acquisition and expression phases of CPP did not alter the development of expression of CPP, suggesting that the EWcp is not involved in conditioned EtOH-seeking behaviors. Inhibition of the EWcp significantly increased body temperature, providing further evidence that the EWcp is involved in thermoregulation.

In **Chapter 3,** I used a chemogenetic approach to selectively modulate neuronal activity in the EWcp and assess the involvement of the EWcp in voluntary oral EtOH drinking. Using the 24-hour 2-bottle choice procedure (2BC), I found that activation of the EWcp decreased EtOH intake. Subsequent studies determined that decreases in EtOH intake due to chemogenetic activation of the EWcp were mediated in part by neurons expressing the vesicular glutamate transporter 2 (Vglut2), as activation of these neurons also decreased EtOH intake.

Together, the experiments in this dissertation demonstrate that the EWcp may be more involved in the regulation of the consumption of EtOH, and not in conditioned EtOH-seeking behaviors. The research presented in this dissertation may inform future treatments for AUD.

Chapter 1: General Introduction

Alcohol Use Disorder

Alcohol use disorder (AUD) is a chronic brain disease observed in an estimated 16 million people in the United states (SAMHDA, 2017). Diagnosis with an AUD requires that individuals meet a certain criterion outlined in the Diagnostic and Statistical Manual of Mental Disorders (DSM5) (American Psychiatric Association, 2013). The severity of an AUD varies depending on the number of these criteria that each individual meets, which include consuming alcohol (ethanol, EtOH) in larger amounts or over longer periods of time than intended, craving or having an urge to use EtOH, and having symptoms of withdrawal (American Psychiatric Association, 2013). Worldwide, an estimated 3 million deaths in 2016, or 5.3% of all deaths, were attributed to harmful use of EtOH (World Health Organization, 2018). Given the detrimental effect of EtOH on both the individual, as well as on society, understanding the neurobiology that drives this disorder is critical to the development of future treatments.

Ethanol's effects on the brain

The transition into an AUD is believed to be driven by EtOH-induced changes in the brain, ranging from those at the molecular level, up to changes in entire systems. Our current understanding of how EtOH affects and subsequently changes the brain, both acutely and after prolonged use, comes from decades of studies both in animals and humans. In particular, EtOH exposure and use has been shown to produce changes in the gamma-amino butyric acid (GABA), glutamate, dopamine (DA) and corticotropin-releasing factor (CRF) systems.

EtOH and GABA

Acutely, EtOH is thought to act on GABA receptors to potentiate GABAergic synaptic inhibition both presynaptically and postsynaptically (Lovinger & Roberto, 2013; Siggins, Roberto, & Nie, 2005). In general, EtOH-mediated postsynaptic potentiation of GABAA

receptor (GABAAR) function, and thus GABAA mediated inhibition, is believed to be mediated by its ability to increase the frequency and duration of channel opening (Tatebayashi, Motomura, & Narahashi, 1998). Presynaptically, EtOH has been shown to increase the frequency of GABAergic inhibitory postsynaptic currents (IPSCs), although the exact mechanisms are still being investigated (Roberto, Madamba, Moore, Tallent, & Siggins, 2003; Theile, Morikawa, Gonzales, & Morrisett, 2008).

Chronic ethanol use has been shown to dramatically alter postsynaptic GABAergic transmission. Indeed, prolonged EtOH use has been shown to decrease GABA-, pentobarbital-, and benzodiazepine-mediated chloride (Cl^-) uptake (Cagetti, Liang, Spigelman, & Olsen, 2003; Morrow, Suzdak, Karanian, & Paul, 1988). Importantly, changes to the basic properties of the GABAAR have been associated with the development of tolerance to the anxiolytic, sedative, and ataxic effects of EtOH (reviewed in: Kumar et al., 2009). Additionally, changes in GABAAR subunit messenger RNA (mRNA) expression following prolonged EtOH use have been observed in a number of brain regions, including the cortex (Devaud, Fritschy, Sieghart, & Morrow, 1997), hippocampus (Cagetti et al., 2003), and the ventral tegmental area (VTA) (Charlton et al., 1997). With regards to the presynaptic effects of chronic EtOH on the GABA system, work from Marissa Roberto and colleagues has demonstrated that GABA release is increased in the central nucleus of the amygdala (CeA) (Roberto, Madamba, Stouffer, Parsons, & Siggins, 2004).

Glutamate

EtOH has been repeatedly shown to inhibit both α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPAR) (Möykkynen, Korpi, & Lovinger, 2003) and N-methyl-D-aspartate NMDAR (Criswell, Ming, Griffith, & Breese, 2003; Lovinger, White, & Weight, 1989)

receptors in the brain, although it is generally accepted that NMDARs are more sensitive to EtOH. As such, it has been postulated that of the glutamate receptors, NMDARs are the most important glutamate receptors mediating a number of EtOH-associated processes in the brain (for review see: Moykkynen & Korpi, 2012). Acutely, EtOH inhibits NMDRs through a non-competitive mechanism and although it is not fully understood, it has been suggested that inhibition of NMDARs occurs in a calmodulin-dependent manner (Anders, Blevins, Smothers, & Woodward, 2000; Lovinger, White, & Weight, 1990). As with NMDARs, the exact mechanism by which acute EtOH exposure inhibits AMPARs remains somewhat unknown, but again inhibition appears to be through non-competitive mechanisms. It has been hypothesized that EtOH inhibits these receptors by altering their desensitization properties (Möykkynen et al., 2003; Möykkynen, Coleman, Keinänen, Lovinger, & Korpi, 2009).

Chronic EtOH has been shown to alter the glutamate system in several ways. First, increased extracellular levels of glutamate have been observed following withdrawal induced through long-term EtOH exposure (Dahchour & De Witte, 2003; Rossetti & Carboni, 1995). Secondly, both the number of NMDRs (Chandler, Sutton, Norwood, Sumners, & Crews, 1997), as well as receptor function have been shown to be increased following chronic exposure to EtOH (Gulya, Grant, Valverius, Hoffman, & Tabakoff, 1991). Lastly, in regard to changes in NMDR-mediated long-term potentiation (LTP), long term exposure to EtOH results in reductions of LTP in hippocampal neurons (Durand & Carlen, 1984; Roberto, Nelson, Ur, & Gruol, 2002) as well as in the amygdala (Stephens et al., 2005).

Dopamine

It is well established that dopaminergic projections originating in the VTA and terminating in the nucleus accumbens (NAcc) are a critical component of the reward system in

the brain. DA levels have been shown to dose-dependently increase in the NAcc following exposure to EtOH (Imperato & Di Chiara, 1986; Yan, 1999; Yim & Gonzales, 2000; Yoshimoto, McBride, Lumeng, & Li, 1992). This increase in striatal DA release appears to be mediated by increased synaptic release of DA, and not via the inhibition of the DA transporter (DAT). Furthermore, EtOH increases DA release in the caudate putamen in mice lacking DAT (Mathews, John, Lapa, Budygin, & Jones, 2006), again indicating that EtOH does not interact with DAT to produce its effects on the mesolimbic reward pathway. In rats, the anticipation of EtOH consumption has also been shown to increase DA levels in the NAcc, which has been suggested as potentially being involved in driving relapse events (Katner, Kerr, & Weiss, 1996; Katner & Weiss, 1999). EtOH exposure has also been shown to elevate DA levels in the CeA (Yoshimoto et al., 2000). In line with the studies listed above, both *in vitro* (Brodie, Pesold, & Appel, 1999; Brodie, Shefner, & Dunwiddie, 1990) and *in vivo* (Didone, Masson, Quoilin, Seutin, & Quertemont, 2016; Foddai, Dosia, Spiga, & Diana, 2004; Gessa, Muntoni, Collu, Vargiu, & Mereu, 1985) studies have demonstrated that EtOH stimulates the firing of DA neurons in the VTA. Together, these findings suggest that the reinforcing effects of EtOH are attributed to its ability to increase DA release in the NAcc via stimulation of DA neurons in the VTA.

Studies in rodents investigating the effects of repeated EtOH exposure on the dopamine system have found decreased DA levels in the NAcc following the cessation of EtOH treatment (Rossetti, Melis, Carboni, & Gessa, 1992; Weiss et al., 1996). Decreased levels of DA have also been observed in the striatum of human AUD patients (Volkow et al., 2007), suggesting that as with other drugs of abuse (for review see; Volkow, Fowler, Wang, Baler, & Telang, 2009), an altered DA system may be a common feature of prolonged EtOH exposure. Furthermore,

decreased levels of DA in the NAcc have also been postulated to drive increased EtOH intake, as rats undergoing withdrawal have been shown to self-administer EtOH until DA in the NAcc has reached pre-withdrawal levels (Weiss et al., 1996). Both tonic and phasic firing of VTA DA neurons has also been shown to be decreased in rats undergoing withdrawal following a chronic EtOH-treatment paradigm (Diana, Pistis, Carboni, Gessa, & Rossetti, 1993; Diana, Pistis, Muntoni, & Gessa, 1995). In addition, the magnitude of EtOH-mediated increases in VTA DA neuron firing has been shown to be altered (Brodie, 2002; Okamoto, Harnett, & Morikawa, 2006) after prolonged exposure to EtOH. Although EtOH is not believed to increase DA release in the NAcc through actions on the DAT (see above), prolonged EtOH exposure either via self-administration (Siciliano et al., 2016) or EtOH vapor (Budygin et al., 2007) can increase DAT function in the NAcc.

Corticotropin-releasing factor

The CRF system has long been implicated as being affected by, and involved in the regulation of, EtOH intake. Neuroadaptations in this system caused by both short-term and long term EtOH exposure have been observed in a number of brain regions, including the bed nucleus of the stria terminalis (BNST) and the CeA. In the CeA, exposure to a short-term binge-like paradigm increases CRF1 receptor (CRF1R) immunoreactivity. Furthermore, the laboratory of Dr. Todd Thiele found that these increases in CRF1R immunoreactivity were accompanied by changes to CRF-mediated GABA release following prolonged binge-like intake (Lowery-Gionta et al., 2012). Functional adaptations to the CRF system in the CeA have also been observed in EtOH-dependent rats. Compared to naïve animals, EtOH-dependent rats displayed increased levels of GABA in the CeA, which was attenuated by intra-CeA administration of a CRF1R antagonist (Roberto et al., 2010). Increases in CRF mRNA levels were also detected in the CeA

of EtOH-dependent animals, providing further evidence for EtOH-induced changes in this brain region. As with GABA, excitatory glutamatergic neurotransmission in the CeA has been shown to be regulated by actions of CRF on CRF1Rs. Specifically, Liu and colleagues demonstrated that CRF activation of CRF1Rs decreased excitatory glutamatergic neurotransmission (Liu et al., 2004). Thus, it is plausible that chronic EtOH may also alter CRF's actions on glutamate within the CeA.

Dysregulation of CRF activity has also been observed in the BNST following prolonged EtOH exposure (Francesconi et al., 2009). The BNST has been particularly implicated in mediating stress-induced EtOH-seeking behaviors (for review see: Silberman & Winder, 2013). Indeed, it has been shown that exposure to stressors that elicit EtOH-seeking behaviors increase CRF mRNA expression in the BNST (Funk, Li, & Lê, 2006). In addition, Huang and colleagues found that microinjections of CRF into the BNST sensitized withdrawal-induced anxiety in rats (Huang et al., 2010).

Summary

Acute and chronic EtOH exposure has profound effects on the GABA, glutamate, DA, and CRF systems in the brain. Understanding how these systems within specific brain regions are involved in EtOH-seeking behaviors is essential for the development of treatments. Importantly, studies in which these systems are deliberately altered using animal models is necessary for future treatment development.

Animal Models used to study AUD

Introduction: EtOH as a reinforcer

Although the initial reasons why a person chooses to consume EtOH may vary, it is assumed that at least in part, the reinforcing effects of the drug drive one's decision to consume and potentially abuse EtOH. Animals, like humans, consume or self-administer drugs of abuse (including EtOH), presumably due to their effects on similar reward systems in the brain and their ability to act as reinforcers. In order to properly understand how the procedures described below are used in the field to examine the neurobiological basis of EtOH intake, EtOH-seeking behaviors, and AUDs, it is important to first describe how EtOH may act as a reinforcer.

Reinforcers, in the Skinnerian view, can be described as an event that follows a specific response and that can subsequently change the probability that the response occurs again in the future. In the context of learning and memory however, EtOH (as well as other drugs of abuse) acts as a reinforcer to promote changes in the probability of a response in several different ways (White, 1996). First, EtOH may act as a reinforcer by activating neuronal substrates that elicit an internal state that an animal perceives as rewarding, thus resulting in approach (or consummatory) behavior. Importantly, external stimuli present while an animal is exposed to the rewarding effects of EtOH have the potential to acquire salience through conditioning. Second, through negative reinforcement, EtOH may act to reduce needs or drives (such as aversive internal states during withdrawal). Finally, EtOH may act as a reinforcer by enhancing the ability of an animal to retain information about the situation in which they had access to EtOH. This memory-enhancing action is derived from the view of Thorndike, who described reinforcers as having an ability to enhance the "stamping-in" of memories (Thorndike, 1898). Of note, EtOH

has indeed been shown to enhance memory-formation (Alkana & Parker, 1979; Parker et al., 1980).

Types of animal models

The procedures described below can be classified into two categories; (1) those that involve Pavlovian conditioned-behaviors, and (2) self-administration (SA) procedures. In Pavlovian-conditioning procedures, the rewarding effects of EtOH can be paired with a neutral context, and after repeated pairings, the context gains salience. In the absence of EtOH, an animal will approach and/or prefer the context previously paired with EtOH. Although some debate exists as to why this approach and/or preference occurs, it has been hypothesized that the context has come to predict the rewarding effects of EtOH, and thus animals spend more time approaching and/or preferring the context. Following this logic, this approach and/or preference can be interpreted as an EtOH-seeking behavior.

The other types of models commonly used can be further divided into those that involve operant and non-operant procedures. In non-operant SA procedures, animals are allowed to consume an EtOH solution *ad libitum*. Although the duration and access schedules vary between specific procedures, animals are not required to complete an action in order to gain access to the drug. Conversely, operant-SA procedures require that animals complete specific actions (lever presses, nose-pokes, etc.). Upon the successful completion of such actions, animals are given access to an EtOH amount or access period pre-determined by the investigator. In some cases, EtOH may also be given intravenously through a cannula or catheter.

In summary, animal procedures used in the field vary greatly in their nature, and thus possess unique qualities that allow for the examination of the various components of EtOH-

seeking and -taking behaviors. These include the conditioned place preference procedure (CPP), operant SA, drinking-in-the-dark (DID) and the 24 h 2-bottle choice procedure (2-BC), which are described in detail below.

EtOH-induced conditioned place preference

AUD is characterized in part by the chronic relapse events that occur even after prolonged periods of abstinence. One the factors that has been implicated in promoting relapse is exposure to environmental stimuli that have previously been paired, through Pavlovian conditioning, with the rewarding or reinforcing effects of drugs. Indeed, in humans, alcohol-associated stimuli can promote desire for alcohol and can lead AUD patients to resume drinking after abstinence (Pomerleau, Fertig, Baker, & Cooney, 1983; Staiger & White, 1991). In both humans (Childs & de Wit, 2016) and animals (Tzschentke, 1998, 2007), CPP has been used to confirm that environmental stimuli can in fact become associated with the reinforcing effects of alcohol and that through these associations, stimuli can acquire salience and promote alcohol-seeking and -taking behaviors. The CPP procedure has several advantages, including technical simplicity and the ability to distinguish between the various phases of learning that are involved in the formation of these associations. Furthermore, CPP has been used to examine how EtOH's reinforcing effects vary depending on the dose as well as on the temporal parameters used during conditioning.

In EtOH-induced CPP (EtOH-CPP), EtOH acts as an unconditioned stimulus (US), while a previously neutral context serves as the conditioned stimulus (CS) (Cunningham, Gremel, & Groblewski, 2006). A typical CPP procedure consists of a habituation or pretest phase during which animals are exposed to the conditioning apparatus and the CS that are to be used (if performing a pre-test). Following habituation, animals undergo the acquisition phase, during

which the CS is repeatedly paired with the US (CS+) while an alternate CS is presented in the absence of the US (CS-) in discrete trials. Animals are then exposed to the both CS+ and CS- in an expression test, in the absence of EtOH. After numerous CS-US pairings, the CS+ gains salience, and when an animal is given a choice between the CS+ and the context not paired with EtOH (the CS-) during a preference test, more time is spent exploring the CS+. The degree of preference for the CS+ can then be used as an indicator of EtOH's reinforcing effects. Conversely, if an animal avoids the CS+ this is an indication of the development of conditioned place aversion (CPA). Previous studies have examined the methodological and temporal parameters that promote the development of CPA or CPP and have subsequently led to a better understanding of EtOH's actions on the brain, and as such merit consideration.

In mice, the order in which the CS and US are presented appears to be a major factor in determining whether CPP or CPA develops (Cunningham, Okorn, & Howard, 1997). When EtOH injections are given immediately before exposure to the CS, the outcome is CPP. In contrast, injection of EtOH immediately after the CS produces CPA in mice (Cunningham & Henderson, 2000; Cunningham, Henderson, & Bormann, 1998). Interestingly, the dose of EtOH appears to affect the strength of CPA in the same manner as in CPP. In regard to dose, Groblewski and colleagues have demonstrated that a wide range of doses can be used to produce place preference in DBA/2J mice, and that differences in the rewarding effects of various doses (0.5 – 4 g/kg) can be distinguished using a CPP dose reference procedure (Groblewski, Bax, & Cunningham, 2008). When compared to saline, a low dose of 0.5 g/kg EtOH appears to be more rewarding. When that same dose (0.5 g/kg) was compared to a higher dose of 1.5 g/kg however, the experimenters were unable to see differences in CPP between the conditioning groups, suggesting that the rewarding properties of those two doses could not be distinguished. When the

standard dose of 2 g/kg (the dose used in Chapter 1) was compared to 0.5 g/kg, or to a higher dose (4 g/kg), a significant conditioning effect was only observed in the 2 g/kg vs. 0.5 g/kg groups, suggesting that mice could distinguish between the rewarding properties of these two doses, but not between 2 g/kg and 4 g/kg. Although this type of EtOH reference-dose examination has only been conducted in DBA/2J mice, differences in place preference between other mouse strains have been reported to both 2 g/kg and 4 g/kg EtOH (Cunningham, 2014). Of note, significant differences in the acquisition of CPP, as well as in the magnitude of CPP have been observed between DBA/2J, and C57BL/6J mice, with DBA/2J mice displaying CPP after fewer trials, as well as CPP of a greater magnitude (Cunningham, Niehus, Malott, & Prather, 1992; Cunningham & Shields, 2018b; Gabriel & Cunningham, 2008). Additionally, the addition of visual-spatial cues does not reduce or eliminate these strain differences (Cunningham & Shields, 2018a). The latter finding suggests that differences in CPP between these two strains is mediated by genetic differences in the strains' sensitivity to the rewarding effects of EtOH, and not by differences in the salience of the cues that have been paired with EtOH.

Compared to SA models, the CPP procedure has a number of advantages. First, significant place preference has been observed after only two conditioning trials, highlighting the relatively short duration of the paradigm (Cunningham & Shields, 2018a; Pina & Cunningham, 2014). Second, potential differences in taste preferences and sensitivities between strains, sexes and ages are avoided, as EtOH is administered systemically via an injection. In line with this, since EtOH is experimenter-administered, one has control over doses and schedules to which animals are exposed. Lastly, the CPP has been effectively used to examine how various manipulations affect the different phases of the procedure. For example, a manipulation that interferes with the acquisition phase of CPP can provide insight into the neuronal mechanisms

that control the acute reinforcing effects of EtOH, as well as the mechanisms that may promote the formation of EtOH-cue associations. In contrast, manipulations that alter or disrupt the expression phase of CPP may provide information regarding the neuronal mechanisms that drive EtOH-cue-seeking behaviors or cue memories. In addition to these advantages, the CPP procedure has some disadvantages that should be considered. First, that EtOH is administered by the experimenter reduces the face validity of CPP procedures, as an animal has no control as to how much EtOH it consumes. In line with this, the CPP procedure lacks the typical increases or escalation of intake over time that can occur in SA studies, which represent loss of control of intake that is seen in the human condition. Lastly, manipulations that alter locomotor activity can have profound effects on CPP, and the subsequent interpretations of the results of these manipulations, particularly as increased locomotor activity during the preference test has been shown to be negatively correlated with CPP expression (Gremel & Cunningham, 2007).

Operant Self-Administration

Operant EtOH SA studies have been successfully used in a number of species, including rats (Samson, Pfeffer, & Tolliver, 1988), mice (Ford, Fretwell, Mark, & Finn, 2007) and monkeys (Meisch & Stewart, 1994). Furthermore, operant SA studies have shown that EtOH can act as a reinforcer (as measured by increased responding) when it is delivered orally (Meisch, 2001) or intravenously (Grahame & Cunningham, 1997). In a typical operant SA study, animals are trained to make a response under a schedule of reinforcement. Animals only receive EtOH after completing a specific number of lever presses or nose pokes (ratio schedule), or after a certain period of time has elapsed (interval schedule). The oral delivery of EtOH can occur either via the "dipper" or "sipper" methods (for review see: Samson, 2000). Briefly, specific amounts of EtOH are delivered into a tray for consumption in the "dipper" method after each completion

of the required response. In contrast, animals that complete the required response in studies utilizing the "sipper" method are given a sipper tube of EtOH for a certain period of time, during which they have free access to the solution. Although it is more common that studies deliver EtOH orally, intravenous delivery of EtOH can be advantageous. For example, Grahame and Cunningham demonstrated that differences in EtOH preference between the C57BL/6J and DBA/2J mouse strains are not apparent when EtOH is administered intravenously, as EtOH had reinforcing effects in both strains.

In general, operant SA models have a number of advantages over other non-operant SA models (such models are described below). To begin with, typical SA procedures can be used to separately analyze the two components (appetitive and consummatory) of motivated behavior (Berridge, 2004). The appetitive component of the procedure is believed to represent EtOH-seeking, while the consummatory component occurs when the animal drinks the EtOH. The ability to separate these two components allows investigators to examine them independently. Indeed, the motivational effects of EtOH can be examined by changing the number of responses required to receive EtOH, as animals may be asked to work more for EtOH. Similarly, changing the EtOH concentration or the amount of EtOH given after successful completion of the requirement can alter an animal's willingness to work for the reward. An additional advantage of such models comes from their ability to provide detailed information regarding the pattern of responding and intake over the course of a single trial, day, week, and or an entire experiment. Furthermore, a discrete cue presented during the delivery of the EtOH can be become associated with the rewarding effects of the EtOH. Once these association have been made, it is possible to examine extinction and cue-induced reinstatement of EtOH-seeking behaviors.

In addition to the numerous advantages of operant SA models, a few key limitations must be noted. First, as the successful completion of a response is required to receive EtOH, consumption of the attained EtOH may affect an animal's ability to successful complete the response in the future. Changes in responding could therefore be attributed to either the effects of the EtOH itself, or to changes in motivation. Furthermore, physiological or pharmacological (among others) manipulations that prevent the animal form successfully completing the required response may be interpreted as having affected motivation, when they could potentially altered motor function. Thus, it is especially important that results are considered and interpreted carefully when using SA procedures. Finally, operant SA procedures may require extensive training, and therefore can be time and labor intensive.

24 h 2-Bottle Choice Procedure

Of the various self-administration models currently used in the field, the continuous access 24 h 2-BC procedure is widely used and studied. In this procedure, rodents are given continuous access to two bottles, one containing water and one containing EtOH. Over the course of the procedure, the concentration of EtOH can either be increased from low (3%) to high (20%) concentrations or may remain constant. Experimenters can calculate EtOH and water intake, as well as preference ratio. When combined with lickometer technology, the procedure can also be used to assess circadian patterns of EtOH intake, as well as spontaneous intake (Giardino et al., 2017; Panksepp, Rodriguez, & Ryabinin, 2017). The 2-BC procedure has been used in the past to examine how differences in intake between mouse strains are driven by genetic determinants. To that extent, several studies have reported large differences in intake between DBA/2J and C57BL/6J mice, where C57BL/6J mice have significantly higher preference for EtOH (Belknap, Crabbe, & Young, 1993; Rodgers, 1966; Yoneyama, Crabbe,

Ford, Murillo, & Finn, 2008). Importantly this difference in preference appears to have remained stable over the 50 years (Wahlsten, Bachmanov, Finn, & Crabbe, 2006).

The 24 h 2-BC procedure is advantageous in that it is relatively non-invasive and easy to implement, as animals are allowed to drink in their home cage and voluntarily. Animals are allowed to drink their desired amount, at their desired schedule, and as such this also gives the procedure high face and construct validity. The 2-BC procedure allows for examination of the long and short term effects of EtOH intake, as well as the negative affective states associated with cessation of EtOH intake; withdrawal behaviors have been reported using this procedure (Smith, Hostetler, Heinricher, & Ryabinin, 2016). One disadvantage of the procedure however is that mice generally do not consume enough EtOH to maintain blood EtOH concentrations (BECs) that amount to intoxication (80 mg/dl). When mice and rats are given 24 h access to EtOH, they tend to spread their EtOH intake over the course of the day, preventing them from maintaining high BECs. Additionally, it has been suggested that in both mice and rats, EtOH intake decreases once an animal has reached peak EtOH metabolism and elimination (Dole, Ho, & Gentry, 1985; Murphy et al., 1986).

Drinking-in-the-dark

As mentioned above, one concern associated with continuous access procedures is that mice generally do not consume enough EtOH in a certain time period to be considered a binge and/or reach intoxication. To address this issue, experiments have attempted to create procedures based on limited access periods during circadian hours when mice are most active (Freund, 1970; Goldstein & Kakihana, 1977). Initial studies provided access to EtOH during the initial hours of the circadian dark cycle in order to take advantage of a period in time where consummatory behaviors in mice are at their highest. An initial study utilizing a brief access period (30 min) 2.5

h into the dark cycle found that mice consumed enough EtOH to reach BECs (250 mg%) considered highly intoxicating (Ryabinin, Galvan-Rosas, Bachtell, & Risinger, 2003). Importantly, behavioral measures of intoxication confirmed that the amount of EtOH consumed using these methods resulted in behavioral phenotypes indicative of alcohol intoxication (Sharpe, Tsivkovskaia, & Ryabinin, 2005).

Based on these initial studies (as well as others), the DID procedure was created and is now commonly used in the field (Rhodes, Best, Belknap, Finn, & Crabbe, 2005). A typical DID experiment is conducted over the course of four days, during which mice are given daily access to EtOH for a limited period of time (2-4 h). During daily access periods, water in the home cage is replaced by a tube containing EtOH. In particular, Rhodes and colleagues found the highest levels on intake and BECs (according to measurements taken on day 4) when mice given access to 20% EtOH for 2 h on days 1-3, and 4 h of access on day 4. Additionally, intake and BEC levels achieved using this method have been shown produce behaviors indicative of intoxication (Rhodes et al., 2007).

One potential limitation of the commonly-used DID procedure is that mice are not given access to water during the EtOH-access periods. When water is presented concurrently with EtOH, mice generally reach lower BECs than when EtOH is presented alone (Rhodes et al., 2007), which has been attributed to changes in absorption rates due to the intake of both water and EtOH. Since EtOH is the only fluid available, it is possible that mice must choose between thirst and consuming the EtOH solution, which results in mice drinking the EtOH. Another limitation of this procedure is that it relies heavily on intake during specific periods of the circadian cycle. When mice have been given access to EtOH later in the dark cycle, intake and BECs have been shown to be a fraction of those reported when animals have access earlier on in

the dark cycle (Lowery-Gionta et al., 2012). Thus, results obtained from studies using this procedure must be considered with respect to circadian cycles and physiological processes that change throughout the course of the day.

Summary

The CPP, SA, DID, and the 24 h 2-BC procedures all possess various advantages and disadvantages. When combined, these procedures can be used to assess the involvement of brain regions in the regulation of EtOH intake and preference, as well as in conditioned EtOH-seeking behaviors. One such region, the centrally-projecting Edinger-Westphal nucleus (EWcp), has been implicated as being a regulator of voluntary EtOH intake, and EtOH-seeking behaviors.

The centrally-projecting Edinger Westphal Nucleus

Historical perspective and nomenclature

Named after Ludwig Edinger and Carl Friedrich Otto Westphal, the Edinger-Westphal (EW) nucleus was described in the 19th century as a small population of midbrain neurons located in the periaqueductal grey, dorsal to the oculomotor nucleus. Independently, Edinger and Westphal published studies characterizing the involvement of these cells in the regulation of various functions of the eye, including pupillary constriction and dilation (Edinger, 1885; Westphal, 1887). Neuroanatomical studies conducted after the work of Edinger and Westphal helped show that constriction of the pupil, as well as pupillary dilation, was mediated by a parasympathetic projection from the Edinger-Westphal nucleus to the ciliary ganglion (CG) (Warwick, 1954), and by the subsequent innervation of the ciliary and pupillary sphincter muscles (Gamlin & Reiner, 1991; Gamlin, Zhang, Clendaniel, & Mays, 1994). Tracing studies in primates conducted in the 20th century, in which retrograde tracers were injected into the CG, continued to build on these findings and added to the idea of the EW as a group of cells with

direct projections to the CG (Akert, Glicksman, Lang, Grob, & Huber, 1980; Burde & Loewy, 1980; Sun & May, 1993). Early on however, neuroanatomical studies using non-primate mammals challenged this concept. In cats and rabbits, various studies found that only a small portion of cells within the defined EW actually project to CG (Loewy & Saper, 1978; Sugimoto, Itoh, & Mizuno, 1978b; Toyoshima, Kawana, & Sakai, 1980). The cat EW was found to send projections to the cerebellum and the spinal cord (Sugimoto, Itoh, & Mizuno, 1978a). In addition, these non-CG-projecting neurons were found to express various neuropeptides, including cholecystokinin (CCK) and substance P (Maciewicz, Phipps, Foote, Aronin, & DiFiglia, 1983; Maciewicz, Phipps, Grenier, & Poletti, 1984). In rodents, the EW is defined as two columns of cells located on either side of the midline, directly between the oculomotor nuclei (Paxinos & Franklin, 2004; Paxinos & Watson, 2013). Within the cytoarchitecturally-defined mouse EW, Weitemier et al. demonstrated that in addition to the preganglionic parasympathetic neurons positive for choline acetyltransferase (ChAT), the mouse EW contains a second population of neurons that express urocortin 1 (Ucn1), but not ChAT (Weitemier, Tsivkovskaia, & Ryabinin, 2005). This second population of neurons was believed to project centrally, and not to the CG, and thus was postulated to be involved in extra-pupillary related functions. Similarly, Ryabinin and colleagues found within the defined human EW, most cells express Ucn1, and not ChAT (Ryabinin, Tsivkovskaia, & Ryabinin, 2005). In light of these neuroanatomical studies, numerous attempts have been made to rename and classify these different populations of neurons. Most recently, Kozicz and colleagues proposed nomenclature that characterized EW neurons based on their connectivity and projection cites (Kozicz et al., 2011). Briefly, the ChAT-positive cells projecting to the CG and involved in oculomotor function were termed as the belonging to the preganglionic EW (EWpg), while the cells

expressing neuropeptides with central projections were classified as belonging to the centrally-projecting EW (EWcp).

Projections of the EWcp

To date, the most comprehensive studies investigating the ascending and descending projections of the EWcp have been performed in rats (da Silva, Torres, Haemmerle, Céspedes, & Bittencourt, 2013; Dos Santos Junior et al., 2015). Using a combination of anterograde tracing techniques, work from the Bittencourt laboratory has provided critical information regarding the neurocircuitry of the EWcp. Using a semi-quantitative comparative analyses, these studies classified the amount of EWcp-originating labeled fibers as being either "few", "moderate", or "many". Within the prosencephalon, regions that displayed moderate levels of labeled fibers included the orbital cortex, the basolateral amygdala (BLA), and the anterior amygdaloid area. Many fibers were detected in the lateral septum (LS), the anterodorsal part of the BNST, and the ventral pallidum. Furthermore, the CeA and the oval part of the BNST displayed the densest amount of fibers. Several regions of the diencephalon showed moderate levels of fibers, including the paraventricular nucleus of the hypothalamus (PVN), as well as a number of regions within the thalamus. Only the lateral hypothalamic area and the reuniens thalamic nucleus displayed high amounts of fibers in the diencephalon. Within the brainstem, a moderate amount of fibers were detected in the ventrolateral and anterior sections of the periaqueductal gray, the substantia nigra parts compacta SNc, and the ventral and dorsal parts of the dorsal raphe nucleus (DRN). Few to moderate fibers were also detected in the VTA. Of note, the EWcp sends dense projections to the BNST and CeA; two brain structures that express high levels of corticotropin releasing factor receptors (CRFRs), and that have been shown to be sensitive to ethanol exposure. The effects of ethanol on the EWcp, as well as the potential involvement of the EWcp

in regulating stress and ethanol intake (described below) could therefore possibly be mediated through interactions between the EWcp and the BNST and/or CeA.

Neuropeptides of the EWcp

Ucn1

Of the several neuropeptides that are expressed in the EWcp, Ucn1 has been studied the most thoroughly regarding its role in the regulation of ethanol intake. Although Ucn1 is primarily expressed in the EWcp, it is also produced in the lateral superior olive, and in the supraoptic nucleus, although at much lower levels (Kozicz, Yanaihara, & Arimura, 1998; Vaughan et al., 1995; Wong et al., 1996). As a member of the corticotropin releasing factor (CRF) family of neuropeptides, Ucn1 binds to both CRF receptors (CRF1R, CRF2R) with greater affinity than CRF itself (Hsu & Hsueh, 2001; Vaughan et al., 1995). Of these two receptors, CRF1Rs are expressed widely throughout the brain, while CRF2Rs are expressed in a more restricted manner, primarily in regions associated with stress, anxiety, and depression, like the BNST, the DRN, and the medial amygdala (MeA) (Dedic, Chen, & Deussing, 2018).

To date, numerous studies have shown that Ucn1-expressing cells within the EWcp are highly sensitive to EtOH administration. Using the immediate early gene (IEG) c-Fos as a marker of neuronal activity, the EWcp has been repeatedly shown to be activated following experimenter-administered ethanol as well as voluntary drinking (Bachtell, Tsivkovskaia, & Ryabinin, 2002a; Ryabinin, Criado, Henriksen, Bloom, & Wilson, 1997; Ryabinin & Wang, 1998; Turek & Ryabinin, 2005b). In rats, the only brain region activated following a limited access period for non-alcoholic beer that was supplemented with ethanol was the EWcp (Topple, Hunt, & McGregor, 1998). Rats given access to non-supplemented non-alcoholic beer, as well as saccharin or water, did not show these increases in c-Fos, suggesting that the activation of the

EWcp was specific to the ethanol. In mice, increased c-Fos expression in the EWcp has been observed following limited-access to sucrose-sweetened ethanol, as well as ethanol alone, but not following sucrose, saccharin, or water (Bachtell, Wang, Freeman, Risinger, & Ryabinin, 1999; Ryabinin, Bachtell, Freeman, & Risinger, 2001; Ryabinin, Wang, Freeman, & Risinger, 1999; Ryabinin, Galvan-Rosas, Bachtell, & Risinger, 2003). Importantly, the amount of ethanol consumed by mice in a limited-access paradigm was positively correlated with the number of c-Fos positive cells in the EWcp (Sharpe et al., 2005). Fos mRNA expression levels have also been found to positively correlate with EtOH intake in longer access paradigms (24-h) (Giardino et al., 2017). Increased neuronal activity in the EWcp has also been observed in prairie voles, a monogamous rodent species known to show high levels of ethanol intake and preference. Indeed, an increase in c-Fos expression in the EWcp has been observed following a 2-h limited access period (Anacker, Loftis, Kaur, & Ryabinin, 2011). Additionally, increased levels of FosB were observed in the EWcp after seven days of 24-h access to ethanol in prairie voles (Walcott & Ryabinin, 2017, 2019) and mice (Bachtell et al., 1999; Ozburn et al., 2012). More recently, Giardino and colleagues demonstrated that Ucn1 expression levels are increased 24 h after animals have been exposed to an intermittent access paradigm in which they had 22-h access to ethanol every other day, suggesting that the EWcp-Ucn1 is also sensitive to long-term exposure to ethanol (Giardino et al., 2017).

In addition to EtOH, several other drugs have been shown to increase c-Fos expression in the EWcp. In rats, increased c-Fos expression has been seen following exposure to either heroin (Singh, McGregor, & Mallet, 2006) or morphine (Singh, Verty, Price, McGregor, & Mallet, 2004). Interestingly, the cannabinoid receptor 1 (CB1) antagonist SR141716 has also been shown to increase c-Fos expression in the EWcp when administered alone. When given in

combination with morphine however, SR141716 did not increase c-Fos expression, indicating that the opioid and cannabinoid systems may interact to modulate neuronal activity in the EWcp (Singh et al., 2004). The EWcp has also been shown to be sensitive to psychostimulants (as measured by increased c-Fos expression), including cocaine and methamphetamine (Spangler, Cote, Anacker, Mark, & Ryabinin, 2009) in both mice and rats.

The role of the EWcp-Ucn1 system in the regulation of ethanol intake is supported by several different lines of work. First, various genetic studies have demonstrated that baseline Ucn1 expression levels are higher in rodent strains with high levels of ethanol intake and preference, compared to those that consume lower levels of ethanol (Weitemier et al., 2005). When animals were selectively bred to drink high levels of ethanol, multiple studies demonstrated that high ethanol intake was accompanied by relatively higher basal levels of Ucn1 immunoreactivity in the EWcp (Bachtell, Tsivkovskaia, & Ryabinin, 2002; Kiianmaa et al., 2003; Turek et al., 2005). Within mice, it is well known that the C57BL/6J strain drinks much more ethanol than DBA/2J mice, and these differences in ethanol intake have also been associated with significantly higher levels of Ucn1 mRNA, as well as a higher number of Ucn1-expressing neurons in the EWcp of C57BL/6J mice (Giardino, Cote, Li, & Ryabinin, 2012; Weitemier et al., 2005). In light of these findings, several studies have utilized the Ucn1 KO line to assess how genetic deletion of Ucn1 alters EtOH intake and preference. Interestingly, Ucn1 KO mice drink lower levels of ethanol than their WT littermates only when they have long-term 24-h access to ethanol (Giardino et al., 2017), and not during a binge-like paradigm (Kaur, Li, Stenzel-Poore, & Ryabinin, 2012). Specifically, when mice were exposed to a 12-day procedure in which they had 24-h access to increasing concentrations of ethanol (10, 20, 40%), Ucn1-KO mice displayed significantly lower levels of intake and preference compared to WT mice

(Giardino et al., 2017). Additionally, Ucn1-KO mice do not develop ethanol-induced place preference, suggesting that Ucn1 is necessary for the formation of EtOH-cue associations (Giardino, Cocking, Kaur, Cunningham, & Ryabinin, 2011). Interestingly, mice lacking functional CRF2Rs also did not develop place preference (Giardino et al., 2011), suggesting that Ucn1 binding on CRF2Rs could facilitate the rewarding properties of ethanol.

Surgical manipulations of the EWcp have provided additional evidence for the involvement of the EWcp in the regulation of ethanol intake. When mice received electrolytic lesions to the EWcp, they consumed significantly less ethanol than sham-operated controls, as well as reduced preference for ethanol (Bachtell et al., 2004). The decrease in intake and preference observed in EWcp-lesioned mice was accompanied by an attenuation of ethanol-induced hypothermia, suggesting that the EWcp may also be involved in the regulation of the physiological effects of ethanol. When the EWcp was lesioned in Ucn1 WT and KO mice, preference for ethanol was decreased only in WT mice, highlighting that Ucn1 expression in the EWcp is necessary for high ethanol preference (Giardino et al., 2011). In contrast, ethanol intake was decreased in both WT and KO animals that received an EWcp lesion. This latter finding suggests that other neuromodulators within the EWcp may also be also contribute to the regulation of ethanol intake. Other potential neuronal systems are discussed below. In addition to lesions of the EWcp, a viral approach has been used to alter Ucn1 expression in the EWcp. Using an RNA interference via gene-transfer-mediated knockdown of Ucn1 expression in the EWcp, Giardino et al. demonstrated that decreased Ucn1 expression can blunt ethanol intake in a long-term intermittent access paradigm, without affecting food or fluid intake (Giardino et al., 2017).

CART

The EWcp is known to express high levels of several other neuropeptides, including cocaine and amphetamine-regulated transcript (CART), CCK, and substance P (SP) (Cservenka, Spangler, Cote, & Ryabinin, 2010; Kozicz, 2003; Maciewicz et al., 1983, 1984). CART is a neuropeptide richly expressed in both the central nervous system and the periphery, and as its name suggests, is responsive to cocaine and amphetamine administration. Indeed, following exposure to cocaine or amphetamine, CART mRNA expression has been shown to be increased in the striatum (Douglass, McKinzie, & Couceyro, 1995). The role of CART in reward and addictive behaviors has been studied and reviewed extensively, particularly in the VTA and the striatum (Vicentic & Jones, 2007). In the EWcp however, fewer studies have attempted to investigate the role of this peptide in relation to reward and addiction. In rats, offspring of dams exposed to alcohol from gestation to weaning show ~2-fold increase in the number of CART-immunoreactive cells in the EWcp, compared to those born to mothers not given alcohol access (Dandekar, Bharne, Borkar, Subhedar, & Kokare, 2019). Interestingly, this difference in the number of CART-positive cells was only visible during weaning, as there were no significant differences during adulthood. The exact consequence of these differences in CART expression remains to be investigated. Differences in CART expression have also been observed between mouse strains that show differing levels of preference for ethanol intake. In C57BL/6J mice, a strain known to exhibit high levels of ethanol intake and preference, shows remarkably higher levels of both CART mRNA and protein, when compared to a strain with lower ethanol intake and preference (DBA/2J) (Giardino et al., 2012). Given the almost uniform co-expression of Ucn1 and CART in the EWcp, and the similar differences in Ucn1 expression between these two strains (see above), one is tempted to hypothesize that these two neuropeptides have related

functions in the regulation of ethanol intake. Future studies assessing the involvement of CART in ethanol-seeking behaviors will provide much needed insight into this idea.

CART has also been shown to be involved in several other neurophysiological processes, including stress and feeding. Notably, a number of studies have suggested that CART within the EWcp may be involved in these processes. Electrolytic lesions of the EWcp drastically reduce food and fluid intake, even following a food-deprivation challenge (Weitemier & Ryabinin, 2005). Furthermore, these reductions in intake were not attributed to changes in taste sensitivity or preference towards palatable flavors. While it cannot be ruled out that the absence of *both* Ucn1 and CART in EW-lesioned mice could be driving this decrease in intake, Ucn1 KO mice do not show altered feeding behaviors, suggesting the involvement other neuropeptides in the EWcp, such as CART (Vetter et al., 2002; Wang et al., 2002). The involvement of CART within the EWcp in feeding is further supported by upregulation of CART mRNA following a systemic injection of leptin (Xu et al., 2014). Leptin is known to reduce food intake through its actions on the leptin receptor (LepRb) in the brain. High levels of LepRb expression have been observed in the EWcp (Xu et al., 2011), and as such it has been hypothesized that leptin acting on receptors expressed on CART neurons in the EWcp may facilitate reductions in food intake.

Substance P

SP is an 11-amino acid neuropeptide that belongs to the tachykinin family of neuropeptides (Chang & Leeman, 1970). Of the three receptors that exist within the tachykinin family, SP is known to preferentially bind to the neurokinin 1 receptor (NK1R) (Ingi, Kitajima, Minamitake, & Nakanishi, 1991). NK1Rs are expressed in a number of regions stress- and reward-related regions in the brain, including the CeA, BNST, paraventricular nucleus of the thalamus (PVT), the DRN, VTA, and the R (LC) (Sandweiss & Vanderah, 2015). SP expression

has been observed in the rodent (Otake, 2005; Skirboll, Hökfelt, Rehfeld, Cuello, & Dockray, 1982) and feline EWcp (Maciewicz et al., 1983; Phipps, Maciewicz, Sandrew, Poletti, & Foote, 1983), suggesting that expression of the neuropeptide in this brain region has been conserved across species. In rodents, substance P and CCK are co-expressed in a sub-population of neurons that have been shown to project to the PVT (Otake, 2005).

A number of studies have implicated the SP-NK1R system in alcohol-related behaviors. When compared to WT mice, NK1R-KO mice have lower EtOH intake levels and do not develop EtOH-CPP (George et al., 2008). Furthermore, George and colleagues demonstrated that mice lacking NK1R appear to be more sensitive to the effects of EtOH, as they require more time to regain the righting reflex after receiving an EtOH injection (2008). Pharmacological antagonism of the NK1R has also been shown to dose-dependently decrease EtOH intake (Thorsell, Schank, Singley, Hunt, & Heilig, 2010). This was not observed in NK1R-KO mice, indicating that this effect is specific to the receptor. The involvement of the SP-NK1R system in EtOH-related behaviors has also been investigated and demonstrated in rats. Indeed, stress-induced reinstatement of EtOH seeking has also been prevented by a systemic injection of an NK1R antagonist given prior to the reinstatement session (Schank et al., 2011). In alcohol-preferring rats, intra-CeA microinfusion of SP reduced responding for EtOH, but not sucrose in an operant SA procedure (Yang et al., 2009). Interestingly, as it known that the EWcp projects to the CeA (a region that expresses NK1Rs), it is possible that the EWcp may be a source of SP onto the CeA.

Cholecystokinin

CCK is a peptide expressed in the gut and the nervous system, including the EWcp (Maciewicz et al., 1984; Rehfeld, 1999). Studies in the early 1970s demonstrated that CCK dose-

dependently decreased food intake in food-deprived rats (Gibbs, Young, & Smith, 1973). Its role as a satiety endocrine neuropeptide has been further supported through a number of studies since then (for review see Rehfeld, 2017). CCK is known to bind to two receptors, CCKA and CCKB (sometimes referred to as CCK1 and CCK2, respectively (Wank, 1995). CCKA receptors are expressed primarily in the periphery, although their expression has been detected in a number of brain regions associated with reward, including the NAcc, VTA, and the BNST (Mercer & Beart, 1997). In contrasts, CCKB receptors are expressed more abundantly in the brain than in the periphery, and are expressed in a number of brain regions, including the cortex, amygdala, and NAcc (Mercer et al., 1996).

In the VTA, CCK has been shown to be colocalized with DA in 80-90% of neurons and terminals containing both CCK and DA have been detected in the NAcc, and the CeA (Hökfelt, Rehfeld, et al., 1980; Hökfelt, Skirboll, et al., 1980). It has been hypothesized that CCK modulates DA function by acting on CCKB receptors in the NAcc, although this relationship is believed to vary depending on specific parts of the NAcc. For example, Vickroy & Bianchi found that DA release is increased by CCK in the caudal NAcc, but inhibited by CCK in the rostral NAcc (Vickroy & Bianchi, 1989). These effects are believed to also be mediated by CCK acting on either CCKA or CCKB receptors. Specifically, CCK acting on CCKA receptors in the NAcc is believed to increase DA release (White & Wang, 1984), while CCK appears to act on CCKB receptors to decrease DA release in the NAcc (Fuxe et al., 1980).

Given the high level of CCK and DA co-expression in the VTA, as well as CCK's modulatory effects on DA in the NAcc, CCK has been hypothesized to play a role in the regulating the rewarding effects of drugs of abuse, including alcohol (Thiele, Navarro, Sparta, Fee, & Cubero, 2003). Systemic injections of CCK have been shown to decrease EtOH, but not

water, intake in rats (Kulkosky & Chavez, 1984; Kulkosky, Foderaro, Sandoval, Cesar, & Marrinan, 1991). In a series of studies, Crespi and colleagues demonstrated that CCK-mediated decreases occurr via the CCKA receptor (Crespi, 1998; Crespi et al., 1997). In addition to potentially regulating EtOH intake, the CCK system has also been shown to be sensitive to chronic EtOH exposure, as rats fed an EtOH-solution for 3 months displayed increased sensitivity to the anorexic effects of CCK (Weatherford, Figlewicz, Park, & Woods, 1993).

Summary

The EWcp expresses a number of neuropeptides believed to be involved in the regulation of EtOH intake. Furthermore, the EWcp projects to a number of brain regions that express receptors for these neuropeptides. Importantly, these regions, including the BNST, CeA, and LS have all been implicated as being altered by, and may modulate EtOH intake and preference. As such, the studies in this dissertation were designed to examine the involvement of the EWcp in the regulation of voluntary EtOH intake and conditioned EtOH-seeking behaviors.

Rationale

Numerous pieces of evidence suggest that the EWcp is both sensitive to, and may regulate, EtOH-intake (see EWcp section above). While these studies have provided invaluable information to our understanding of the neurobiology underlying EtOH-seeking behaviors, additional questions remain to be answered. Thus, the overarching goal of this dissertation was to examine the role of the EWcp in EtOH intake and EtOH-seeking behaviors. With regards to conditioned EtOH-seeking behaviors, the current literature has not yet determined the specific involvement of the EWcp in the acquisition or expression of place-preference for an EtOH-paired context. Some evidence does exist however, for Ucn1's involvement in conditioned EtOH-seeking behaviors, potentially through its actions on CRF2Rs (Giardino et al., 2011).

Given the limited number of studies that have attempted to investigate this region's involvement in these behaviors, **Chapter 2** assessed the potential involvement of the EWcp in either the acquisition or expression of EtOH-CPP. The CPP procedure has been used numerous times to assess the potential involvement of a number of brain regions in the development of conditioned-EtOH seeking behaviors (Bechtholt & Cunningham, 2005; Groblewski, Ryabinin, & Cunningham, 2012; Pina & Cunningham, 2017; Pina, Young, Ryabinin, & Cunningham, 2015; Young, Dreumont, & Cunningham, 2014). Thus, **Chapter 2** utilized a pharmacological approach that allows for the inhibition of the EWcp during these two distinct phases of CPP, therefore making it possible to examine the potential involvement of the EWcp in the formation and/or recollection of EtOH-context associations.

In contrast to the limited number of studies investigating the role of the EWcp in conditioned EtOH-seeking behaviors, several published reports have shown that the EWcp is involved in voluntary EtOH intake. These studies have used techniques such as lesions (Bachtell et al., 2004; Giardino et al., 2011) and viral knockdown (Giardino et al., 2017) of the EWcp to demonstrate that EtOH intake can be altered by manipulating the EWcp. Recent advances in the field of neuroscience have resulted in the development of tools that allow for more precise spatial and temporal modulation of neuronal populations (Sternson & Roth, 2014). Specially, these techniques can be used to reversibly activate or inhibit neurons to examine the effects of these manipulation on behaviors (Rogan & Roth, 2011). Given our knowledge of the EWcp's involvement in EtOH intake, and the possibility of modulating neurons with precise temporal resolution, **Chapter 3** was designed and conducted to investigate the effects of EWcp modulation on voluntary EtOH intake. Specifically, **Chapter 3** used a variety of Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) in combination with the 24-h

2-BC procedure. The 2-BC procedure was chosen because acute (and reversible) changes in EtOH intake due to our manipulations could be measured and observed over the course of the day, as opposed to a DID procedure.

Together, the series of experiments described in the following chapters were designed and conducted to better understand the involvement of the EWcp in the appetitive (EtOH-seeking) and consummatory (EtOH intake) EtOH behaviors. Pre-clinical models and procedures such as the ones used here could provide invaluable information that could be used to better understand the human condition. By understanding the neurobiology that drives conditioned EtOH-seeking and EtOH intake, it may be possible to one day provide better treatments for AUD patients.

Chapter 2: Effects of Pharmacological Inhibition of the EWcp on CPP Acquisition and Expression in Male DBA/2J Mice.

This chapter is adapted from a submission under revision (as of 11/11/19) in the journal Alcohol.

Zuniga, A., Ryabinin A. E., & Cunningham, C.L. (*In Revision)*. Effects of pharmacological inhibition of the centrally-projecting Edinger-Westphal nucleus on ethanol-induced conditioned place preference and body temperature. Alcohol.

Introduction

Alcohol use disorder (AUD) is a chronic disease characterized by compulsive alcohol intake, negative emotional affect during periods of abstinence, and repeated relapse events (American Psychiatric Association, 2013). Through Pavlovian conditioning, environmental stimuli or cues that are temporally proximal to the intoxicating effects of alcohol can become associated with alcohol, and can subsequently trigger conditioned responses (Chaudhri, Sahuque, & Janak, 2008; Cunningham & Noble, 1992; Duncan, Alici, & Woodward, 2000; Morales, Varlinskaya, & Spear, 2012; Remedios, Woods, Tardif, Janak, & Chaudhri, 2014). Of note, exposure to these cues is believed to promote relapse, even following prolonged periods of abstinence (Ciccocioppo, Angeletti, & Weiss, 2001; Ciccocioppo, Lin, Martin-Fardon, & Weiss, 2003; Nie & Janak, 2003). Indeed, in abstinent AUD patients, exposure to olfactory cues elicits a number of physiological and psychological responses, including elevated heart rate, salivation, desire to drink, and withdrawal symptoms (Pomerleau et al., 1983; Staiger & White, 1991).

In rodents, the conditioned place preference (CPP) procedure has been readily used to assess the rewarding and aversive effects of drugs (Liu, Le Foll, Liu, Wang, & Lu, 2008; Tzschentke, 1998, 2007). In this procedure, a context acts as a conditioned stimulus (CS+), and through repeated pairings with an unconditioned stimulus (such as alcohol), strong preference for the CS+ develops. Importantly, the nature of this form of classical conditioning allows for separate examinations of how distinct brains regions may be involved in the acquisition, expression, and/or extinction of these cue-drug associations. Indeed, previous work has shown that microinfusion of the dopamine D1-receptor antagonist SCH23390 into the nucleus accumbens (NAcc) during CPP acquisition prevents the development of place preference to an ethanol-paired context (Young et al., 2014). In contrast, the same study found that D1-receptor

antagonism in the NAc during expression testing had no effect on CPP. Pharmacological methods have been used in combination with the place preference procedure to assess the involvement of numerous other brain regions in ethanol-cue associated behaviors, including the bed nucleus of the stria terminalis (BNST) (Pina, Young, Ryabinin, & Cunningham, 2015; Pina & Cunningham, 2017), the ventral tegmental area (VTA) (Bechtholt & Cunningham, 2005), and the medial prefrontal cortex (mPFC) (Groblewski, Ryabinin, & Cunningham, 2012). Although these studies have all provided critical insight into the neurobiology of ethanol-seeking behaviors, additional work examining how other brain regions may be involved is essential. One area in particular, the centrally-projecting Edinger-Westphal nucleus (EWcp), has been hypothesized to be involved in the regulation of ethanol-seeking behaviors, and could prove to be a viable target for treatment moving forward.

The EWcp is a midbrain region situated along the midline in the perioculomotor area, between the VTA and the dorsal raphe nucleus (DRN). In contrast to the cholinergic parasympathetic neurons that form the pre-ganglionic EW (EWpg), the EWcp is the main source of urocortin 1 (Ucn1) (Kozicz et al., 1998; Wong et al., 1996), a neuropeptide belonging to the corticotropin-releasing factor (CRF) family, within the brain. Previous work has implicated the CRF system as being involved in the regulation of ethanol intake, particularly through its role in mediating negative affective states during withdrawal (Becker, 2012; Menzaghi et al., 1994; Phillips, Reed, & Pastor, 2015; Zorrilla, Heilig, de Wit, & Shaham, 2013). Given that Ucn1 has greater binding affinities to both CRF1 and CRF2 receptors than CRF itself (Hsu & Hsueh, 2001; Vaughan et al., 1995), Ucn1 within the EWcp has been hypothesized to be involved in the regulation of ethanol intake and preference. The potential involvement of the EWcp in ethanol-seeking behaviors was supported by early reports indicating that the EWcp is sensitive to ethanol

administration. Indeed, both experimenter-administered ethanol and self-administration studies have shown that the EWcp consistently shows increased levels of c-Fos following exposure to ethanol (Bachtell, Wang, Freeman, Risinger, & Ryabinin, 1999; Ryabinin, Bachtell, Freeman, & Risinger, 2001; Ryabinin, Criado, Henriksen, Bloom, & Wilson, 1997; Ryabinin & Wang, 1998; Ryabinin, Galvan-Rosas, Bachtell, & Risinger, 2003; Sharpe, Tsivkovskaia, & Ryabinin, 2005; Smith, Li, Cote, & Ryabinin, 2016; Topple, Hunt, & McGregor, 1998). In addition, the EWcp is involved in the regulation of ethanol intake, as electrolytic lesions (Bachtell, Weitemier, & Ryabinin, 2004; Giardino, Cocking, Kaur, Cunningham, & Ryabinin, 2011), as well as viral knockdown of the Ucn1 within the EWcp (Giardino et al., 2017), decrease ethanol intake and preference.

To date, only one study has investigated the role of the EWcp in conditioned ethanol-seeking behaviors. Using a Ucn1-KO transgenic line, Giardino et al. examined the involvement of Ucn1 within the EWcp in EtOH-CPP (Giardino et al., 2011). When compared to WT littermates, Ucn1-KO mice displayed no preference for the ethanol-paired context, indicating that Ucn1 is necessary for place preference. The use of a transgenic KO mouse line however, does not allow for one to distinguish between Ucn1's involvement in the various phases of CPP, as mice are lacking Ucn1 throughout development and during the entire procedure. As such, we sought to pharmacologically inhibit the EWcp during the acquisition and expression phases of conditioning, in order determine its role in either the formation or expression of conditioned EtOH-seeking behaviors.

Since the physiological and neurobiological effects of EWcp inhibition have not been previously investigated, two additional experiments were conducted to assess the effects of EWcp inhibition on body temperature, as well as on EtOH-induced c-Fos expression in the

EWcp. Importantly, previous genetic and lesion studies have suggested that the EWcp is involved in the regulation of body temperature, including ethanol-induced hypothermia (Bachtell et al., 2002b, 2004; Turek & Ryabinin, 2005a). Together, the findings presented here provide evidence for the EWcp's more limited role in regulating ethanol-seeking behaviors, as well as for its potential role in thermoregulation.

Materials and Methods

General Methods

Animals

Male, eight-week old DBA/2J mice (The Jackson Laboratory, CA) were housed in groups of four and were allowed to habituate to the animal room for one week prior to undergoing stereotaxic surgeries. Mice were kept on a 12:12 h light-dark cycle with lights on at 7:00 AM. After recovering from surgery (see below) mice were housed two to a cage for the remainder of all experiments. Standard rodent chow and water were available *ad libitum* throughout the duration of the study. All procedures were approved by the Oregon Health & Science University IACUC.

Drugs

Ethanol (20% v/v) was prepared from a 95% stock solution in 0.9% sterile saline, and was injected intraperitoneally (IP) at a dose of either 2 g/kg (volume, 12.5 mL/kg) or 3 g/kg (volume, 18.75 mL/kg). Using concentrations based on previous work in our lab (Pina et al., 2015), the $GABA_A$ and $GABA_B$ agonists muscimol (0.1 mM, Sigma-Aldrich, MO) and baclofen (1.0 mM, Sigma-Aldrich, MO) were dissolved in 0.9% saline and a cocktail (muscimol + baclofen, M+B) of the two drugs (100 nL) was microinfused into the EWcp over the course of 60

s. Injectors were then left in place for an additional 30 s in order for the drugs to completely diffuse into the EWcp.

Stereotaxic Surgery and Cannula Implantation

For all experiments, mice were anesthetized with 5% isoflurane delivered in oxygen via a precision vaporizer (Datex Ohmeda, WI), and subsequently secured into a stereotaxic frame (Kopf Instruments, CA). Once in the stereotaxic frame, mice were given a subcutaneous injection of the non-steroidal anti-inflammatory drug carprofen (0.5 mg/kg, 10 ml/kg) and maintained under 1-2% isoflurane anesthesia. The EWcp was targeted using coordinates (A/P -3.45 mm from bregma, M/L -1.20 mm, D/V -3.5 mm) based on previous studies in the Ryabinin lab (Giardino et al., 2017) and on the standard mouse brain atlas (Paxinos and Franklin, 2001). A single burr hole was drilled 3.45 mm from bregma, and 1.2 mm from the midline. In order to avoid the sagittal sinus, a single guide cannula (2.5 mm, 26 ga, Plastics One, VA) was implanted 2.0 mm above the EWcp at a 20-degree angle. Guide cannulae were held in place using Durelon carboxylate cement (3M, MN) anchored with stainless steel screws. Custom dummy cannulae (2.5 mm, 32 ga, Plastics One, VA) were then lowered and placed into the guide cannulae so as to prevent clogging. Following the surgery, mice were given additional daily subcutaneous injections of carprofen (0.5 mg/kg, 10 ml/kg) for 3 days, and were allowed to recover for 4-7 days before the start of an experiment.

Piercing

In all experiments, 24-48 h prior to the first intracranial infusion, a 3.5 mm stylet was lowered into the EWcp in order to minimize any behavioral effects associated with the initial lowering of an internal cannula (Gremel & Cunningham, 2009). For Exp. 1 and 2, this was done

24 h before the habituation and preference test, respectively. For Exp. 3, this was done during the habituation session.

Conditioning Apparatus

Conditioning was conducted using an apparatus described in detail by Cunningham and colleagues (Cunningham, Gremel, & Groblewski, 2006). Briefly, eight conditioning boxes (30 x 15 x 15 cm) were individually enclosed in larger, well-ventilated chambers (Coulbourn Instruments, Model E10-20, dimensions: 56.1 x 46 x 39.4 cm) in which sound and light were both attenuated. The conditioning boxes were each equipped with six sets of infrared photodetectors (5 cm apart, 2.2 cm above the floor) allowing for real-time activity measurements and the detection of an animal's position. The conditioning floors used here consisted of two interchangeable halves that were either a *grid* or a *hole* pattern. *Grid* floors were made up of 2.3 mm stainless steel rods, mounted to acrylic sides in 6.4 mm intervals. *Hole* floors were made from stainless steel sheets perforated with 6.4 mm holes in a staggered manner (9.5 mm apart). These floors have been used extensively in our laboratory, and numerous studies have shown that DBA/2J mice will develop robust place conditioning using these two floor types, while showing an equal preference for both floors initially (Cunningham et al., 2003).

Experiment 1: Inhibition of the EWcp during EtOH CPP Acquisition

General Procedure

Exp. 1 was conducted to examine the effect of inactivation of the EWcp (via M+B) on CPP acquisition. Following recovery from surgery, mice were assigned to M+B (n = 24) or vehicle groups (n =24), as well as to GRID+ (G+) or GRID- (G-) subgroups (n = 12/subgroup). Mice in the G+ subgroup received 2 g/kg EtOH [CS+] while on the grid floor, and the hole floor

was paired with saline [CS-]). Conversely, mice in the G- subgroup received EtOH while on the hole floor, and saline was paired with the grid floor. All mice were exposed to an unbiased, one-compartment conditioning procedure that consisted of three distinct phases: habituation (one session), conditioning (two CS+ sessions, two CS- sessions), and preference test (one preference test).

Conditioning Procedure

In Exp. 1, habituation occurred 24 h after the piercing session and consisted of a single session in which mice were handled and gently scruffed for 90 s as they would be during a microinjection session, but nothing was microinfused. Mice were then immediately returned to their home cage, and 30 min later, mice were given a single IP injection of saline and placed in the apparatus on white paper floor for 5 min. This habituation session was conducted to familiarize the animals to both the microinjection and CPP procedure, as well as to the CPP apparatus itself.

As the goal of Exp. 1 was to determine the effect of EWcp inhibition on the acquisition of EtOH CPP, M+B or vehicle was microinfused into the EWcp only during this phase. Previous work in our lab has shown that the handling associated with microinfusion, as well as the amount of time between microinfusion and the start of a conditioning trial can impede the development of place preference in control animals (Young et al., 2014). Based on those studies, mice received a microinfusion of either M+B or vehicle 30 min prior to CS+ trials. During CS- trials, 30 min before the conditioning trial, mice were exposed to a sham infusion procedure, in which a dummy microinjector (2.5 mm, 32 ga) was inserted into the guide cannulae for an equivalent

period of time, but no fluid was infused. The order in which mice were exposed to each trial type (CS+ or CS-) was counterbalanced between treatment and conditioning subgroups.

Twenty-four h after the last conditioning session, mice were weighed and injected with saline immediately prior to being placed in the apparatus for a 30-min preference test on split floors. The positioning of each floor during testing (left vs. right) was counterbalanced within treatment and conditioning subgroups. Immediately after the preference test, mice were sacrificed, and brains were collected for placement verification.

Experiment 2: Inhibition of the EWcp during EtOH CPP expression

General Procedure

Exp. 2 examined the effect of EWcp inhibition on CPP expression during a preference test. As with Exp. 1, following recovery from surgery, mice were assigned to M+B (n = 28) or Vehicle (n = 28) groups, as well as to G+ and G- subgroups (n = 14/subgroup). The one-compartment unbiased procedure consisted of a single 5-min habituation session, eight 5-min conditioning sessions (four CS+, 4 CS-) and a single 30-min preference test.

Conditioning Procedure

During habituation, mice were weighed and injected with saline immediately prior to being placed on white-paper floors for a single 5-min session. During conditioning, mice were weighed and given a single injection of either 2 g/kg EtOH (CS+ days) or saline (CS- days) immediately before being placed in the apparatus on the appropriate floor. Twenty-four h after the last conditioning session, mice were gently scruffed and received a single 100 nL microinfusion of either M+B or saline over the course of 60 sec, with an additional 30 sec allocated to complete diffusion of the drug or vehicle. Previous work in our lab has shown that

handling immediately prior to preference testing has no effect on CPP expression (Bechtholt, Gremel, & Cunningham, 2004), and as such mice received a saline injection and were placed in the apparatus immediately after the microinfusion for a 30 min test on a floor made up of both floor types. Following the preference test, mice were euthanized, and brains were collected for placement verification.

Experiment 3: Inhibition of the EWcp and ethanol-induced hypothermia

Exp. 3 examined the effect of acute inactivation of the EWcp on ethanol-induced hypothermia using parameters based on previous studies (Bachtell et al., 2004). Following the habituation session, baseline rectal temperatures were assessed, immediately followed by a 100 nL microinfusion of either M+B (n = 12) or vehicle (n = 12) into the EWcp. Thirty min later, rectal temperatures were once again assessed, followed by an IP injection of either EtOH (3 g/kg, 18.75 mL/kg) or saline. Rectal temperatures were subsequently measured 15 and 30 min after the EtOH injection (45 and 60 min after microinfusion). Mice were then euthanized, and brains were collected for placement verification.

Experiment 4: Inhibition of the EWcp and ethanol-induced c-Fos in the EWcp

Exp. 4 was conducted to determine if inhibition of the EWcp via M+B was sufficient to prevent the increase in c-Fos induction reported in previous studies (Bachtell et al., 2002, Smith et al., 2016). Twenty-four h following the habituation session, mice received a single 100 nl microinfusion into the EWcp of either M+B (n = 6) or vehicle (n = 4), followed by an IP injection of EtOH (2 g/kg, 12.5 mL/kg) 30 min later. Ninety min after the microinfusion, mice were sacrificed, and brains were collected for c-Fos immunohistochemistry.

Histology

Brain extraction and processing

In all experiments, mice were sacrificed via CO_2 inhalation, brains were extracted, post-fixed for 24 h in 2% paraformaldehyde/phosphate-buffered saline (PBS) and cryopreserved in 20-30% sucrose/PBS. Brains were sliced at 30 μm and were processed for placement verification and c-Fos immunohistochemistry.

Placement Verification

For all experiments, coronal sections containing the EWcp (-3.16 mm to -4.0 mm from bregma) were collected and stained with 0.1% thionin in order to verify cannula placements.

c-Fos immunohistochemistry

For each animal, 5-7 sections encompassing the EWcp were washed three times in PBS and then rinsed with 0.3% peroxide in PBS. Sections were then blocked in 5% normal goat serum in PBS and 0.3% Triton X-100, followed by an overnight incubation with a 1:15000 rabbit polyclonal c-Fos antibody (Sigma-Aldrich, MO) in PBS/Triton X-100 and 0.1% bovine serum albumin. The following day, sections were incubated in a biotinylated anti-rabbit secondary. Finally, a Vectastain ABC Kit (Vector, CA) and a metal enhanced DAB Kit (Thermo Scientific, MA) were used. Slices were mounted, dehydrated and then coverslipped.

Statistical Analysis

Activity data for Exp. 1 and 2 were averaged across trials. For both experiments, activity was analyzed via a two-way ANOVA with Treatment (M+B vs. Vehicle) as a between-group factor and Trial Type (CS+ vs. CS-) as a within-group factor. Place preference was defined as a

significant difference in time spent on the grid floor between conditioning subgroups. Time spent on the grid floor was analyzed using a two-way ANOVA with Treatment (M+B vs. Vehicle) and Conditioning Subgroup (G+ vs. G-) as between-group factors. Locomotor activity during the preference test was analyzed using a one-way ANOVA using Treatment as the factor. For Exp. 3, temperature measurements were converted into a change-from-baseline value, and these data were analyzed using a two-way ANOVA for the first time point (30 min after mice received the microinfusion), with Microinjection (M+B vs. Vehicle) and Treatment (EtOH vs. Saline) as between-group factors. The two time points after the IP injection of either EtOH or saline were analyzed via a three-way repeated measures (RM) ANOVA with Time as the within-subjects factor, and Microinjection, and Treatment as the between-subjects factors. For Exp. 4, a single c-Fos cell-count value was calculated for each animal by averaging the cell counts across the 5-7 sections that encompassed the EWcp in each animal. Thus, each animal had a single c-Fos cell count value for the EWcp. C-Fos data were analyzed using a one-way ANOVA with Microinjection (M+B vs. M+B Miss vs. Vehicle) as the between group factor.

Results

Experiment 1: Inhibition of the EWcp during CPP Acquisition

Injector Placement

Fig. 1C depicts the placements of the microinfusion injectors within the EWcp, based on thionin staining conducted following the conclusion of the experiment. Thirteen mice were excluded from the analysis due to lost headcap, while five mice were excluded for an incorrect placement of the injector. The final sizes for conditioning subgroups ranged from 6-8 per subgroup. Exact group numbers are depicted within individual bar graphs.

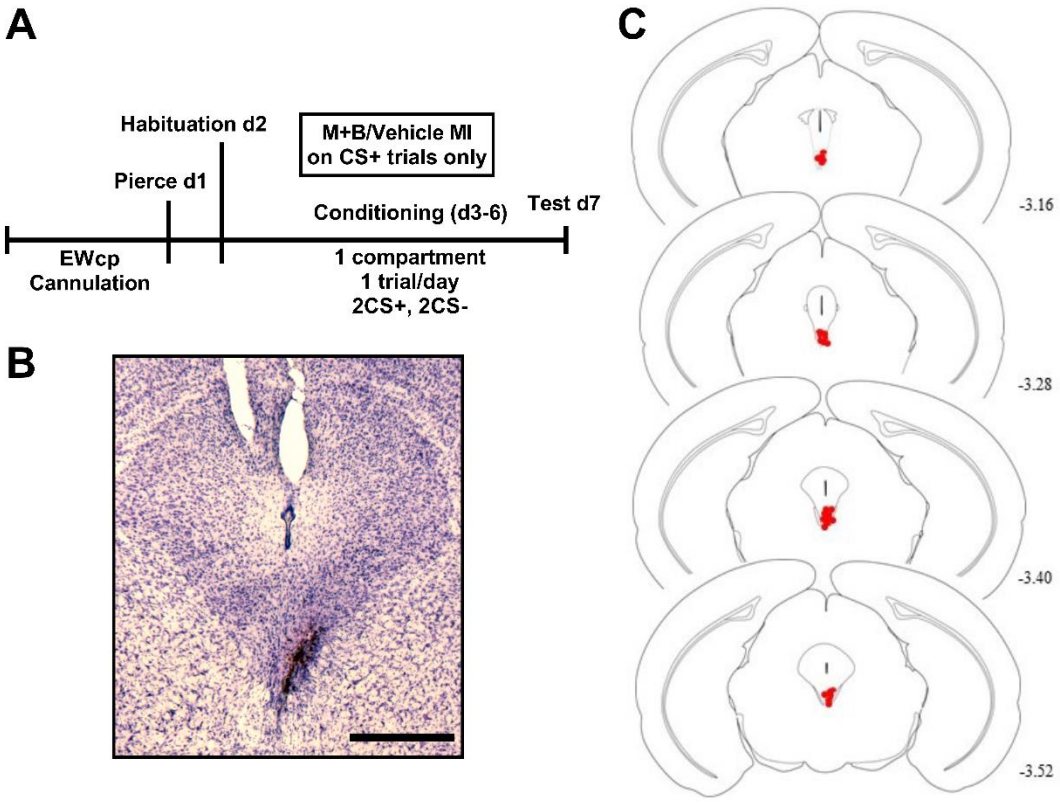

Fig. 1. Procedural timeline and successful cannulation of the EWcp in Exp. 1.
A cannula was placed above the EWcp, and following recovery, mice received either 100 nL of Muscimol (0.1 mM) + Baclofen (1.0 mM) (M+B) or saline prior to CS+ trials. A) Procedural timeline for Exp. 1. B) Brightfield image of thionin staining used to confirm cannula placement location. C) Cannula location placements for mice included in the study. Scale bar denotes 500 µM.

Conditioning Activity

Mean conditioning activity rates collapsed across the two conditioning trials of each type are depicted in Fig. 2A. Pharmacological inhibition of the EWcp during CS+ trials throughout conditioning did not alter the stimulatory effects of ethanol, nor did it alter general locomotion during CS- trials. Indeed, mice in both groups were significantly more active during CS+ trials, compared to CS- trials, and the differences in activity between trial types did not differ between

groups. A two-way ANOVA (Trial Type x Treatment) confirmed these observations, as only a significant main effect of Trial Type was detected [$F(1, 56) = 138.0$, $p < .0001$). Neither the main effect of Treatment ($p = .71$) nor the Trial Type x Treatment interaction ($p = .61$) was significant.

Preference Test

Fig. 2B-C depict the mean activity counts and times spent on the grid floor during the preference test. As can be seen, pharmacological inhibition of the EWcp during the acquisition phase did not have any effect on the development of place preference, or on locomotor activity during the preference test. No significant differences in test activity rates between mice treated with vehicle and M+B during conditioning were detected during the test. A two-way ANOVA (Conditioning Subgroup X Treatment) revealed a main effect of Conditioning Subgroup [$F(1, 26) = 36.6$, $p < .001$], indicating that mice in the G+ subgroup spent significantly more time on the grid floor than G- mice, regardless of the treatment they received during conditioning. The main effect of Treatment ($p = .68$) and the Conditioning Subgroup X Treatment interaction ($p = .13$) were not significant.

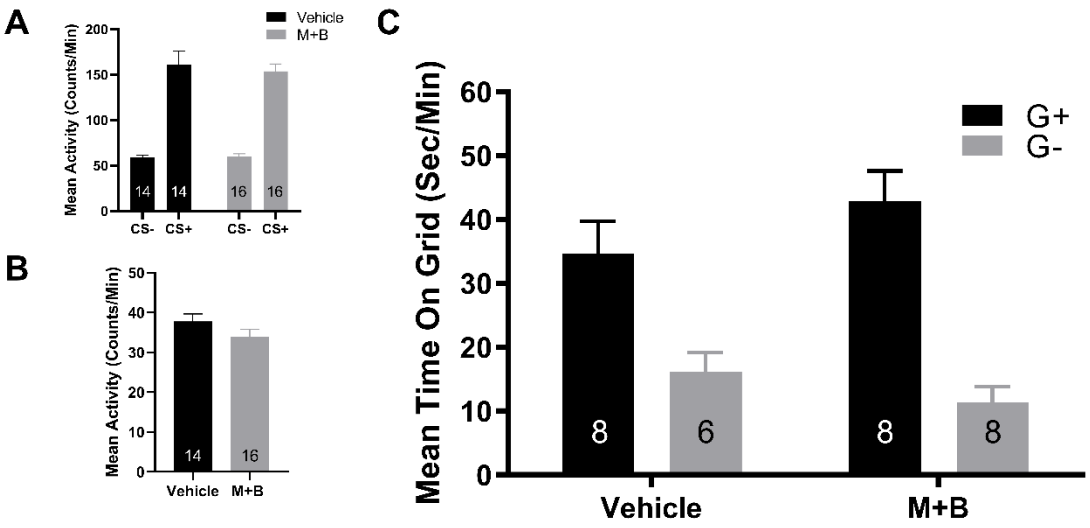

Fig. 2. Pharmacological Inhibition of the EWcp does not alter the acquisition of EtOH-CPP.
Following recovery from stereotaxic surgery and cannula implantation, mice received a single microinfusion of 100 nL of Muscimol (0.1 mM) + Baclofen (1.0 mM) (M+B) or vehicle into the EWcp prior to CS+ trials. A) Mice were more active during CS+ trials than during CS- trials, and no differences in activity were detected between M+B and vehicle groups. B) A history of M+B did not alter locomotor activity during the preference test. C). Significant place preference was detected in both the M+B and vehicle groups, but there was no effect of M+B on CPP.

Experiment 2: Inhibition of the EWcp during CPP Expression

Injector Placement

Microinfusion injector placements within the EWcp are shown in Fig. 3C. In Exp. 2, 20 mice were excluded from the final analysis for lost headcap (n = 9), histological error (n = 2), or for incorrect placement of the injector (n = 9). Final sizes for conditioning subgroups ranged from 8-10 per subgroup. Exact group numbers are depicted within individual bar graphs.

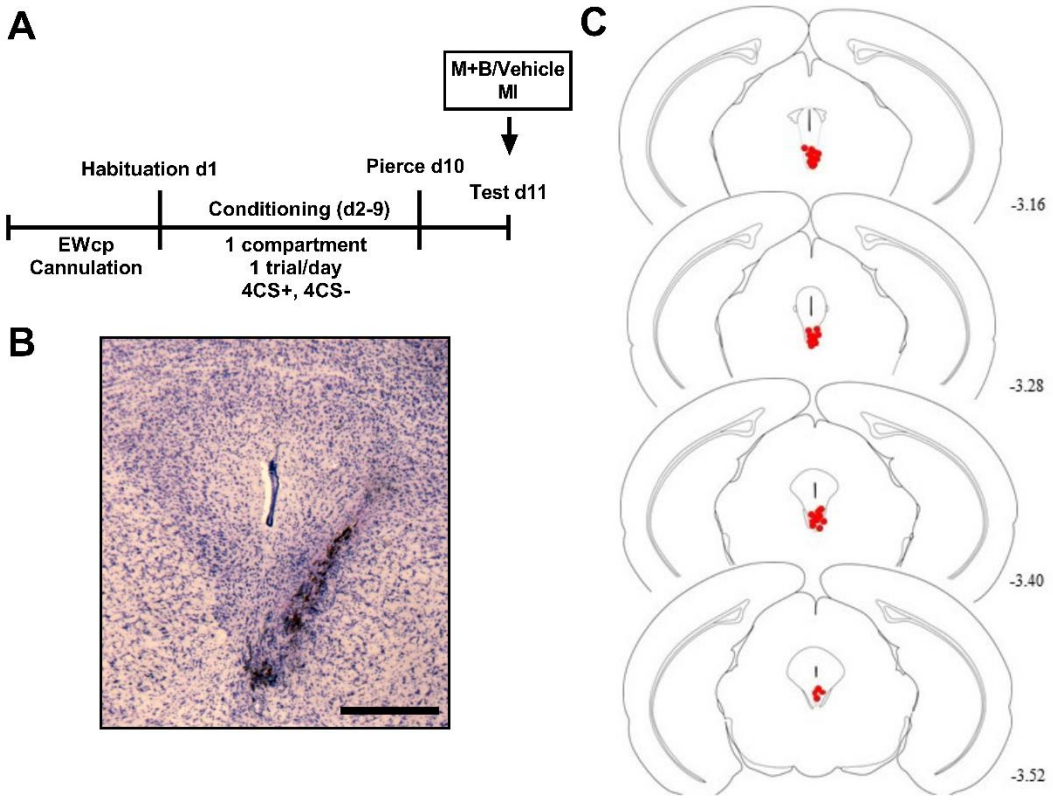

Fig. 3. Procedural timeline and successful cannulation of the EWcp in Exp 2.
A cannula was placed above the EWcp, and following recovery, mice received either 100 nL of Muscimol (0.1 mM) + Baclofen (1.0 mM) (M+B) or saline immediately prior to the preference test. A) Procedural timeline for Exp. 1. B) Brightfield image of thionin staining used to confirm cannula placement location. C) Cannula location placements for mice included in the study. Scale bar denotes 500 µM.

Conditioning Activity

Mean conditioning activity rates collapsed across the four conditioning trials of each type are shown in Fig. 4A. Mice were significantly more active on CS+ trials, compared to CS- trials. Additionally, mice assigned to receive either M+B or saline during the preference test did not differ in their sensitivity to the stimulatory effects of ethanol during conditioning. Indeed, a two-way ANOVA (Trial Type x Treatment) revealed a main effect of Trial Type [$F(1, 68) = 648.1$, $p < .0001$], but not of Treatment ($p = .59$). No significant Trial Type x Treatment interaction was detected ($p = .67$).

Preference Test

Mean activity counts as well as mean time spent on the grid floor during the 30-min preference test are presented in Fig. 4B-C. As can be seen, inhibition of the EWcp during preference testing had no effect on locomotor activity or on CPP expression. Indeed, the saline and M+B groups both displayed significant place preference that did not differ from one another. In support of this, a two-way ANOVA (Conditioning Subgroup X Treatment) revealed a main effect of Conditioning Subgroup [$F (1, 32) = 37.0$, $p < .001$], demonstrating that the amount of time spent on the grid floor between G+ and G- subgroups was significantly different in both treatment groups. The main effect of Treatment ($p = .41$) and the Conditioning Subgroup X Treatment interaction ($p = .19$) were not significant. Lastly, activity rates between the two groups

did not differ significantly during the 30-min preference test indicating that inhibition of the EWcp during expression testing did not alter locomotor activity.

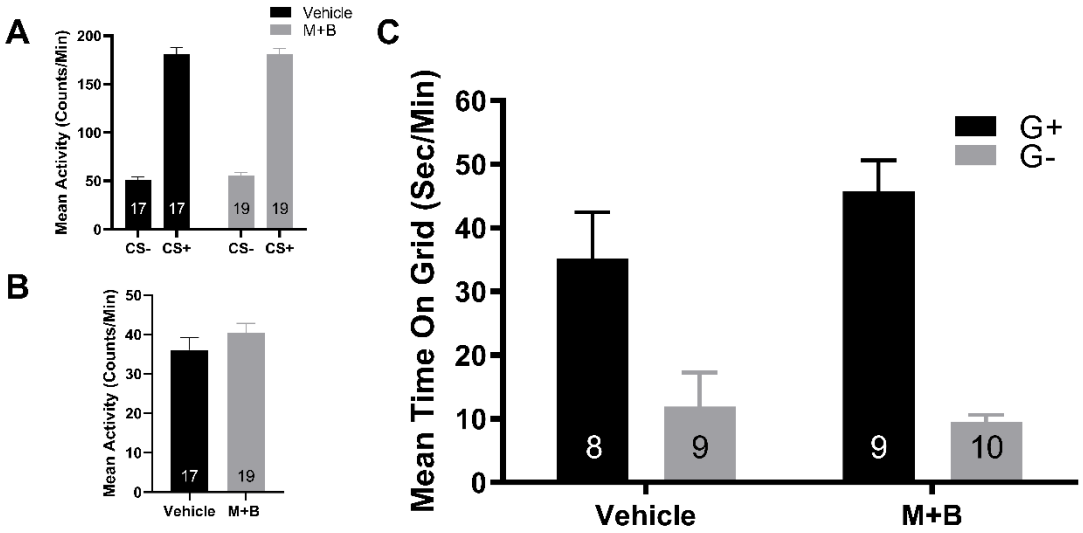

Fig. 4. Pharmacological Inhibition of the EWcp does not alter the expression of EtOH-CPP. Following recovery from stereotaxic surgery and cannula implantation, mice received a single microinfusion of 100 nL of Muscimol (0.1 mM) + Baclofen (1.0 mM) (M+B) or vehicle into the EWcp prior to the place preference test. A) Mice were significantly more active during CS+ trials, compared to CS- trials. No differences were detected between groups assigned to receive M+B or vehicle during the preference test. B). Pharmacological inhibition of the EWcp did not alter locomotor activity during the preference test. C. Significant place preference was detected in both the M+B- and vehicle-treated groups, but inhibition of the EWcp did not alter the expression of CPP.

Experiment 3: Inhibition of the EWcp and ethanol-induced hypothermia

Injector Placement

Microinfusion injector locations within the EWcp are shown in Fig. 5A. Three mice were excluded from the analysis due either to lost headcap (n =1) or for incorrect placement of the injector (n = 2). Final group sizes ranged from 5-6 per group. Exact group numbers are depicted within individual bar graphs in Fig. 5C-D.

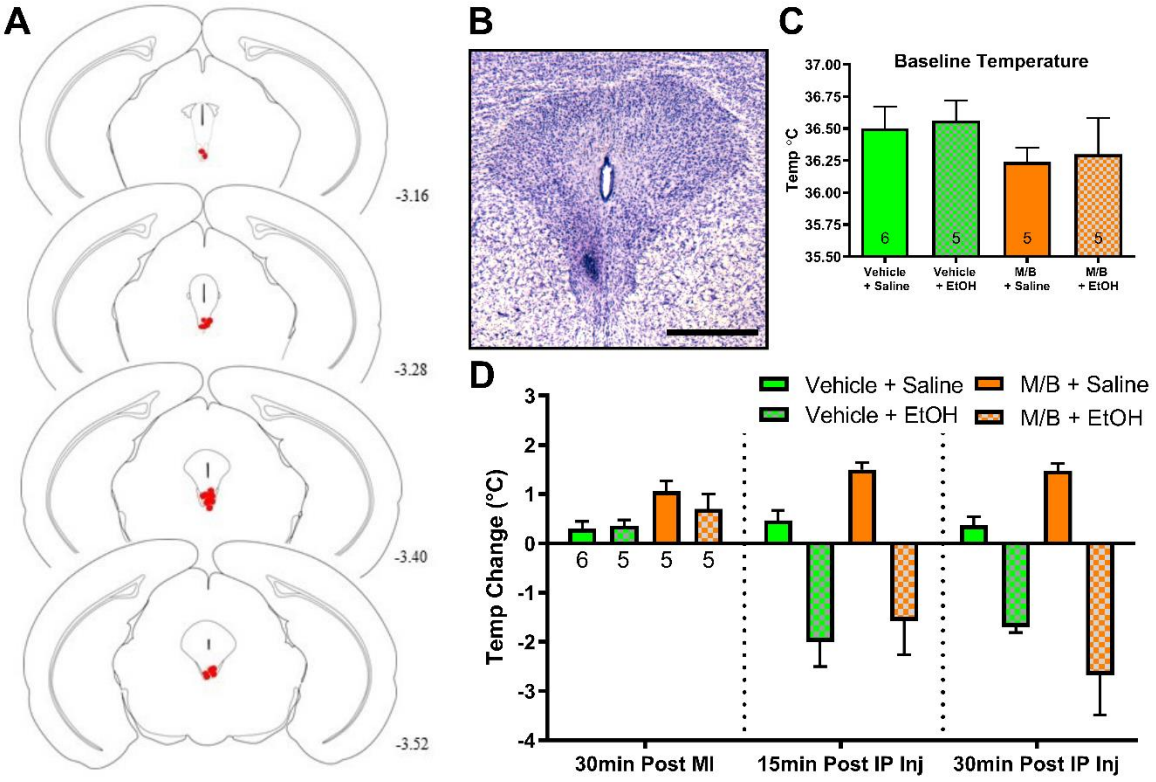

Fig. 5. Pharmacological inhibition of the EWcp increases body temperature.
Following recovery from stereotaxic surgery and cannula implantation, mice received a single microinfusion of 100 nL of Muscimol (0.1 mM) + Baclofen (1.0 mM) (M+B) or vehicle into the EWcp, 30 min prior to receiving a single IP injection of either 3 g/kg EtOH or saline. Rectal temperatures were measured 30, 45, and 60 min post microinfusion. A) Cannula location placements for mice included in the study. B) Brightfield image of thionin staining used to confirm cannula placement location. C) No differences in baseline temperature were detected between groups. D) Microinfusion of M+B increased body temperature significantly in mice given a saline injection. Scale bar denotes 500 µM.

Body Temperature

As there were no differences in baseline rectal temperature (Fig. 5C) between groups, data for the three time points are presented as change-from-baseline values in Fig. 5D. As can be seen, 30-min after mice received the microinfusion, inhibition of the EWcp via M+B increased body temperature significantly (30 min post MI). When mice were then given an IP injection of 3 g/kg EtOH, body temperatures significantly decreased, compared to mice that received a saline

injection (15 min Post IP inj.). In support of this, a two-way ANOVA found that there were no significant differences in baseline temperatures between groups (Microinfusion x Treatment), as no significant main effects or interactions were detected. In contrast, a two-way ANOVA (Microinfusion x Treatment) for temperature 30 min after the microinfusion revealed a significant main effect of Microinfusion, demonstrating that inhibition of the EWcp significantly increased body temperature when compared to vehicle [$F(1, 17) = 7.3$, $p < .05$]. When the two time points after the IP injection were analyzing using a three-way RM ANOVA (Time x Microinfusion x Treatment) a main effect of Treatment was detected, confirming that EtOH significantly decreased body temperature. Additionally, a significant Time x Microinfusion x Treatment three-way interaction was detected. In order to better understand this interaction, individual two-way ANOVAs were conducted for each of the two post-IP injection time points (15 min post IP inj., 30 min post IP inj.). These ANOVAs showed main effect of Treatment 15 [$F(1, 17) = 42.2$, $p < .0001$] and 30 min [$F(1, 17) = 59.7$, $p < .0001$] after the IP injection, again indicating that the decrease in body temperature in EtOH-treated mice was significant. Furthermore, a significant Microinfusion x Treatment interaction was detected 30, but not 15 min after the EtOH injection [$F(1, 17) = 6.7$, $p < .05$]. A Bonferroni post-hoc analysis for this interaction revealed that although EtOH significantly decreased body temperature in both microinfused groups, the difference in temperature between EtOH- and saline-treated mice was greater in M+B-treated animals, than in mice that received a vehicle microinfusion. No other significant main effects or interactions were detected at any of the time points.

Experiment 4: Inhibition of the EWcp and ethanol-induced c-Fos

Injector Placement

One mouse was excluded from the analysis due to a lost headcap. The three mice with incorrect injector placements were included in the analysis as negative controls (M+B Miss). Final group numbers are shown within Fig. 6D.

c-Fos Expression in the EWcp following an EtOH injection

Representative brightfield photographs illustrating c-Fos immunoreactivity are presented in Fig. 6A-C. The mean number of c-Fos positive cells in the EWcp in mice that received a microinfusion of either M+B or vehicle prior to an EtOH injection are presented in Fig. 6D. As can be seen, inhibition of the EWcp via M+B 15 min prior to a 2 g/kg IP EtOH injection reduced the number of c-Fos positive cells in the EWcp. Importantly, this effect was not seen in mice with incorrect injector placements (M+B Miss). In support of this, a one-way ANOVA revealed a main effect of Treatment [$F(2, 6) = 119.5$, $p < .0001$]. A Bonferroni post-hoc analysis revealed that the M+B group was significantly different than both the Vehicle ($p < .0001$), and the M+B Miss ($p < .0001$) group, but that the Vehicle and M+B Miss groups were not significantly different ($p = .52$).

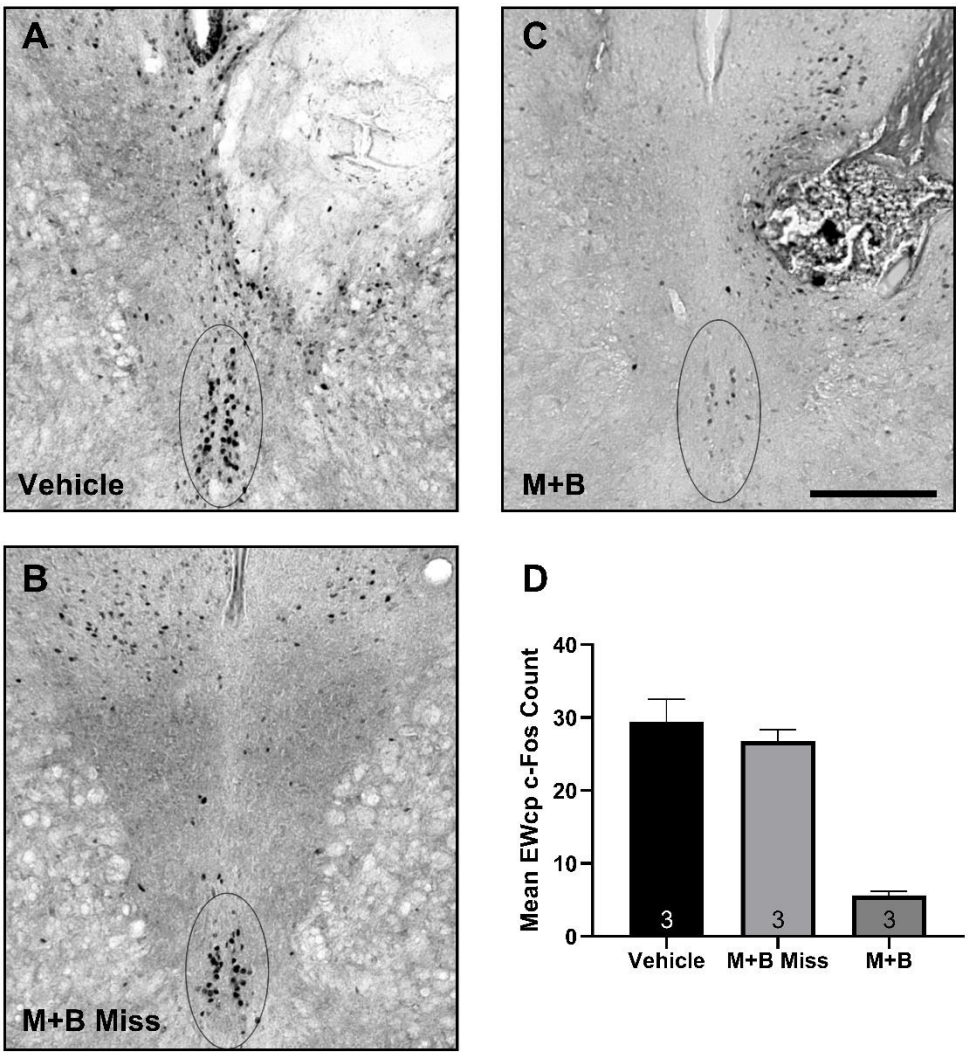

Fig. 6. Pharmacological inhibition of the EWcp attenuates EtOH-induced c-Fos expression. Following recovery from stereotaxic surgery and cannula implantation, 100 nL of Muscimol (0.1 mM) + Baclofen (1.0 mM) (M+B) or saline were microinfused into the EWcp 30 min prior to an EtOH injection (2 g/kg IP). A). Representative photomicrograph of cannulae placement and c-Fos expression in vehicle-treated mice. B.) Representative photomicrograph of cannulae placement and c-Fos expression in M+B-treated mice with incorrect cannulae placements. C) Representative photomicrograph of cannulae placement and c-Fos expression in M+B-treated mice D.) Pretreatment with M+B prevents the EtOH-induced increase in c-Fos expression in the EWcp. Scale bar denotes 200 µM.

Discussion

The studies presented here demonstrate that pharmacological inhibition of the EWcp alters body temperature in the absence of EtOH, prevents EtOH-induced c-Fos expression in the EWcp, but does not affect the acquisition or expression of EtOH-induced CPP. More specifically, we found that intra-EWcp microinfusion of the GABAAR and GABABR agonists muscimol and baclofen (M+B) during the conditioning or preference test phases of CPP was not sufficient to alter place preference. Additionally, these studies show that inhibition of the EWcp increases body temperature in the absence of ethanol, indicating that the EWcp may be involved in temperature regulation. Lastly, microinfusion of M+B into the EWcp attenuated the increased expression of c-Fos following an ethanol injection.

To date, these are the first studies examining the effect of acute pharmacological inhibition of the EWcp on the acquisition and expression of EtOH-induced CPP. Here, we report that inhibition of the EWcp does not alter either the acquisition or the expression of EtOH-induced CPP. It could be theorized that inhibition of the EWcp during the acquisition phase did not alter the development of place preference because the 30-min delay between the microinfusion and the conditioning trial was too long, and as such lower concentrations of the drug were present in the EWcp during conditioning. The fact that we see decreased c-Fos expression in M+B-treated mice (Exp. 4) following an EtOH injection (using the same dose and timeline) suggests that this is not the case. In regard to the expression of CPP, it is possible that inhibition of the EWcp following 4 conditioning trials is simply not sufficient to alter place preference due to the fact that place preference is near-asymptotic after that many conditioning trials (Groblewski, Bax, & Cunningham, 2008). Future experiments inhibiting the EWcp during preference testing but following fewer trials could help elucidate this possibility. When

considered in light of the numerous reports showing that the EWcp is involved in the regulation of self-administration, our data suggest that the EWcp may not be involved in mediating conditioned responses to EtOH-paired cues. In support of this, when c-Fos expression across the brain was analyzed following exposure to EtOH or an EtOH-paired cue, the EWcp showed increased c-Fos expression after exposure to EtOH, but not following exposure to the EtOH-paired cue (Hill, Ryabinin, & Cunningham, 2007). Interestingly, our data are somewhat at odds with previous work showing that mice lacking Ucn1 do not develop place preference for an EtOH-paired floor (Giardino et al., 2011). The use of different genotypes between studies, as well as the compensatory mechanisms associated with KO mice make it difficult to compare these two findings. Even so, the potential involvement of the various neuropeptides expressed in the EWcp in conditioned behaviors requires further attention moving forward.

Our finding that inhibition of the EWcp increases body temperature in the absence of EtOH adds to previous literature that has implicated the EWcp in thermoregulation. Indeed, acute exposure to warm (34º C) or cold (10º C) ambient temperatures increases c-Fos expression in the EWcp (Bachtell, Tsivkovskaia, & Ryabinin, 2003). Furthermore, Ucn1 expression in the EWcp is positively correlated with baseline body temperature and negatively correlated with EtOH-induced hypothermia in the male F2 offspring of C57BL/6J and DBA/2J mice, and is higher in one of the replicates of HOT versus COLD mice, strains selectively bred for differences in their hypothermic response (Bachtell, Tsivkovskaia, & Ryabinin, 2002). Lastly, electrolytic lesions of the EWcp blunt the hypothermic response following an injection of EtOH (Bachtell et al., 2004). Given the known Ucn1-positive projections to the DRN, and the known involvement of DRN in thermoregulation (Ginefri-Gayet & Gayet, 1993; Hale, Dady, Evans, & Lowry, 2011; Ishiwata et al., 2016), one is tempted to hypothesize that the hyperthermic results

presented here are mediated through an EWcp-DRN mechanism. This specific pathway has previously been shown to be involved in modulating body temperature, as intra-DRN microinfusion of Ucn1 significantly decreases body temperature (Turek & Ryabinin, 2005a). Furthermore, it has been hypothesized that EtOH-induced hypothermia may be mediated by inhibitory effects of Ucn1 on the DRN (Turek & Ryabinin, 2005b). Thus, our finding that acute inhibition of the EWcp increases body temperature, in combination with the hypothermic effects of Ucn1 in the DRN, suggests that the EWcp may be acting to regulate body temperature by modulating neuronal activity in the DRN.

Robust increases in c-Fos expression in the EWcp following administration of EtOH have been reported numerous times, across various models and species (Anacker, Loftis, Kaur, & Ryabinin, 2011; Bachtell, Wang, Freeman, Risinger, & Ryabinin, 1999; Ryabinin, Wang, Freeman, & Risinger, 1999; Ryabinin, Galvan-Rosas, Bachtell, & Risinger, 2003; Sharpe, Tsivkovskaia, & Ryabinin, 2005; Smith, Li, Cote, & Ryabinin, 2016; Walcott & Ryabinin, 2017). Here, we report that microinfusion of GABAAR and GABABR agonists into the EWcp prior to an ethanol injection prevents this increase in c-Fos expression. The GABAAR agonist muscimol has been shown to decrease the firing activity of neurons in the locus coeruleus (Jin, Cui, Zhong, Jin, & Jiang, 2013), as well as in midbrain regions, including the DRN (Kim et al., 2018) and the VTA (Theile, Morikawa, Gonzales, & Morrisett, 2011). Similarly, decreases in cell firing rates, as well as in burst activity have been observed when the GABABR agonist baclofen has been applied to cells in the substantia nigra (Engberg, Kling-Petersen, & Nissbrandt, 1993; Erhardt, Andersson, Nissbrandt, & Engberg, 1998) and VTA (Y. Chen, Phillips, Minton, & Sher, 2005), as well as direct hyperpolarization of neurons when applied in the DRN (Chieng & Christie, 1995). Given these findings, we hypothesized that microinfusion of

a combination of muscimol and baclofen would have similar effects on neurons in the EWcp. Previous work has suggested that increased c-Fos expression in the EWcp following an EtOH injection is mediated through activation of the MEK1-2 and ERK1-2 pathway (Bachtell et al., 2002a). As it is known that Ca^{2+} influx, either through voltage-dependent ion channels or receptor-mediated Ca^{2+} channels, activates ERKs through its actions on the Ras pathway (Finkbeiner & Greenberg, 1996; Grewal, York, & Stork, 1999; Rosen, Ginty, Weber, & Greenberg, 1994), GABAAR and GABABR activation in the EWcp via M+B could prevent c-Fos expression by preventing Ca^{2+} influx. Somewhat in contrast to our findings, previous work had led to the hypothesis that GABAAR activation is necessary for increased c-Fos expression in EWcp neurons, as inhibition of GABAAR prevented EtOH-induced c-Fos expression in the EWcp (Bachtell et al., 2002a). Furthermore, GABAAR positive modulators also result in increased c-Fos expression in the EWcp, although at lower levels than EtOH (Bachtell et al., 2002a). Importantly however, both the GABAAR inhibitors and positive modulators were administered systemically via an IP injection in this previous study. That systemic inhibition of GABAAR prevents EtOH-induced c-Fos expression, in a similar manner as intra-EWcp inhibition via muscimol and baclofen suggests that EtOH's ability to increase c-Fos in the EWcp might be mediated by upstream regions. Systemic inhibition of GABAA receptors may prevent EtOH's actions in brain regions that project to the EWcp, thus preventing the EtOH-induced c-Fos expression.

In summary, the present studies demonstrate that pharmacological inhibition of the EWcp is not sufficient to alter EtOH-induced CPP, suggesting that the EWcp may play a more selective role in regulating the self-administration of EtOH, and not conditioned EtOH-seeking behaviors. Additionally, our data provide further evidence for the EWcp's involvement in thermoregulation,

as inhibition via a microinfusion of M+B significantly increased body temperature. Lastly, we report that inhibition of the EWcp prevents EtOH-induced increased in c-Fos expression, further elucidating the mechanisms that may mediate EtOH's effects in the EWcp.

Chapter 3: Effects of Chemogenetic Activation of the EWcp on Voluntary EtOH Drinking in C57BL/6J Mice

Acknowledgements: Dr. Monique L. Smith contributed to Exp. 5 in this chapter. She performed the stereotaxic surgeries, as well as the cell counts presented in Fig. 7D.

Introduction

Alcohol use disorder (AUD) is a progressive disease with detrimental consequences for both the affected individual and society as a whole. Globally, 3.3 million people die from alcohol-related causes annually, making it the fifth leading cause of premature death and disability (Lim et al., 2012). Transition into compulsive alcohol use is hypothesized to be due to alcohol-induced adaptations within several neural circuits, including stress regulatory systems (Koob, 2013). As a member of the corticotropin releasing factor (CRF) family of neuropeptides, the Urocortin 1 (Ucn1) system has been implicated as a mediator of the stress response as well as of ethanol- (EtOH) seeking behaviors.

Ucn1 is known to bind to both CRF1R and CRF2R with a higher affinity than CRF itself (Vaughan et al., 1995). Within brain, the primary source of Ucn1 is the centrally-projecting Edinger-Westphal nucleus (EWcp). The EWcp is a midbrain structure located along the midline between the most caudal sections of the ventral tegmental area (VTA) and the rostral dorsal raphe nucleus (DRN) (Dos Santos Junior et al., 2015; Kozicz et al., 2011). The EWcp sends projections to a number of brain areas involved in motivated behaviors, including the LS, BNST, and CeA (Dos Santos Junior et al., 2015). The EWcp is distinct from the preganglionic Edinger-Westphal nucleus (EWpg), which has peripheral projections and is involved in oculomotor adaptation (Kozicz et al., 2011).

Using c-Fos expression as a marker of neuronal activation, it has been shown that oral EtOH self-administration, as well as IP injections of EtOH and exposure to EtOH vapor, induce neuronal activity in the EWcp (Anacker et al., 2014; Bachtell et al., 1999; Ryabinin et al., 2001, 1997; Smith et al., 2016; Walcott & Ryabinin, 2019). In addition to its induction by EtOH, it is becoming evident that the EWcp may also play an important role in the regulation of alcohol

consumption. Accordingly, electrolytic lesions of the EWcp attenuate EtOH consumption in C57BL/6J mice (Bachtell et al., 2004; Giardino et al., 2011), and genetic deletion of Ucn1 decreases ETOH intake and prevents the development of EtOH-induced CPP (Giardino et al., 2011). In addition, viral knock-down of Ucn1 within the EWcp attenuates EtOH intake (Giardino et al., 2017). Further support comes from genetic studies, indicating that majority of mouse and rat strains selectively bred to prefer alcohol have higher levels of Ucn1 in the EWcp than alcohol-avoiding strains (Bachtell et al., 2002b; Fonareva et al., 2009; Turek et al., 2005). These findings clearly indicate that EWcp-Ucn1 neurons contribute to alcohol consumption and reward (as measured by CPP). Yet, the techniques used to obtain this evidence are subject to developmental compensations and/or unintended effects caused by cannulations and lesions.

Recent advances in chemogenetic technology have provided the field of neuroscience with new tools that can be used to modulate neuronal activity without the limitations associated with cannulations and lesions. Specifically, Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) have been used to inhibit or activate specific neuronal populations. Moreover, many published studies have used DREADDs to affect behavior (For review see: Alexander et al., 2009; Armbruster, Li, Pausch, Herlitze, & Roth, 2007) DREADDs are mutant muscarinic acetylcholine receptors that trigger G-protein-coupled signaling when clozapine-N-oxide (CNO) is bound (Armbruster et al., 2007). Given the potential involvement of the EWcp in the regulation of EtOH intake, and the ability to acutely control neuronal activity via CNO and DREADDs, the current studies elucidate the functional outcome of manipulative activity of EWcp neurons on EtOH drinking utilizing DREADDs during 24-h access 2-bottle choice (2-BC) drinking.

Materials and Methods

General Methods

Subjects

For Exp. 5, adult male C57BL/6J (B6) mice (The Jackson Laboratory, CA, 7-8 weeks upon arrival) were housed 3-5 per cage upon arrival and allowed to acclimate to the animal colony for 7 days prior to surgery. Male Ucn1 WT and KO mice used for Exp. 6 were littermates from heterozygous matings. Ucn1 KO mice contained a deletion of exon 2 of the Ucn gene and created in a 12961/SvJ x B6 background (Vetter et al., 2002); they have since been backcrossed for over 15 generations onto B6 background. Male and female Vglut2-Cre and WT littermates were generated from Vglut2-ires-Cre knock-in on a B6 background (Jackson Lab stock # 028863) and C57BL/6J (Jackson Lab stock # 000664) breeding pairs purchased from the Jackson Laboratory. Following acclimation to the animal colony (for Exp. 5) or after reaching adulthood (Exp 6,7), all mice were subjected to stereotaxic surgery (described below), individually housed, and allowed to recover for 7-10 days prior to the start of the experiment. Following recovery, mice were transferred to the experimental room with a 12/12 reverse light/dark cycle (lights on at 9:00 PM) for a 7-day acclimation period prior to the initiation of the experiments. All housing rooms were temperature and humidity controlled and food and water were available *ad libitum*. All protocols were approved by the Oregon Health & Science University animal care and use committee and performed within the National Institutes for Health Guidelines for the Care and Use of Laboratory Animals, as well as the Guidelines for the Care and use of Mammals in Behavioral Research.

Drugs

EtOH solutions were prepared in tap water from 95% ethyl alcohol. CNO (1 mg/kg, Sigma-Aldrich, MO) was made fresh daily by being dissolved in 0.5% DMSO (Sigma-Aldrich, MO) in sterile saline.

Stereotaxic surgery and viral infusions

7 to 10 days prior to the start of each experiment, mice were transported to a suite for stereotactic surgery. Mice were anesthetized via 5% isoflurane delivered in oxygen via a precision vaporizer (DatexOhmeda, WI). Following induction, mice were maintained under 1-2% isoflurane anesthesia and secured in a stereotaxic frame. Once in the stereotaxic frame, mice were given a subcutaneous injection of the non-steroidal anti-inflammatory drug carprofen (5 mg/kg) to aid recovery. The EWcp was targeted using a 20 ^{o} angle so as to avoid the sagittal sinus (coordinates: A/P -3.45 mm from bregma, M/L -1.20 mm, D/V -3.5 mm) based on previous studies (Giardino et al., 2011, 2017). A single burr hole was drilled 3.45 mm from bregma, and 1.2 mm from the midline, and a stainless-steel injector (32 ga) attached to a 1 μl Hamilton syringe via polyethylene tubing was lowered above the EWcp. Mice in Exp. 1 received a 150 nL infusion of excitatory AAV8-hSyn-hM3D-Gq-mCherry (hM3Dq), inhibitory AAV8-hSyn-/hM4D-Gi-mCherry (hM4Di) or control AAV8-hSyn-gfp (GFP) DREADD (Catalog #: 50474-AAV8, 50475-AAV8, 50465-AAV8, Addgene, MA). All mice in Exp. 2 received 150 nL of the excitatory (AAV8-hSyn-hM3Dq) virus (Catalog # 50474-AAV8, Addgene, MA) while all mice in Exp. 3 received 150 nL of the Cre-dependent AAV8-hSyn-DIO-HA-hM3D-Gq-IRES-mCitrine (DIO-hM3Dq) virus (Catalog # 50454-AAV8, Addgene, MA). In all 3 experiments, viral infusions were conducted over the course of 5 min. Injectors were left in place for 10 min and extracted over the course of 5 min. Following recovery from anesthesia, mice were individually housed and transported back to the animal colony for 7 days to allow for viral gene

transduction and expression and recovery from surgery. In the three experiments outlined below, we examined the effects of chemogenetic modulation of the EWcp in separate groups of mice transduced with the excitatory, inhibitory, or gfp-containing virus (Exp. 5), the excitatory virus (Exp. 6), and the cre-dependent excitatory virus (Exp.7) In all three experiments, mice were transported to the experimental room in individual housing and were allowed to acclimate to a reverse light dark cycle for 7 days prior to alcohol access. For all experiments, injections were considered a "hit" when neuronal expression of the virus was limited to < 1.0 mm total diameter including/surrounding the EWcp. When spread of the virus was beyond this, it was considered a "miss". This procedure led to an 85% success rate. Any misses were excluded from analysis.

Exp. 5: Chemogenetic modulation of the EWcp

Exp. 5A: Effect of chemogenetic inhibition or activation on c-Fos expression in the EWcp

We first conducted a pilot experiment testing whether CNO or vehicle injection affected levels of c-Fos immunoreactivity in mice expressing the hM4Di or hM3Dq DREADD in the EWcp. Following recovery from surgery, a subset of mice were given a single injection of CNO (1 mg/kg IP) or vehicle and were then euthanized via CO_2 inhalation 2 h later.

Exp. 5B: Effect of chemogenetic modulation of the EWcp on EtOH intake

24-h access 2-Bottle Choice (2-BC)

Exp. 5B was conducted to examine the effects of chemogenetic inhibition or activation on EtOH intake. As described above, all mice received a stereotaxic infusion of an AAV carrying either the hM4Di, hM3Dq, or the GFP construct. During the acclimation period, mice were allowed 24-h access to two 25ml glass cylinder bottles with metal sipper tubes (containing

water) on either side of the cage, with food evenly spread across the cage top. Mice underwent 12 days of drinking, during which they received 24-h access to two bottles: one containing tap water and one containing increasing concentrations of EtOH (3-10%) dissolved in tap water. Each concentration (3, 6, 10% EtOH v/v in tap water) was available for 4 days during the initial escalation period. All bottles were introduced and fluid levels were recorded on a daily basis at 2 h into the dark cycle. The locations of the bottles on the cages (left vs. right) were alternated daily to avoid the potential confound of an inherent side preference.

CNO Administration

Mice received a single IP injection of CNO (1 mg/kg; 10 mL/kg) or vehicle (0.5% DMSO in saline) at the beginning of the dark cycle on day 13, following 12 days of 24 h access to EtOH and water. Treatment groups were counterbalanced based on EtOH intake during the 12 days of drinking. Following the IP injection of CNO or vehicle, mice were given access to two bottles, one containing 10% EtOH, and the other water, and intake was measured at 4 h post injection.

Exp. 6: Chemogenetic activation of the EWcp in Ucn1 WT and KO mice

24-h access2-BC

Exp. 6 was designed and carried out in order to characterize the involvement of Ucn1 in hM3Dq-mediated changes in EtOH intake. As such, Ucn1 WT and KO mice were all transduced with the hM3Dq DREADD as described above. Following recovery from surgery and acclimation, mice were exposed to the same 24-h 2-BC described in Exp. 5B. Briefly, for 12 days, mice were given 24-h access to two bottles: one containing tap water and one containing EtOH (3-10%) dissolved in tap water. Daily intake levels were measured and recorded 2 h into

the dark cycle, and bottles locations were switched daily so as to prevent the development of a side preference.

CNO Administration

As with Exp. 6, mice in Exp. 6 received a single IP injection of CNO or vehicle at the beginning of the dark cycle on day 13, following 12 days of 24 h access to EtOH and water. Within WT and Ucn1 KO mice, treatment groups were counterbalanced based on EtOH intake during the 12 days of drinking. Following the IP injection of CNO or vehicle, mice were given access to two bottles, one containing 10% EtOH, and the other water, and intake was measured at 4 h post injection. A timeline of the experimental procedure can be found in Fig. 9A.

Exp. 7: Chemogenetic activation of Vglut2-expressing neurons in the EWcp

24-h access2-BC

Using a Cre-dependent DIO-hM3Dq DREADD, in combination with Vglut2-Cre and WT mice, Exp. 7 examined the role of EWcp Vglut-2-expressing neurons in the modulation of EtOH intake. As with Exp. 5 and 6, mice in Exp. 7 had 24h access to two 25 mL glass cylinder bottles with metal sipper tubes (containing water) on either side of the cage, with food evenly spread across the cage top. Following habituation to the bottles, mice were given 24 h access to bottles for 12 days, containing EtOH (3, 6, 10% EtOH v/v in tap water) or tap water (see Fig. 11A and 12A). We next assessed the effects of chemogenetic activation of the EWcp on sucrose intake and preference. Mice were given 7 days of access to two 25 mL glass tubes with only water following the last day of CNO administration (day 15). Following the water-only period, mice were given 4hr access to 2% sucrose, in addition to a bottle containing water, to assess baseline

sucrose intake and preference. 24 h later, mice were again given 4 h access to 2% sucrose and water. 48 h later, mice were given their last access period to 2% sucrose.

CNO Administration

For Exp. 7, all mice were given both vehicle and CNO IP injections, spread 48 h apart. Following 12 days of 24 h access to EtOH and water all mice were given an IP injection of CNO or vehicle at the beginning of the dark cycle on day 13. Intake was then measured 2 h, 4 h, and 24 h after the IP injection. Following an additional 24 h "wash-out", during which mice had continued access to both EtOH and water, mice were given a second IP injection of the treatment opposite of what they initially received, at the beginning of the dark cycle. That is, if mice received CNO initially, they received vehicle the second time, and vice versa. Intake was measured 2 h, 4 h, and 24 h after the injection. Mice were then given an additional seven day "wash-out" period during which they only had access to water. Following this period, mice were given an IP injection of CNO or vehicle at the beginning of the dark cycle, and were then given access to 2 bottles, one containing 2% sucrose, and the other water for 4 h. Intake was measured 2 h and 4 h after the injection. After the 4 h period, mice were again given access to only water. 48 h later, mice were given a second injection (opposite of what they received the 1st sucrose day), and again had access to 2% sucrose and water for 4 h, during which intake was measured at the 2 and 4 h timepoints. 48 h later, a subset of Vlgut2-Cre mice were given one final injection of either CNO or vehicle in order to investigate the effects of CNO administration of c-Fos expression in hM3Dq-expressing neurons. These mice were euthanized 2 h after the CNO or vehicle treatment. The timelines for each behavioral experiment are displayed within the first panel of each figure.

Brain Extraction and Immunohistochemistry

On the final day of each experiment, mice were euthanized via CO_2 inhalation, brains were extracted, post-fixed for 24 h in 2% paraformaldehyde/ phosphate-buffered saline (PBS) and cryopreserved in 20% sucrose (in PBS), followed by 24 h in 30% sucrose (in PBS). Brains were sliced at 30 μm and processed for Ucn1, mCherry, mCitrine, HA, Vglut2, and c-Fos immunohistochemistry. The subset of mice used for the c-Fos analysis in Exp 5 and 7 were sacrificed 2 h after they received an injection of CNO or vehicle. Unless noted otherwise, all steps were performed in 0.3% Triton-X/Tris-buffered saline (TBS) and preceded by three washes in TBS. The sections were rinsed for 30 min in 1% sodium borohydride in TBS, and blocked in 5% normal donkey serum (Jackson Laboratories) for 45 min. The tissue was then incubated with 1:5000 goat polyclonal Ucn1 antibody (Santa Cruz), 1:15000 rabbit polyclonal c-Fos antibody (Sigma-Aldrich, MO), 1:1000 rabbit polyclonal influenza hemagglutinin (HA) antibody, 1:850 rabbit or goat polyclonal green fluorescent protein antibody (for mCitrine detection) (Abcam, MA), 1:850 rabbit polyclonal Vglut2 antibody (Synaptic Systems, Germany), or 1:2500 rabbit polyclonal DS-Red (for mCherry detection) (Clontech, CA). This was followed by 1 h incubations with AlexaFluor 555-labeled and AlexaFluor 488-labeled secondary antibodies (raised in donkey) (Invitrogen Thermo Fisher, MA). Finally, sections were washed with PBS, mounted on gelatinized slide and coverslipped with Prolong Gold (Invitrogen Thermo Fisher, MA). Co-localization of immunoreactivity was quantified manually (see Statistics sections below) using a Leica DM4000 microscope.

Statistics

Based on the appropriate concentration, EtOH consumption in mL was converted to grams and divided by the animal's body weight to give daily intake scores expressed in grams per kilogram (g/kg). Sucrose and water consumption were divided by the animal's body weight to give values expressed in mL/kg. The initial 12-day drinking data were averaged over the 4 days of access to each concentration, such that a single value for 3, 6, and 10% were calculated for each animal. These data were then analyzed via ANOVAs for each concentration, with Sex (male or female), Virus (DREADD or gfp) or Genotype (Ucn1-WT or KO, WT or Vglut2-Cre) as the between groups factors. On treatment days for Exp. 5 and 6, drinking data were analyzed via a two-way ANOVA with Virus and Treatment (Exp. 5) or Genotype and Treatment (Exp. 6) as the between group factors. Bonferroni post-hoc analyses were conducted when appropriate. For Exp. 7, intake data following treatment were analyzed via a three-way repeated measures (RM) ANOVA with Treatment as the within groups factor, and Sex and Genotype as the between groups factors. For c-Fos cell-count analyses, a single c-Fos value was calculated for each animal by averaging the cell counts across the 5-7 sections that encompassed the EWcp in each animal. Thus, each animal had a single c-Fos cell count value for the EWcp. For Exp. 5 c-Fos data were analyzed using a two-way ANOVA with Virus (hM3Dq vs hM4Di) and Treatment (CNO vs vehicle) as the between group factors. In Exp 7, c-Fos data were analyzed using a Student's t-test comparing CNO- and vehicle-treated mice. All data are expressed as mean + standard error of the mean (SEM).

Results

Exp. 5A: Expression and activation of DREADDs in the EWcp

To investigate whether activity of EWcp neurons could be manipulated by chemogenetic approaches, we transduced EWcp with hM4Di or hM3Dq DREADDs using AAV8. EtOH-naïve

mice were given a CNO or vehicle injection (Fig 7A) and were sacrificed 2 h post injection for c-Fos analyses. As expected, a single injection of 1.0 mg/kg CNO (but not vehicle) led to activation of c-Fos in nearly all hM3Dq-transduced neurons of EWcp and surrounding area 2 h after the injection (Fig.7C). In contrast, significant changes in c-Fos were not detected in hM4Di-transduced neurons. Quantitative analysis (Fig. 7D) confirmed this observation. Specifically, a two-way ANOVA found main effects of Virus [$F(1, 15) = 30.8$, $p < .0001$] and Treatment [$F(1, 15) = 28.6$, $p < .0001$], as well as a significant Virus x Treatment interaction [$F(1, 15) = 28.1$, $p < .0001$]. A Bonferroni-adjusted post-hoc analysis for this interaction confirmed that CNO significantly increased the number of c-Fos-positive cells only in mice tranduced with the hM3Dq DREADD ($p < .001$). To our surprise, we found no mCherry expression in Ucn1-expressing neurons, suggesting that this AAV serotype did not transduce Ucn1-neurons in the EWcp. When we compared the exact location of Ucn1-positive neurons to that of mCherry-expressing neurons, we found that these neurons formed two distinct populations within the EWcp (Fig. 7E-F).

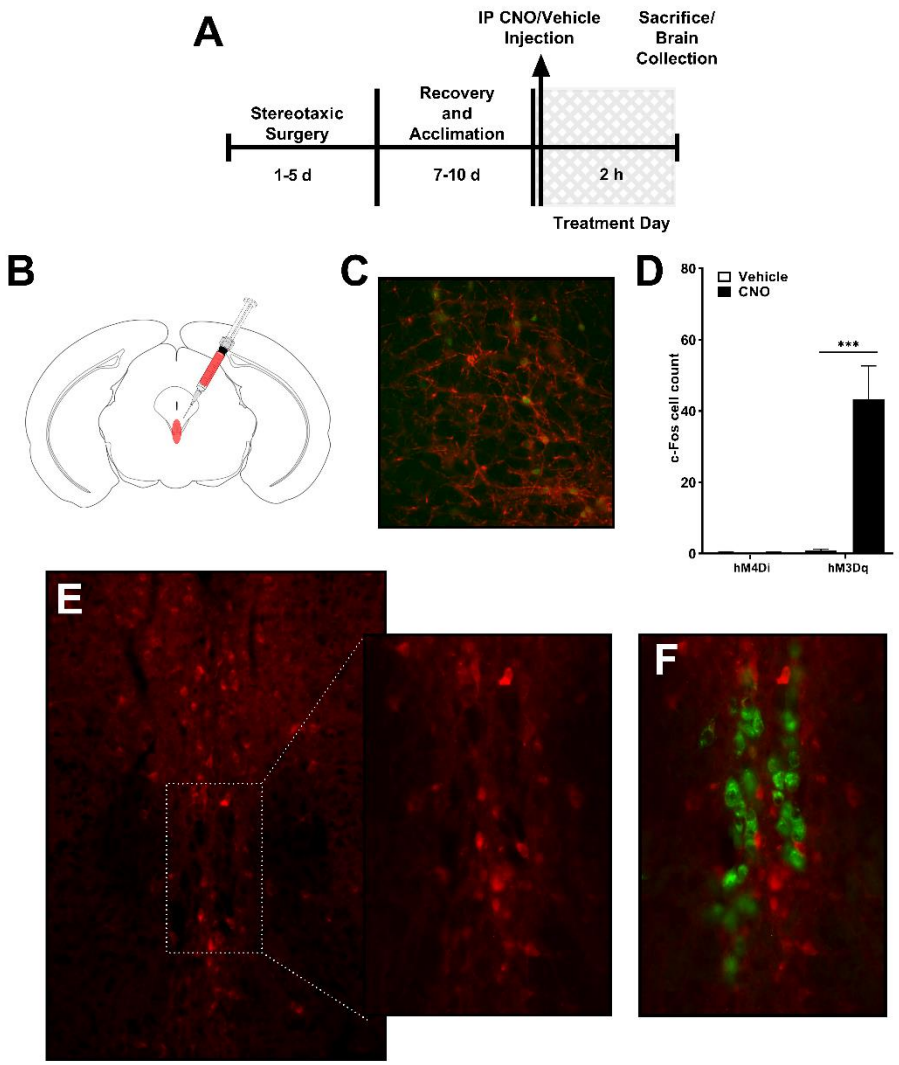

Fig. 7. Procedural timeline and transduction of neurons in the EWcp in male C57BL/6 mice.

Mice in Exp. 5A received an infusion of an AAV carrying either the hM3Dq or the hM4Di DREADD. Mice were given a single CNO injection (1 m/kg) and sacrificed 2 h later in order to analyze CNO-induced changes in c-Fos immunoreactivity. A) Experimental timeline, shaded region represents time period after CNO or vehicle treatment. B) Representative illustration demonstrating the angled approach used to target the EWcp. C) Representative photomicrograph of c-Fos (green) and mCherry (orange) co-expression in the EWcp of a hM3Dq-transduced mouse 2 h after a single injection of CNO. D). This CNO injection led to highly significant activation of c-Fos in the EWcp compared to vehicle and mice transduced with the hM4Di, which displayed little to no c-Fos activation. E) Representative immunofluorescent image of hM3Dq expression in the EWcp. F) Immunofluorescent image of hM3Dq expression, as indicated by mCherry (orange) and Ucn1 (green) in the EWcp, demonstrating no overlap between Ucn1 expression and the hM3Dq DREADD. ****, $p < .0001$.

Exp. 5B: Effect of chemogenetic modulation of the EWcp on 10% EtOH Intake

Exp. 5B was conducted in order to examine the effect of chemogenetic inhibition or activation on EtOH intake. Average EtOH and water intake levels over the course of the 12-day drinking period, as well as on the treatment day are depicted in Fig 8. There were no differences in intake based on the type of virus mice were transduced with, regardless of the EtOH concentration mice were drinking (Fig. 8B). Following the 12 days of 24 h 2-BC access, mice were given a single IP injection of either CNO or vehicle, and 4-h EtOH intake was measured. Activation of the EWcp in hM3Dq-transduced mice via an injection of 1.0 mg/kg CNO significantly decreased EtOH intake (Fig. 8C). CNO did not alter EtOH intake in GFP- or hM4Di-transduced mice. Lastly, CNO did not alter water intake in any of the viral groups during the 4-h period (Fig. 8D). In support of this observation, the only significant statistical finding was detected in the two-way ANOVA for EtOH intake 4 h-post injection, where a significant Virus x Treatment interaction was detected [$F(2,27) = 5.0$, $p < .05$]. A Bonferroni-adjusted post-hoc analysis found that there was a significant difference in EtOH intake between CNO- and Vehicle-treated mice only in mice transduced with the hM3Dq DREADD ($p = .009$). No other main effects or interactions were detected.

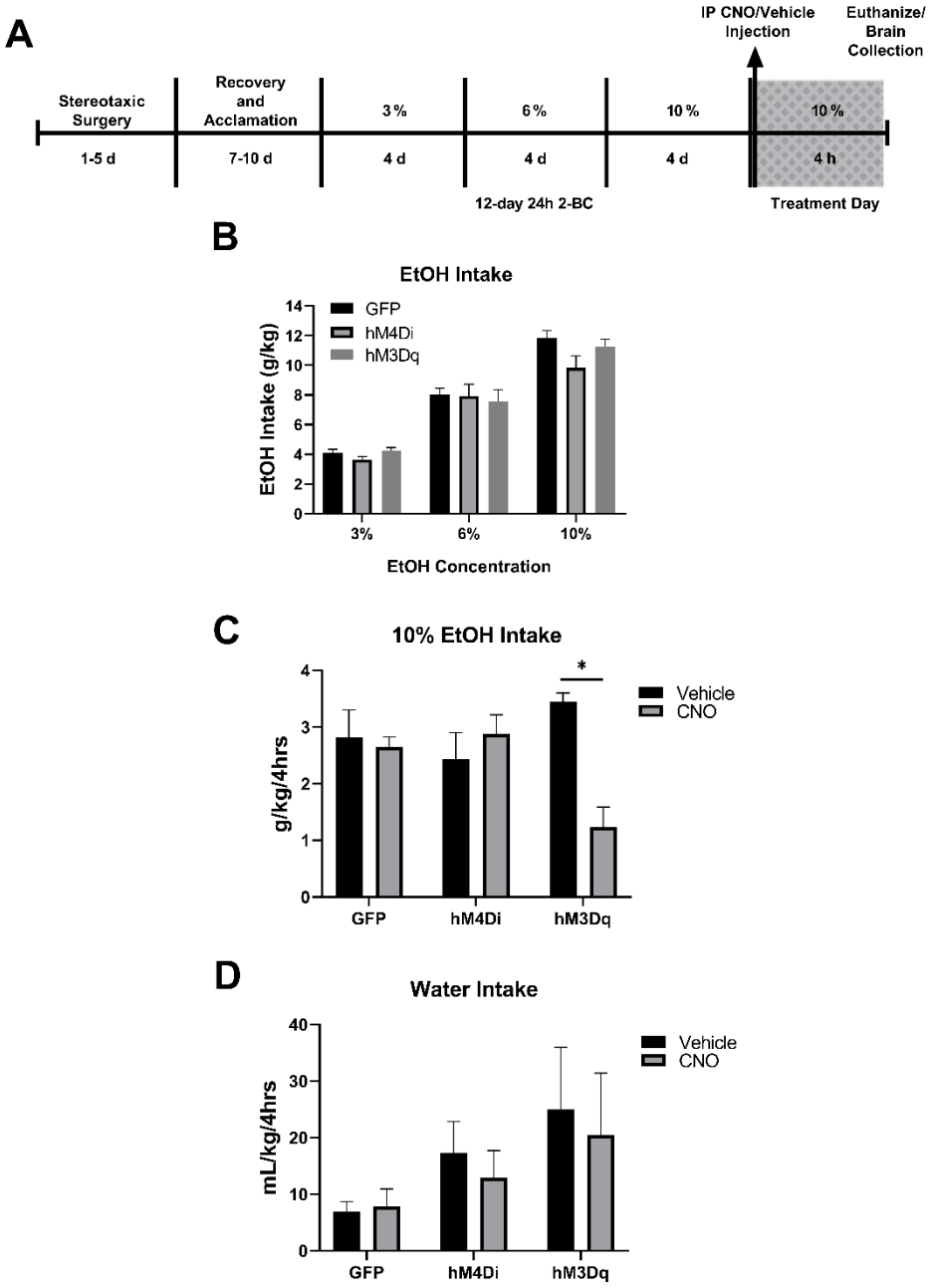

Fig. 8. Chemogenetic activation of the EWcp attenuates 10% EtOH intake.
Following recovery from surgery and acclimation, mice transduced with either the GFP-control, hM4Di, or hM3Dq DREADD were allowed access to increasing concentrations of EtOH (3-10%) prior to treatment with CNO or vehicle. A) Experimental timeline, shaded region represents intake periods after CNO or vehicle administration. B) No differences in 24-h EtOH (g/kg EtOH) intake between mice transduced with the GFP, hM4Di or hM3Dq viruses were detected at any of the EtOH concentrations. C) On the fifth day of 10% EtOH access, an injection of CNO (1.0 mg/kg) led to a significant decrease in EtOH intake in hM3Dq-transduced

mice compared to vehicle-treated mice. No significant differences in intake were detected in GFP- or hM4Di-transduced mice. D) There were no differences in water intake in any group following injection with CNO or vehicle.

Exp. 6: Effect of chemogenetic activation of the EWcp on 10% EtOH intake in Ucn1 WT and KO Mice

Based on our finding that chemogenetic activation of non-Ucn1 expressing neurons in the EWcp decreased EtOH intake, we next sought to determine if Ucn1 expression was required for this hM3Dq-mediated decrease in intake by using Ucn1 KO and Ucn1 WT mice. Fig. 9 depicts the average EtOH and water intake over the course of the 12-day drinking period, as well as EtOH and water intake 4 h post injection of CNO. During the 12-day 2-BC procedure, there were no significant differences in EtOH intake between Ucn1 WT and KO mice, at any of the EtOH concentrations (Fig 9B). As can be seen however, Ucn1 WT mice drank slightly more than Ucn1 KO mice during the 4 h window following treatment (Fig. 9C). Activation of the EWcp via an injection of CNO significantly decreased 10% EtOH intake in both and WT and Ucn1 KO mice 4-h post injection, compared to mice that received a vehicle injection (Fig. 9C). In contrast, CNO did not affect water intake in either genotype (Fig. 9D). A two-way ANOVA for EtOH intake post-treatment found a main effect of Treatment [$F(1,16) = 11.6$, $p < .01$], confirming that CNO decreased EtOH intake in both genotypes. A main effect of Genotype that was trending towards significant ($p = .07$) was also detected indicating that although EtOH intake between genotypes was not significantly different, Ucn1 WT mice did tend to drink more during the 4 h period. No other significant main effects or interactions were detected.

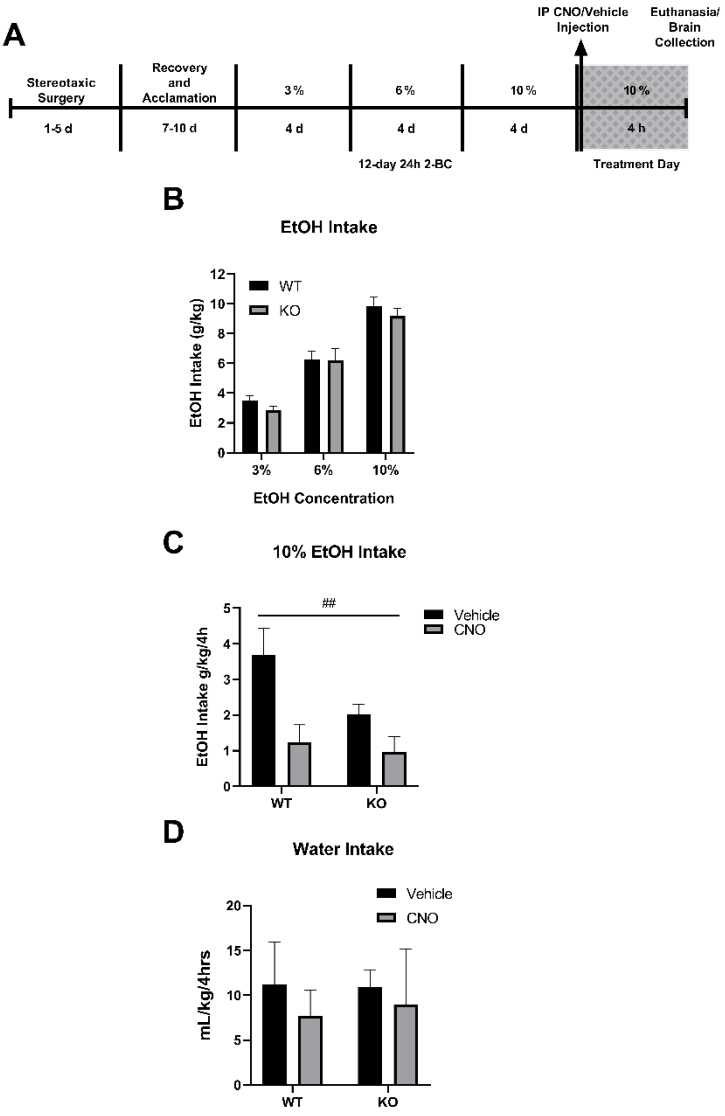

Fig. 9. Chemogenetic activation of the EWcp inhibits 10% intake in WT and Ucn1 KO mice.

Following recovery from hM3Dq injection and acclimation, WT and Ucn1 KO mice were allowed access to increasing concentrations of ethanol (EtOH, 3-10%) prior to treatment with CNO or vehicle. A) Experimental timeline, shaded region represents EtOH intake period after CNO or vehicle administration. B) g/kg EtOH intake did not significantly differ at any concentration during the 12-day drinking period when comparing WT and KO mice. C) Injection of CNO (1.0 mg/kg, IP.) significantly attenuated g/kg EtOH intake in both WT and Ucn1 mice. D) CNO did not alter water intake in either Ucn1 WT or KO mice. ##; main effect of treatment, $p < 0.01$.

Exp. 7A: Expression of Cre-dependent DREADDs in the EWcp of Vglut2-Cre mice

Based on our results from Exp. 5 and 6, demonstrating that activation of non-Ucn1-expressing cells in the EWcp decreases EtOH intake, we next sought to identify the neurochemical nature of the non-Ucn1-expressing neurons transduced by AAV8 viruses in EWcp. A search of the Allen Brain Atlas, indicated presence of Vglut2 mRNA in the vicinity of EWcp (Lein et al., 2007), suggesting that these neurons are glutamatergic. Therefore, in the next experiment we tested effects of chemogenetic activation of Vglut2-expressing neurons of EWcp on EtOH consumption. For this purpose we used AAV8 viruses to transduce neurons in the EWcp with a DIO-hM3Dq DREADD in Vglut2-cre mice. Following this transduction, robust expression of the DIO-hM3Dq in the EWcp was observed in Vglut2-Cre mice, as determined by mCitrine expression (Fig 10D). WT mice did not express the DIO-hM3Dq (Fig 10C). Subsequent fluorescent immunohistochemistry for the hM3Dq-fused influenza hemagglutinin (HA) tag (Fig. 10E), a marker of receptor expression, and for Ucn1 (Fig. 10F), demonstrated that Ucn1-positive neurons did not express the Cre-dependent DIO-hM3Dq DREADD (Fig. 10G), further indicating that Ucn1- and Vglut2-expressing neurons are two distinct populations of neurons in the EWcp.

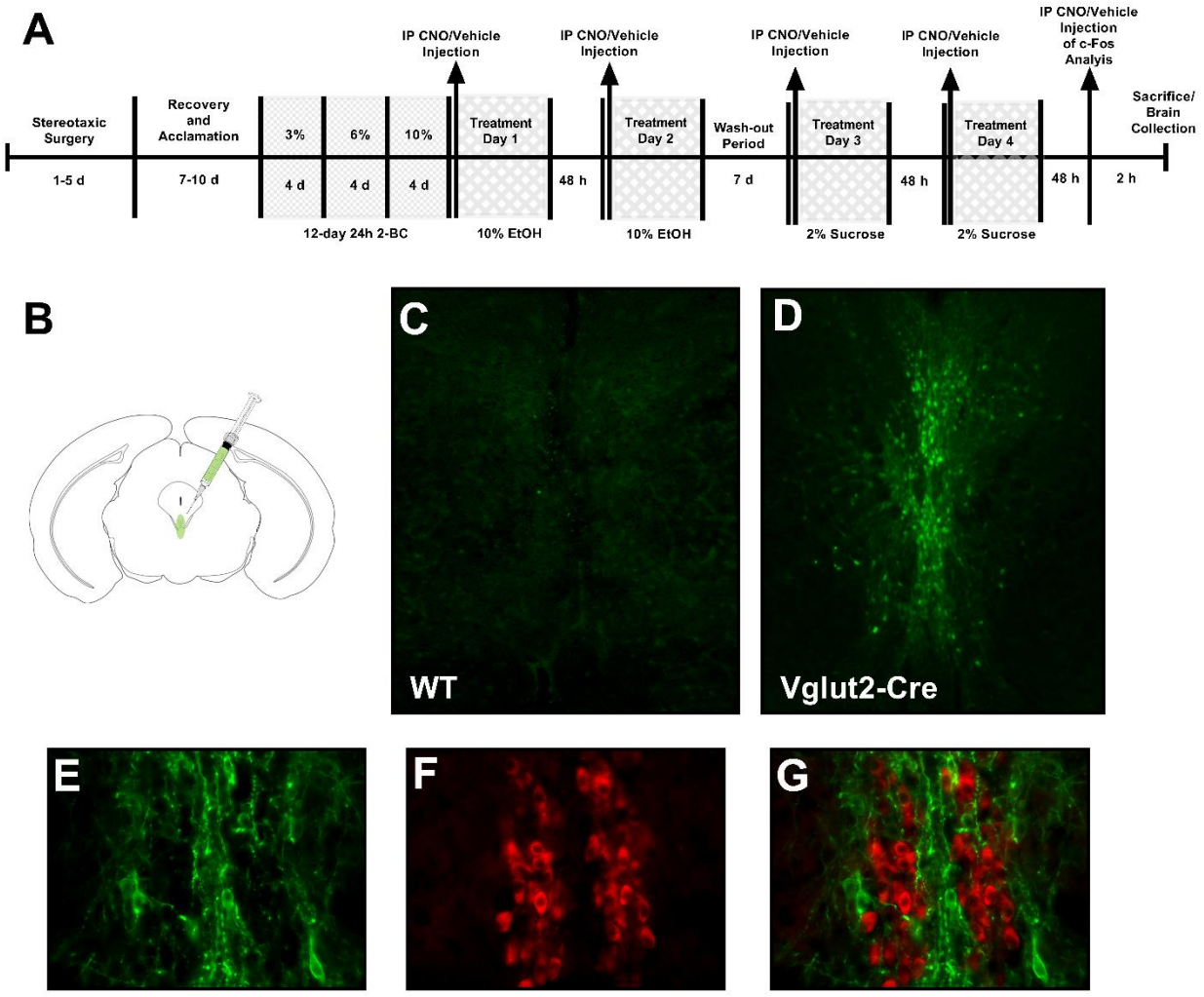

Fig. 10. Procedural timeline and Cre-dependent transduction of neurons in the EWcp of Vglut2-Cre mice.

Male and female WT and Vglut2-Cre mice received a stereotaxic infusion of a Cre-dependent DIO-hM3Dq DREADD in the EWcp. A) Experimental timeline used in Exp. 3, shaded area represent intake periods after CNO or vehicle administration B) Representative illustration demonstrating the angled approach used to target the EWcp. C) Representative photomicrograph displaying the lack of DIO-hM3Dq expression in the EWcp of WT mice. D) Immunofluorescent image illustrating successful DIO-hM3Dq expression in the EWcp of Vglut2-Cre mice. Fluorescent immunocytochemistry for the hM3Dq-fused HA epitope tag (green, E) and for Ucn1 (red, F) revealed that Ucn1-positive cells did not express the hM3Dq-fused HA tag (G).

Exp.7B: Effects of chemogenetic activation of Vglut2-expressing EWcp neurons on 10% EtOH Intake

Average EtOH intakes over the course of the 12-day drinking period, as well as average intakes of EtOH, water, and sucrose throughout the treatment phase are presented in Fig. 11. Throughout all 12 days of drinking, female mice drank significantly more EtOH than males (Fig. 11A). There were however, no genotype-dependent differences in EtOH intake, indicating that Cre-expression did not alter baseline drinking behavior. When EtOH intake was measured 2-h after treatment of CNO and vehicle, Vglut2-Cre mice expressing hM3Dq in the EWcp displayed a strong reduction in EtOH intake when treated with CNO (Fig. 11B). This decrease was specific to EtOH, as CNO treatment did not alter water (Fig. 11C) or sucrose intake (Fig. 11D). In addition, Vglut2-Cre mice appeared to drink more EtOH and less water during this period than their WT littermates. Lastly, when Vglut2-expressing mice were given a final IP injection of either CNO or vehicle, increased c-Fos expression was observed in CNO-treated mice (Fig. 11F). In support of these observations, individual two-way (Sex x Genotype) ANOVAs for each EtOH concentration during the 12-day period revealed significant main effects of Sex at every concentration [3%: $p < .01$, 6, 10%: $p < .001$] but no main effects of Genotype, nor any Sex x Genotype interactions. A three-way RM ANOVA (Treatment x Sex x Genotype) for EtOH intake 2 h-post treatment revealed a Main effect of Genotype [$F(1,34) = 11.1$, $p < .01$] and of Treatment [$F(1,34) = 32.1$, $p < .0001$], as well as a significant Genotype x Treatment interaction [$F(1,34) = 14.1$, $p < .001$]. During the 2-h period, female mice tended to drink more than males, although this was not significant ($p = .06$). Bonferroni post-hoc analyses revealed a significant decrease in EtOH intake when mice were treated with CNO in male ($p < .0001$) and female ($p < .001$) Vglut2-Cre mice only. The RM ANOVA for water intake only detected a main effect of Genotype [$F(1, 34) = 5.2$, $p < .05$], where WT mice drank more water during the 2-h period than

Vglut2-Cre mice. No main effects or interactions were detected when analyzing the sucrose intake data. Finally, a student's t-test confirmed that CNO significantly increased the number of c-Fos-positive neurons in Vglut2-Cre mice, compared to those given a vehicle injection (Fig. 11F) [t (6) = 7.4, $p < .001$].

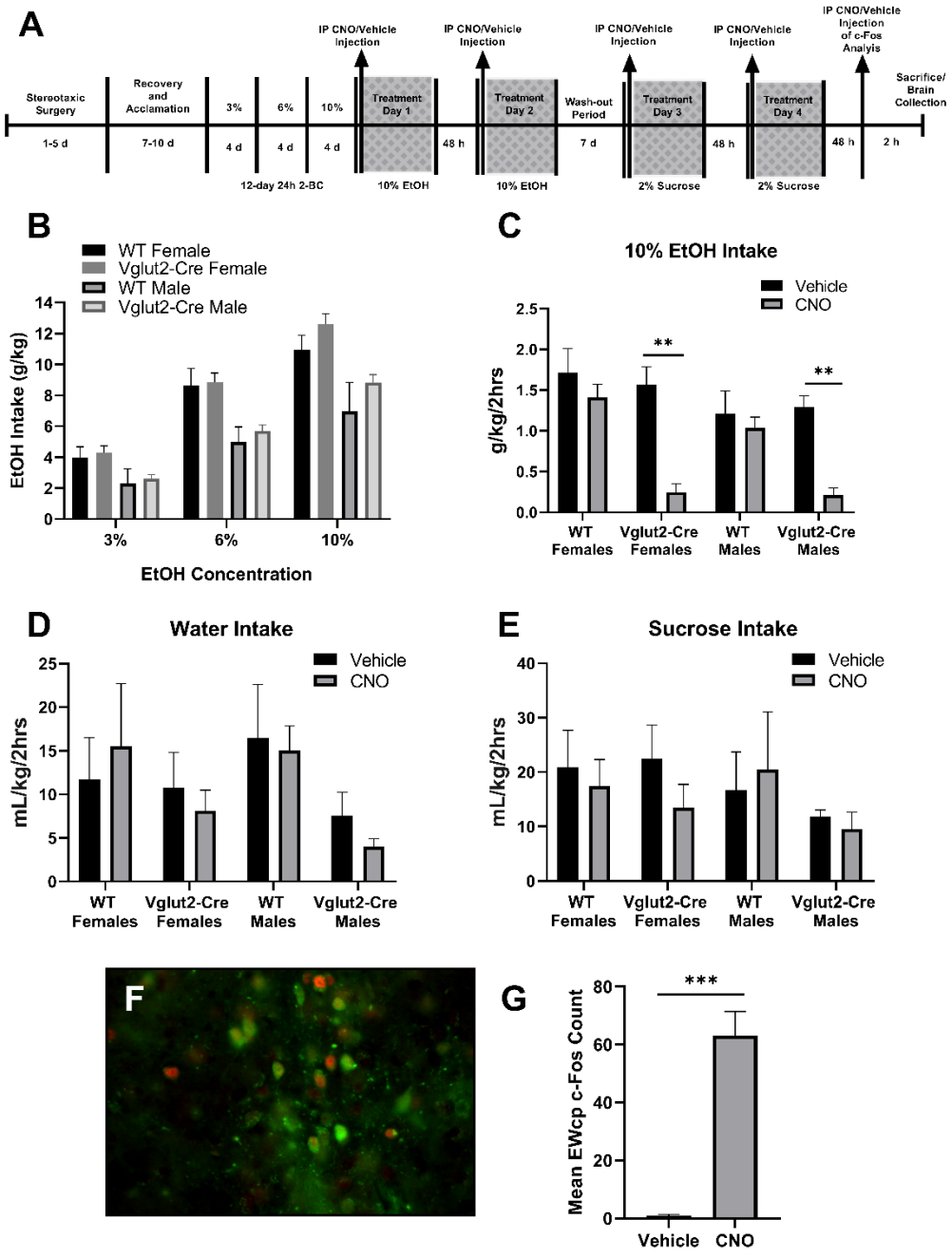

Fig. 11. Chemogenetic activation of EWcp glutamatergic neurons decreases 10% EtOH intake.
Following recovery from DIO-hM3Dq injection and acclimation, WT and Vglut2-Cre mice were allowed access to increasing concentrations of ethanol (EtOH, 3-10%) prior to treatment with CNO or vehicle. A) Experimental timeline used in Exp. 3, shaded area represent intake periods after CNO or vehicle administration. B) Over the course of the 12 day 2-BC procedure, female mice drank significantly more EtOH than males. C) Injection of CNO (1.0 mg/kg, IP) significantly attenuated g/kg EtOH intake in Vglut2-Cre but not WT mice. D) Injection of CNO (1.0 mg/kg, IP) did not alter water intake in WT or in Vglut2-Cre mice. E) Activation of the EWcp via a CNO injection did not alter sucrose intake. F) Immunofluorescent image of mCitrine (green) and c-Fos (red) co-expression in the EWcp of Vglut2-Cre mice. G) c-Fos expression was significantly increased in Vglut2-cre mice given an injection of CNO, compared to vehicle-treated mice. **; $p < 0.01$, ***; $p < 0.001$.

Discussion

The current studies demonstrate that acute activation of the EWcp leads to significant decreases of EtOH drinking in the 2-BC procedure. Surprisingly, we find that this decrease is not mediated by Ucn1-containing neurons, but by separate Vglut2-positive neurons in this brain area. These findings call for attention to this understudied population of neurons.

As described in the introduction, a number of studies using varied approaches have identified the EWcp as highly sensitive to EtOH and involved in regulation of EtOH self-administration. Our demonstration that chemogenetic activation, but not inhibition, of the EWcp leads to decreased EtOH intake expands this evidence. Surprisingly, however, immunohistochemical analyses of the EWcp found no mCherry, and thus no DREADD expression, in Ucn1-positive cells, suggesting the involvement of another population of EWcp neurons in the regulation of EtOH intake. In a subsequent experiment using Ucn1 WT and KO mice we found that chemogenetic activation of the EWcp decreased EtOH intake in both genotypes. That a hM3Dq-mediated decrease in EtOH intake was observed in Ucn1 KO mice again suggested that a separate population of neurons was involved in regulation of EtOH

consumption. Based on data from the Allen Brain Institute demonstrating abundant expression of Vglut2 in the EWcp (Lein et al., 2007), we next transduced Vglut2-Cre mice with a Cre-Dependent DIO-hM3Dq DREADD to selectively activate Vlgut2-expressing neurons in the EWcp. Activation of Vglut2-expressing neurons decreased EtOH intake, in both male and female mice, clearly indicating that this population of neurons is important for regulating EtOH consumption.

Vglut2-expressing neurons in the EWcp have not been previously studied in relation to actions of addictive substances. Interestingly, a recent study by Zhang and colleagues found that Vglut2 neurons in the vicinity of the EWcp, also known as the perioculomotor area (pIII), are important for the regulation of non-rapid eye movement (NREM) sleep (Zhang et al., 2019). It is possible that some of the Vglut2 neurons studied here and those studied by Zhang et al. may be the same populations of neurons. Importantly, the decrease in intake following activation Vglut2 neurons in our study could not be due to potential sleep-promoting effects of these neurons as the decrease in intake was selective for EtOH, and was not observed for water or sucrose intake. On the other hand, Vglut2 neurons of pIII form at least two subpopulations of neurons, containing either cholecystokinin (CCK) or calcitonin gene-related peptide alpha (CALCA), both of which regulate NREM sleep (Zhang et al., 2019). Future work is needed to further characterize the involvement of these specific subpopulations of EWcp Vglut2 neurons in EtOH intake.

While our finding points to the importance of an understudied Vglut2 population of EWcp neurons, the lack of contribution of Ucn1 neurons to the observed effect seemingly contradicts previous literature showing that Ucn1 neurons are capable of regulation EtOH intake. Indeed, selective breeding for alcohol intake results in increased Ucn1 expression in most selectively bred lines of mice and rats (Bachtell et al., 2002b; Fonareva et al., 2009; Turek et al.,

2005), electrolytic lesions of EWcp decrease EtOH preference in Ucn WT (Giardino et al., 2011), but not Ucn KO mice, and Ucn1-directed RNA interference in EWcp inhibits escalated EtOH intake in B6 mice (Giardino et al., 2017). Interestingly, these prior studies point to EWcp Ucn1 neurons promoting EtOH intake, while activation of EWcp Vglut2 neurons in our study inhibits EtOH intake. Thus, these two populations of neurons appear to regulate EtOH in opposite directions.

Although the difference in EtOH intake between Ucn1 WT and KO mice during the 4 h period post-treatment was only trending towards significance, it is important to consider this difference in the context of previous studies using Ucn1 KO mice. Recently, Giardino and colleagues demonstrated that the differences in EtOH intake between Ucn1 WT and KO mice were most apparent during the first 4 h of the dark cycle (Giardino et al., 2017), similar to what we report here. The decision to use this specific time window was made based on previous work showing that C57BL/6J mice consume high levels of EtOH during the first several hours of the dark cycle (Freund, 1970; Goldstein & Kakihana, 1977; Kurokawa, Akino, & Kanda, 2000; Panksepp et al., 2017). Future experiments in which mice are given CNO later on in the dark cycle, when the difference in intake between genotypes is not as apparent could potentially address this issue.

Vglut2 is a marker of glutamatergic neurons. (Vigneault et al., 2015). The involvement of the glutamate system, and/or changes in glutamate transmission during and after ethanol intake have been extensively reported in various regions that receive input from the EWcp, including the CeA (Roberto et al., 2004; Zhu, Bie, & Pan, 2007), LH (Chen, Barson, Chen, Hoebel, & Leibowitz, 2013; Wei et al., 2015) and the BNST (Kash, Baucum, Conrad, Colbran, & Winder, 2009; Wills et al., 2012). Given our findings, it is thus possible that the EWcp may be a source of

glutamatergic input to these regions, and that increased activation of glutamatergic neurons in the EWcp may decrease EtOH intake through their projections to any or all of these regions. Alternatively, one must consider that the decrease in intake observed after activation of Vglut2-expressing neurons is mediated by the interaction between these neurons and those that express Ucn1 in the EWcp. Specifically, as it is known that the majority of EtOH-activated neurons in the EWcp are Ucn1-positive (Anacker et al., 2014; Bachtell et al., 2002b; Ryabinin et al., 2003), understanding how these two populations of neurons interact may provide insight into how EtOH-seeking behaviors are mediated by the EWcp.

Ultrastructural work has shown that both D- and L-type asymmetrical synapses make contact with Ucn1 neurons in the EWcp (Van Wijk et al., 2009), and as asymmetrical synapses are believed to be excitatory (DeFelipe & Fariñas, 1992), one possibility is that these glutamatergic neurons may act to regulate Ucn1-expressing neurons in the EWcp. Furthermore, Van Wijk and colleagues reported that the sizes of vesicles found on these asymmetrical contacts were consistent with those that have been shown contain glutamate (Van Wijk et al., 2009). Although Ucn1-neurons were not transduced by the AAV carrying the hM3Dq receptor in Exp. 1, it is possible that some of the observed effect on EtOH intake occurs via interactions between transduced neurons and Ucn1-expressing neurons. Future electrophysiological studies investigating how neuronal activity in Ucn1-expressing neurons is altered following activation *or* inhibition of these glutamatergic neurons will undoubtedly provide much needed additional information.

Given the likely complexity of potential interactions between Vglut2 neurons and Ucn1 neurons of the EWcp, it would be important to investigate the effects of selective chemogenetic manipulation of EWcp Ucn1 neurons on EtOH intake. In fact, this was the original intent of our

study. However, to our surprise, we found a lack of DREADD expression in these neurons, suggesting an inability of the AA8 serotype to transduce Ucn1 neurons. One possible explanation for the lack of mCherry expression in Ucn1-expressing neurons is that these neurons in the EWcp do not express the receptors required for AAV transduction. Importantly, our pilot experiments also showed lack of transduction of Ucn1 neurons following injections of AAV2, AAV5 and AAVDJ viral serotypes (data not shown). To date, the exact molecular mechanisms involved in AAV transduction of cells have not yet been deciphered. The laminin receptor has been shown to be a potential receptor for AAV8 as well as AAV2, 3 and 9. In addition, recent studies pointed to a universal receptor, KIAA0319 (or AAVR), as essential for cell binding for multiple AAV serotypes. (Pillay et al., 2016). Currently, it is not known if Ucn1 neurons in the EWcp express AAV receptors, and future work investigating this possibility is required. This technical challenge makes manipulations of EWcp neurons using standard chemogenetic tools difficult and requires development of alternative approaches. Interestingly, a prior RNA viral interference experiment showed that Ucn1 neurons can be transduced with lentiviruses (Giardino et al., 2017). Future studies could use lentiviral vectors to manipulate activity of EWcp Ucn1 neurons.

The EWcp is more complex than originally envisioned and contains several subpopulations of cells, other neuromodulators that are known to be present in this area will also need to be taken into account when considering the role of the EWcp in EtOH self-administration. Besides Ucn1, the EWcp is also known to express high levels of the peptides cocaine- and amphetamine-regulated transcript (CART), cholecystokinin (CCK) and nesfatin-1, as well as delta-like 1 homologue, and receptors for the peptide ghrelin (Cservenka et al., 2010; Giardino et al., 2012; Goebel-Stengel, Wang, Stengel, & Taché, 2011) . These molecules are all

known to regulate food consumption, mood and reward (Ayada, Toru, & Korkut, 2015; Murray, Tulloch, Gold, & Avena, 2014; Rogge, Jones, Hubert, Lin, & Kuhar, 2008; Skibicka & Dickson, 2013; Spencer et al., 2012). As such, our findings in Ucn1 KO mice may be mediated by the activation of a subpopulations of EWcp neurons expressing a number of these neuropeptides. Systemic (IP) administration of CCK has been shown to inhibit EtOH intake (Geary, Wolfe, Polidori, Policani, & Massi, 2004; Kulkosky, Wise, Brandt, & Chavez, 2004), thus it is possible that the decrease in intake observed here is mediated by the release of CCK. Interestingly, intracerebroventricular injections of CCK did not alter EtOH intake, suggesting that peripheral CCK may be mediating this effect (Geary et al., 2004). Beyond its potential involvement in regulating EtOH intake, CCK is primarily known to suppress food intake and promote satiety (Bi & Moran, 2002). As activation of Vglut2-expressing neurons did not alter sucrose intake, and administration of CCK has been shown to decrease sucrose intake (Yamaguchi, Yasoshima, & Shimura, 2017), it is likely that our observed results are not primarily mediated by the release of CCK. Even so, the potential involvement of CCK on the decrease in EtOH intake observed here cannot be ruled out and requires further attention in future studies.

In summary, the studies presented here demonstrate that chemogenetic activation of non-Ucn1-expressing neurons in the EWcp decreased EtOH intake. Furthermore, Vglut2-expressing neurons were found to mediate this effect, as specific activation of these neurons decreased EtOH intake while not affecting water or sucrose consumption. Together, our studies suggest that Vglut2-expressing neurons may regulate EtOH intake, and as such the EWcp may be a promising future therapeutic target.

Chapter 4: General Discussion

Summary of Findings

The studies presented in Chapter 2 used a pharmacological approach to assess the role of the EWcp in conditioned EtOH-seeking behaviors. In separate experiments, the EWcp was pharmacologically inhibited via the GABAAR and GABABR agonists muscimol and baclofen, respectively, during the acquisition and the expression phases on EtOH-CPP. Inhibition of the EWcp did not alter place preference in either of the two experiments, suggesting that this manipulation was either not sufficient to produce detectable changes in place preference, or that the EWcp is not involved in the formation or expression of EtOH-cue associations, or in conditioned ethanol-seeking behaviors. Additional experiments were conducted to assess the molecular and physiological effects of EWcp inhibition. In these experiments muscimol and baclofen were microinfused into the EWcp prior to an EtOH injection, and the effects of this manipulation on EtOH-induced c-Fos, as well as on thermoregulation, were examined. These studies demonstrate that inhibition of the EWcp increases body temperature, indicating a role for the EWcp in thermoregulation. Furthermore, muscimol and baclofen prevented the increased expression of c-Fos that is observed in the EWcp following an injection of EtOH. Based on the behavioral findings in the CPP experiments, in combination with previous work suggesting that the EWcp regulates EtOH intake, it is possible that the EWcp may play a more selective role in modulating of EtOH intake. This possibility was further explored in Chapter 3.

Chapter 3 focused on using a standard 24 h 2-BC EtOH drinking procedure to assess the effects of chemogenetic modulation of neuronal activity in the EWcp on EtOH intake. Specifically, using DREADDs, the EWcp was either inhibited or activated in Exp. 5, and a significant decrease in EtOH intake was observed only in animals transduced with the excitatory DREADD. The following experiment (Exp. 6) replicated these findings in both Ucn1 WT and

KO mice. Lastly, Exp. 7 targeted a glutamatergic population of neurons in the EWcp. When a Cre-dependent excitatory DREADD was microinfused into the EWcp of Vglut2-Cre and WT mice, activation of the EWcp once again decreased EtOH intake, but only in Vglut2-Cre mice. Importantly, activation of glutamatergic neurons in the EWcp did not alter either water or sucrose intake, highlighting the specificity of this manipulation.

Involvement of the EWcp in conditioned EtOH behaviors

In the first set of experiments, in which the EWcp was pharmacologically inhibited during the acquisition phase, M+B did not have any effect on any of the behavioral measures analyzed. Prior work has demonstrated that lesions of the EWcp do not alter EtOH-induced changes in locomotor activity, or EtOH-induced sedation (Bachtell et al., 2004). In light of this, our finding that EWcp inhibition prior to CS+ trials did not affect locomotor activity further suggests that the EWcp may not be involved in regulating EtOH-induced locomotor behaviors. When place preference was tested on the final day of the experiment, we again saw no differences in locomotor activity, indicating that a history of M+B did not have long-term effects on locomotor activity, even in the absence of EtOH. Previous work has demonstrated that the handling associated with local drug microinfusion during conditioning can have profound effects on the development of place preference (Young et al., 2014). In order to minimize handling-induced effects on CPP, we microinfused M+B 30 min prior to each CS+ trial. Although the effects of microinfusion directly before CS+ trials were not tested here, our finding that place preference was observed in our control (Vehicle) group suggests that the decision to implement this 30-min delay was somewhat successful.

The main finding from these acquisition studies was that inhibition of the EWcp did not alter the acquisition of EtOH-induced CPP. This result can be interpreted in several different ways. First, it is possible that the methodological parameters, such as the dose of EtOH and of M+B, used in the experiments were not appropriate to produce detectable changes in the development of CPP. Second, it could also be that the EWcp is not involved in the formation of EtOH-cue associations. The dose of M+B used here was chosen based on previous experiments in the BNST (Pina et al., 2015). This dose was used in a separate experiments (Exp 4), and was found to inhibit EtOH-induced increases in c-Fos expression in the EWcp. It is therefore unlikely that the dose of M+B was not the driving force in the lack of effect seen in Exp 1. Even so, experiments using various doses of M+B could have effects on CPP not seen here, and as such merit consideration in the future. The dose of EtOH used here has been shown to be optimal for inducing place preference in this strain of mice (DBA/2J), using similar parameters used here (Cunningham et al., 1992; Cunningham & Shields, 2018). It is possible however, that using this dose did not allow the detection of subtle M+B-induced changes in CPP. Using a lower dose, such as 0.5 g/kg, which mice do find rewarding (Groblewski et al., 2008), could potentially address this issue.

It must also be considered that the lack of an effect seen in M+B treated mice on CPP acquisition is an indication that the EWcp is not involved in the acquisition of EtOH-CPP. Giardino and colleagues demonstrated that mice lacking Ucn1, which is primarily expressed in the EWcp, do not develop EtOH place preference. In order to ensure that lack of Ucn1 did not simply alter learning in general, the development of CPA between Ucn1 WT and KO mice was also examined. Interestingly, Ucn1 KO mice did develop strong CPA. The authors concluded that lacking Ucn1 prevents mice from making an association between the rewarding effects of

EtOH and a cue. Although it is difficult to compare the findings presented here to those of Giardino and colleagues, they are somewhat at odds with each other. Compensatory mechanisms associated with Ucn1 deletion may underlie the differences between previous studies and the results presented here. For example, the Ucn1 KO line used in the 2011 experiments have been shown to have decreased levels of CRF2R mRNA in the LS, a brain region thought to be involved in the regulation of EtOH intake (Vetter et al., 2002). Furthermore, Vetter et al. reported that CRF mRNA expression was decreased in the BNST (Vetter et al., 2002). Importantly, inhibition of the BNST prevents the expression of EtOH CPP (Pina et al., 2015). Currently it is unknown to what extent CRF within the BNST plays a role in the acquisition or expression of EtOH-CPP, although there is evidence that CRF signaling in the BNST is enhanced during stressors that promote the reinstatement to EtOH seeking (Funk, Li, & Lê, 2006; Lê et al., 2000). It is therefore possible that the lack of EtOH CPP in Ucn1 KO mice reported previously is due in part to changes in the CRF system elsewhere in the brain.

The second study in Chapter 2 focused on examining the role of the EWcp in the expression of EtOH-CPP. Preference for an EtOH-paired context has been hypothesized to represent the conditioned reinforcing properties of the drug (Cunningham, Groblewski, & Voorhees, 2011). Over the course of conditioning, the animal learns to associate the context with the rewarding effects of EtOH, and therefore spends more time exploring that context. Manipulations within the brain that decrease the amount of time spent on the EtOH-paired floor can therefore be used as indicators of brain regions that mediate conditioned behaviors. Here, we found that a single microinfusion of M+B given immediately prior to preference testing did not alter the expression of CPP, suggesting that the EWcp is not involved in the regulation of conditioned EtOH-seeking behaviors.

In their 2007 study, Hill and colleagues demonstrated that exposure to an EtOH-paired CS+ can increase c-Fos expression in different regions throughout the brain (Hill et al., 2007). Exposure to the CS+, and examination of c-Fos expression occurred after 6 conditioning trials, and as such the authors suggested that any regions showing increased levels of c-Fos after CS+ exposure may be involved in the expression of the an EtOH-induced conditioned response, rather than the acquisition. Of the regions examined, increases in cue-induced c-Fos were observed in the anterior portion of the VTA, the CeA, and the BNST. Increased c-Fos expression in the EWcp however, was only observed following an EtOH injection and exposure to the CS+. When considered in light of these findings, our results in Exp 2 suggest that the EWcp is likely not involved in the expression of conditioned EtOH-seeking behaviors. Again, one must consider that the dose and number of trials used here (2 g/kg, 4 trials) did not allow for the detection of M+B-mediated changes. The combination of this dose, and this number of trials produces place preference that is asymptotic (Groblewski et al., 2008), and as testing the effects of M+B on CPP expression following a fewer number of trials, and/or with a lower dose of EtOH may allow for detectable changes in CPP expression.

Involvement of the EWcp in voluntary EtOH drinking

Chapter 3 used a chemogenetic approach to modulate neuronal activity in the EWcp. The effects of such modulation on EtOH self-administration were then analyzed using a 2-BC procedure. In Exp. 5, we found that chemogenetic activation of the EWcp via the excitatory (hM3Dq) DREADD significantly decreased EtOH intake. Conversely, we found no changes in intake in mice that were transduced with the inhibitory (hM4Di) or the control (GFP) DREADDs. Ucn1 within the EWcp has been implicated in the regulation of EtOH intake through

numerous studies (Bachtell et al., 2002b, 2004; Fonareva et al., 2009; Giardino et al., 2011, 2017; Turek et al., 2005). Given these previous studies, the original intent of Exp. 5 was to use DREADDs to acutely activate and inhibit Ucn1-expressing neurons in the EWcp. Surprisingly, we found that the AAV used to deliver DREADDs to neurons in the EWcp (AAV8) did not transduce Ucn1-expressing neurons, as indicated by the lack of expression of the mCherry tag protein in Ucn1-positive cells. Thus, we concluded that the effect observed in Exp. 5 was presumably driven by the activation of these non-Ucn1-expressing neurons in the EWcp. Importantly, an increase in c-Fos expression was observed in these cells in hM3Dq-transduced mice, indicating that this manipulation did indeed have a physiological effect. In addition to the use of AAV8, several other serotypes were used, and their abilities to transduce Ucn1-expressing neurons were tested in several pilot experiments. Of these serotypes (AAV2, AAV5, AAVDJ), none appeared to transduce Ucn1 neurons in the EWcp (data not shown). AAV2, 5 and 8 have all been shown to require the recently discovered AAVR (also known as KIAA0319L) for transduction (Pillay et al., 2016), and as such it is possible that Ucn1-expressing cells do not express this receptor. AAVDJ was created by the DNA shuffling method using the libraries of 8 AAV serotypes (Grimm et al., 2008), and it was believed that its high efficacy was due to its strong binding to a primary receptor, as well as to secondary entry receptors. As with the other serotypes used, it may be that Ucn1 cells do not express the receptors necessary for AAVDJ transduction. Understanding whether the lack of receptors on Ucn1 neurons does indeed prevent successful AAV transduction will require further investigation in the future. In non-human primates, electron microscopy work has recently shown that the mCherry tag can prevent the expression of hM4Di, but not hM3Dq DREADDs (Galvan et al., 2019). Removing the mCherry tag, and instead fusing the hM4Di DREADD with an HA tag appeared to resolve this issue, as

strong immunogold labeling was subsequently detected. In Exp. 5, we saw no expression of either the hM4Di or hM3Dq DREADD, and although to date there have not been any reports in mice of changes in DREADD expression due specifically to the presence of a fluorescent tag, this potential issue must also be considered in the future. Of note, a lentiviral approach has been successfully used in Ucn1-expressing neurons in a prior study (Giardino et al., 2017), and as such could be used in future studies to transduce Ucn1 neurons in the EWcp.

The inability to deliver DREADDs to Ucn1 neurons in the EWcp via the AAVs used here, in combination with the stark decrease in EtOH intake observed in Exp. 5 suggests that a separate population of neurons in the EWcp, that do not express Ucn1, is also involved in the regulation of EtOH intake. One must consider however, that the decrease in intake is mediated in part by Ucn1, potentially via an interaction between hM3Dq- and Ucn1-expressing neurons in the EWcp. As such, Exp. 6 incorporated Ucn1 WT and KO mice to investigate the role of Ucn1 in the hM3Dq-mediated decrease in EtOH intake observed in Exp. 6. In agreement with a recent report by Giardino and colleagues (2017), we found that Ucn1 WT and KO mice did not differ in their EtOH intake during the 12 days of 24-h 2BC, during which they had access to 3, 6, and 10% EtOH. Interestingly, EtOH intake was lower in Ucn1 KO mice during the 4 h period after CNO treatment, although this decrease did not reach significance. Again, this is in agreement with recently published work, showing that Ucn1 WT and KO mice differed most in their intake during the first 4 h of the dark cycle (Giardino et al., 2017). Although not tested here directly, differences in intake do not appear to be driven by differences in physiology or behavior, as these two genotypes have been shown to not differ in sweet or bitter taste reactivity, EtOH-induced loss-of-righting-reflex, baseline anxiety-like behavior, or impulsive-like behaviors (Giardino et al., 2017).

When CNO was administered to Ucn1 WT and KO mice transduced with the hM3Dq DREADD, we observed a decrease in EOH intake in both genotypes. The decrease in intake in Ucn1 KO mice suggests that other EWcp neuropeptides and/or neurotransmitters, such as CART and CCK, may be involved in mediating this decrease in intake. CART expression in the EWcp has been shown to be affected by EtOH exposure (Dandekar et al., 2019), and baseline CART expression has also been shown to be higher in B6 mice, compared to DBAs (Giardino et al., 2012). As such, it is possible that CART may be involved in mediating decreases in EtOH intake. Future work modulating CART, in both Ucn1 WT and KO mice may help elucidate role of the EWcp-CART system in EtOH-seeking behaviors.

In Exp 5 and 6, chemogenetic activation of the EWcp decreased EtOH intake, and as noted above, this effect was observed even though Ucn1-neurons in the EWcp were not transduced. The use of a viral construct incorporating the human synapsin 1 (hSyn) gene promotor suggests that the population of cells that were transduced (and thus mediate the observed decrease) are indeed neurons and not glia, as the addition of this promoter has been shown to restrict adenoviral vector expression to neurons (Kügler, Kilic, & Bähr, 2003). In the adult nervous system, Vglut2 has generally been considered a marker of glutamatergic neurons (Vigneault et al., 2015), and has been detected in the thalamus, hypothalamus, midbrain, and brainstem (Fremeau et al., 2001; Herzog et al., 2001; Kaneko & Fujiyama, 2002; Yamaguchi, Sheen, & Morales, 2007). Additionally, Vglut2 expression has been detected in the EWcp, and recently a subset of these Vglut2-expressing neurons have been shown to be involved in regulating non-rapid-eye-movement sleep (NREM). The use of a Vglut2-Cre line was further supported by our own immunohistochemical evidence indicating that Ucn1-neurons do not express Vglut2. Given the presence of Vglut2 in the EWcp, Exp. 7 used a Cre-dependent hM3Dq

DREADD in combination with a Vglut2-Cre transgenic mouse line, in order to specifically activate glutamatergic neurons in the EWcp. The use of a transgenic mouse line expressing Cre in a cell-type-specific manner, in combination with a vector requiring Cre for sense translation, can and has been used to selectively transduce specific populations of neurons in the brain (Andreoli, Marketkar, & Dimitrov, 2017; Atasoy, Aponte, Su, & Sternson, 2008; Rinker et al., 2017; Smith, Bucci, Luikart, & Mahler, 2016). Here, we found that activation of glutamatergic neurons in the EWcp via a CNO injection significantly decreased EtOH intake. Importantly, this effect was specific to EtOH, as neither water nor 2% sucrose intake were affected. When expression of the hM3Dq receptor was analyzed both via immunohistochemical detection of the HA tag fused to the hM3Dq, as well as mCitrine expression, we saw no expression of either on Ucn1-expressing neurons. As with Exp 5, there was a clear distinction between DREADD-expressing and Ucn1-neurons. Importantly however, the use of the Vglut2-Cre line provides evidence that glutamatergic neurons in the EWcp are involved in the regulation of EtOH intake. Currently, it is not known how these two populations of neurons interact with each other, or with other brain regions. The neurophysiological effect that the activation of Vglut2-positive neurons has on Ucn1-neurons is particularly interesting and should be investigated in the future. Even though the current experiments do not clearly define how these two populations interact and affect each other, our current understanding of EWcp neurocircuitry may provide hypothetical mechanisms by which activation of the EWcp may reduce EtOH intake. An illustration of some of the currently known projections from the EWcp, based on a number of tracing studies, is depicted in **Fig. 12**. These known projections may provide additional therapeutic targets in the future.

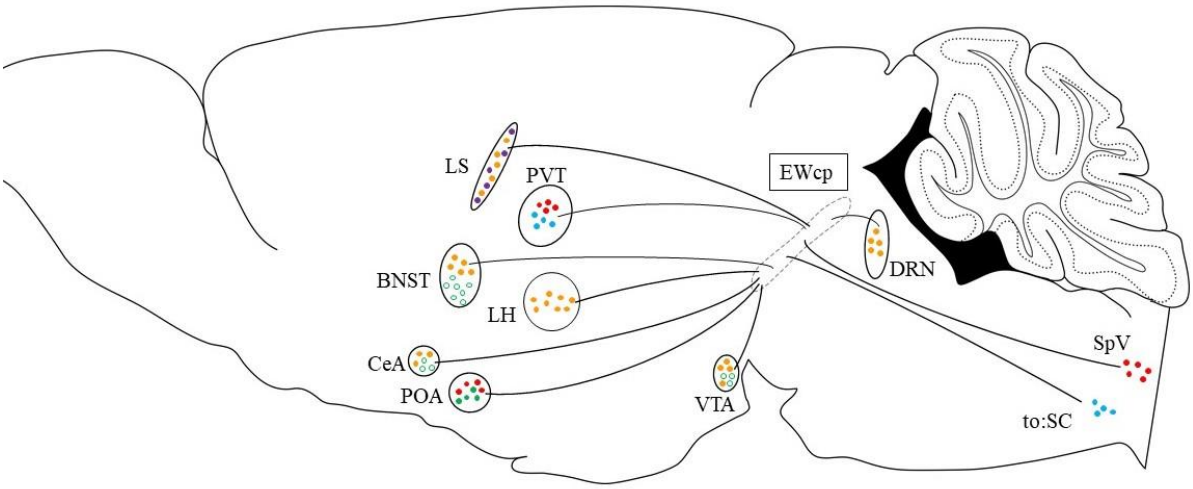

Fig. 12. Schematic of central projections arising from the EWcp.
Efferents originating in the EWcp project to a number of regions through the brain. These regions receive specific neuropeptidergic and neurotransmitter inputs from the EWcp. Filled circles denote known peptides and neurotransmitter known to be released in that area from the EWcp, while open circles denote projections not yet confirmed. Orange circles; Ucn1, purple circles; CART, blue circles; substance P, red circles; CCK, green circles; glutamate. BNST; bed nucleus of the stria terminalis, CeA, central nucleus of the amygdala, EWcp; centrally-projecting Edinger Westphal nucleus, DRN; dorsal raphe nucleus, LH; lateral hypothalamus, LS; later septum, POA; preoptic area, PVT; paraventricular nucleus of the thalamus, SC; spinal cord, SpV; trigeminal nucleus, VTA; ventral tegmental area.

Although the LS has been previously implicated in regulating consummatory behaviors (Olivo, Caba, Gonzalez-Lima, Rodríguez-Landa, & Corona-Morales, 2017; Sweeney & Yang, 2016), a limited number of studies have also provided evidence supporting its involvement in EtOH-seeking behaviors, particularly through Ucn1 projections form the EWcp. Indeed, Ryabinin and colleagues demonstrated that microinfusion of Ucn1 into the LS significantly decreases EtOH intake (Ryabinin, Yoneyama, Tanchuck, Mark, & Finn, 2008). Decreases in intake were observed both when Ucn1 was administered during initial exposures to EtOH, as well as during after an animal had established EtOH intake levels, suggesting that intra-LS Ucn1 can inhibit the acquisition and maintenance of EtOH drinking. In addition, differences in the

EWcp-LS system have also been suggested to mediate differences in intake between strains that consume high and low levels of EtOH (Bachtell, Weitemier, et al., 2003). Additional evidence comes from examination of c-Fos expression following EtOH oral self-administration, which has showed that c-Fos expression is suppressed in the LS of B6 mice following a 30-min access period (Ryabinin et al., 2003). Finally, the LS may be also involved in regulating EtOH's dopamine-elevating effects in the NAcc, as microinfusion of tetrodotoxin into the LS has been shown to prevent increases in NAcc dopamine following exposure to EtOH (Jonsson, Morud, Stomberg, Ericson, & Söderpalm, 2017). In light of these studies, one must consider the possibility that activation of glutamatergic cells in the EWcp may suppress EtOH intake via the activation of Ucn1 neurons, and the subsequent release of Ucn1 in the LS. The presence of asymmetrical synapses on Ucn1 neurons in the EWcp suggests that their activity is regulated by an excitatory source (Van Wijk et al., 2009). Although the exact origin of this input is not known, it is possible that the glutamatergic neurons presented here may be a source of excitatory neurotransmission onto Ucn1 neurons. Activation of the hM3Dq DREADD has been shown to increase synaptic vesicle release via the production of diacylglycerol and inositol triphosphate (IP3) (Pei, Rogan, Yan, & Roth, 2008). Thus, it can be postulated that increased release of glutamate following the activation of these neurons could subsequently activate Ucn1 neurons in the EWcp. Following this logic, Ucn1 release in target regions, such as the LS, could result in decreased EtOH intake. It is clear however, that there are numerous questions and a substantial amount of work that must be addressed in regards to the EWcp-LS system and its involvement in EtOH seeking behaviors.

The EWcp has been shown to send dense projections to the BNST and the CeA, two regions highly implicated in the regulation of EtOH intake. Of these two regions, the CeA in

particular could be involved in this EWcp-activation mediated decrease in intake. Activation of CRF2 receptors in the CeA has been shown to increase alcohol consumption in non-dependent rats (and conversely decreases intake in dependent rats) (Funk & Koob, 2007). In the CeA, acute and chronic EtOH exposure has been shown to greatly affect the function of both gluatamate and GABA systems, primarily through CRFR-mediated mechanisms (Roberto et al., 2010; Roberto et al., 2004). Thus, it is possible that activation of this pathway via Gq-coupled DREADDs may substitute for one of these effects of alcohol, thus decreasing the need for alcohol consumption. To what degree different elements of the EWcp neurocircuit are recruited to act within the CeA likely depends upon an intricate interaction of experimental variables such as the length of alcohol access, the amount of alcohol consumed and the level of EWcp activation. This the EWcp-CeA pathway is particularly promising in regards to its role in mediating EtOH intake, and future experiments targeting this pathway may provide novel therapeutic targets.

Limitations and Future Directions

Preclinical research has historically overlooked the use of females in most studies (Clayton & Collins, 2014; Clayton, 2015). Unfortunately, the work presented here failed to properly consider sex in all but one experiment. Recently, Cunningham and Shields showed that male and female DBA/2J mice do not differ in either the rate of acquisition or magnitude of EtOH-CPP expression, suggesting that there are no sex differences in ethanol-reward sensitivity (Cunningham & Shields, 2018). It should be noted however, that sex-dependent differences in EtOH-intake have been observed in Ucn WT and KO mice (Giardino et al., 2017), and therefore it is possible that the results presented here in studies using only male mice may be specific to that sex. Future work in which both males and females are used will undoubtably be needed.

Although Exp. 7 demonstrated that neurons expressing Vglut2 in the EWcp are involved in regulating EtOH intake, very little is known about these neurons. Immunohistochemical work in the future will be needed to know what other neuropeptides are expressed in these Vglut2-neurons. A better understanding of these neurons may provide future targets to decrease EtOH intake, particularly as we know that the EWcp projects to numerous regions believed to be involved in the regulation of EtOH intake. Combined, the studies of these two chapters indicate that the EWcp is not involved in the regulation of conditioned EtOH-seeking behaviors, but rather may play a more vital role in regulating voluntary EtOH intake. Of note, the work presented here highlights the potential involvement of a glutamatergic population of neurons in the EWcp. Future experiments assessing the involvement of these neuron in EtOH-related behaviors, using procedures such as the drinking-in-the-dark (DID) and CTA will provide additional information regarding its role in mediating other aspects of EtOH consumption, including binge drinking and the aversive effects of EtOH. Understanding how the EWcp may regulate EtOH through its interactions with other brain regions must also be considered, and experiments addressing these interactions will be important for our understanding of EtOH-seeking behaviors.

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