

HYPOCRETIN/OREXIN AND THE VENTRAL MIDBRAIN: TOPOGRAPHY
AND FUNCTION ASSOCIATED WITH PSYCHOSTIMULANT-TAKING AND
AFFECT

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ABSTRACT

Abuse of psychostimulants including cocaine and new synthetic formulations remains an international public health problem and economic burden. Addiction develops consequential to positive and negative drives that underlie “getting” and “staying” high. Dopamine (DA), arising from ventral tegmental area (VTA), projects to ventral striatal targets to encode reward signals and reward prediction. Mesolimbic DA is implicated in both the immediate rewarding effects of psychostimulants, and its hypoactivity underlies negative affect as drug levels decline. Accordingly, modulating inputs to midbrain DA possesses capacity to mediate positive/rewarding and negative/aversive effects of drugs. Hypocretin/orexin (hcr/ox) is a family of excitatory hypothalamic peptides that projects widely throughout the central nervous system including to VTA DA cells, and hcr/ox mediates brain reward function and motivation for self-administered drugs. Notably, the first-in-class hcr/ox receptor antagonist (suvorexant) was approved for management of insomnia in the summer of 2014. Also within the past decade, the caudal division of VTA (termed “tail of VTA” and “rostromedial tegmental nucleus [RMTg]”) was detailed for its ability to negatively regulate VTA DA. Functionally, stimulation of the GABA-producing RMTg population encodes aversion and responds to aversive cues. Curiously, anatomy work depicts the hypothalamus as a principal input to the RMTg although the cellular phenotypes and functions of hypothalamic projections to RMTg have not been fully resolved.

Work in this thesis was designed to map hcr/ox projections to VTA and RMTg in effort to understand functionally-relevant topographical arrangement. In preliminary assessments, we test for the first time the ability of suvorexant to modulate reward and

reinforcement associated with psychostimulant use in rats. Additionally, we profile how self-administered cocaine and “bath salt” synthetic cathinone 3,4-methylenedioxypyrovalerone (MDPV) influence affective states in rats by measuring ultrasonic vocalizations (USVs) and comparing patterns of responding. Subsequently, we test the ability of suvorexant to influence MDPV-taking and affective changes that promote self-administration. Finally, we utilize direct-site pharmacology to assess the degree to which hcr/ox transmission within VTA and RMTg contributes to motivated responding for and affective processing of self-administered cocaine across two doses. Specifically, we hypothesized that intra-VTA suvorexant would suppress drug-taking by reducing the rewarding value of self-administered cocaine, whereas intra-RMTg hcr/ox peptide injection would suppress drug-taking by increasing aversive value of self-administered cocaine.

We observed that systemic suvorexant effectively reduces motivated cocaine-taking, and that this reduction relates in part to reductions in subjective reward of self-administered cocaine as interpreted by reductions in positively-valenced 50-kHz USVs. Retrograde tracing supports that hcr/ox projects to both VTA and RMTg without discernible topographical arrangement. Target-site pharmacology finds that intra-VTA suvorexant has no appreciable effects on motivated cocaine-taking but tends to elevate 50-kHz USVs during the pre-drug “anticipation” time epoch in low-dose cocaine self-administering rats (0.375 mg/kg/inf). While intra-RMTg hcr/ox pre-treatment sparsely affected USVs, 0.3 nmol/hemisphere hcr/ox significantly enhanced cocaine-taking in low-dose cocaine self-administering rats, and, in high-dose (0.750 mg/kg/inf) cocaine

self-administering rats, intra-RMTg hcr/ox significantly suppressed responding when pre-treated with 1.0 and 3.0 nmol/hemisphere.

Collectively, studies within this thesis promote the use of hcr/ox receptor antagonists as adjunct pharmacotherapy in managing psychostimulant use disorders, although the circuitries through which aberrant motivated behaviors are modulated are not entirely clear. Future work will need to be performed to understand how hcr/ox transmits to neurochemically-defined cell populations residing within VTA and RMTg—these pathways are recruited for processing stimuli as “rewarding” and “aversive” which are critical contributors in the development of substance use disorders and other psychiatric disorders characterized by dysregulated reward processing.

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DEDICATION

See “Acknowledgements”.

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CHAPTER 1 – BEHAVIORAL EFFECTS AND NEUROBIOLOGY OF PSYCHOSTIMULANT DRUGS OF ABUSE

1.1) *Discovery of cocaine and abuse potential of psychostimulant drugs*

Cocaine was isolated in the mid-1800s by German chemist Albert Niemann from the coca leaf (*Erythroxylon coca*) native to South America which is chewed for its ability to allay fatigue. In 1865, cocaine's chemical structure was resolved and, in the late 1880s, academicians Halsted and Hall contributed to the discovery of cocaine as an injectable nerve block anesthetic compared to relatively unsuccessful efforts using nitrous oxide and ether for systemic anesthesia. A surge of international interest in cocaine's anesthetic properties, including from Halsted and Hall, was brought about following successful demonstration of its use prior to corneal surgery during a German Ophthalmological Society meeting in 1884 by Carl Koller (for discussion, see Goerig et al. 2012). Thereafter, Halsted used injectable cocaine throughout “thousands” of dental and oral clinical procedures, but formal publication of these findings failed due to manuscript incomprehension and incompleteness. His colleagues at the time recognized coincident “erratic social and professional behavior” (López-Valverde et al. 2011) which was found to stem from self-experimentation and eventual dependence on cocaine, although at the time Surgeon General William Hammond considered cocaine use as nothing more serious than coffee consumption (e.g., Olch and Williams 1975). Hall, possessing competence and prowess in rigorous scientific research, left his New York academic position in 1889 for private practice in Santa Barbara, California. In 1895, Hall wrote to Halsted on the matter of his abrupt exit and delayed communication: “It is now quite a long time since I receive a letter from you and a very kind one. I am not sure that I ever

answered it, for at that time I was only pulling myself together after a long period of misery, the causes of which I do not need to describe... I am like an artist banished from Paris... my greatest grief is my isolation.” (Hall 1895). Hall died two years later. For Halsted, treating cocaine dependence meant turning to morphine and alcohol. Halsted missed academic lectures, and his attendance at New York Surgical Society meetings dropped precipitously. Olch and Williams (1975) described Halsted’s personality as shifting from “a relative extrovert” to “a recluse who frequently avoided social intercourse”. Indeed, it appears Halsted maintained regular use of morphine until his death in 1932. The contributions of Halstead and Hall in detailing cocaine’s anesthetic utility remain paramount as do reports of cocaine’s apparent abuse potential.

Today, cocaine use disorder is recognized as a psychiatric disease per the Diagnostic and Statistical Manual of Mental Disorders. Criteria for diagnosis of substance use disorders include: (i) inability to reduce consumption, (ii) feelings of craving/strong desire, (iii) inability to successfully perform work-related tasks, (iv) reduced engagement in social/occupational/recreational activities, (v) tolerance as defined by increased use to produce desired effect, and (vi) withdrawal symptom emergence upon cessation (American Psychiatric Association 2013). In 2016, the National Survey of Drug Use and Health reported a 16.60% lifetime prevalence risk of cocaine use among adult men and women, although it is clear that not all instances of use lead to development of dependence. Cocaine overdoses were responsible for ~6,500 recorded deaths in 2015 alone—a 62% increase from 2010 (CDC Wonder 2017). Common methods of administration include absorption of cocaine hydrochloride powder through nasal passages (“snorting”) or smoking the freebase form (“crack”) through a

glass pipe both of which elicit sympathomimesis and subjective euphoria which wane and devolve, in turn, to negative affect and craving together with somatic withdrawal symptoms. While opiates and synthetic opioids (fentanyl and fentanyl analogs) have surged in abuse this past decade and have led to alarming death rates, Shiels and colleagues (2017) showed that more overdoses are, in fact, attributed to cocaine among black men and women compared to heroin and synthetic opioids across all 3-year time periods charted from 2000 to 2015. Despite remaining a decades-long public health problem, the Food and Drug Administration (FDA) has not approved a single medication for managing cocaine use disorder, and behavioral intervention remains the only available treatment for those attempting to quit.

1.2) Novel psychoactive substances mimic cocaine, are potently rewarding and abused

To compound the societal problems associated with cocaine abuse, clandestine laboratories in the past 15 years have synthesized new agents to mimic effects of popularly abused “parent” drugs phenylisopropylamine (amphetamine), Δ^9 -tetrahydrocannabinol (THC; active agent in cannabis), and cathinone—an endogenous alkaloid that, similarly to cocaine, is the principal psychoactive ingredient in leaves of a perennial shrub (“khat”) that are chewed for stimulant-like effects. From self-reports, khat leaves are also chewed for subjective improvements in concentration and libido (Numan 2004). Unlike cocaine, purified cathinone does not itself appear as prone to abuse. Capitalizing on comparable biochemistry, home laboratories altered structural motifs of cathinone to produce synthetic agents which have been classified under the umbrella of “novel psychoactive substances” (NPS). The most popular “synthetic cathinones” include 4-methylmethcathinone (mephedrone), 3,4-

methylenedioxypropylamphetamine (MDPV) and the empathogenic agent 3,4-methylenedioxymethcathinone (methyone).

The stark rise in synthetic cathinone production and aggressive marketing arrived on an international scale beginning around 2008. Around then, synthetic cathinones (and other synthetic drug formulations) were advertised and sold online in packages marked “not for human consumption” although users purchased these “plant food” and “bath salt” products for recreational use—this mode of distribution bypassed regulations imposed by the FDA. In 2010, 3,200 calls were made to the Poison Control Centers from emergency departments related to synthetic drug abuse including from “bath salt” products. By the end of 2011, the number of calls made rose beyond 13,000 (AAPCC 2012). Administering synthetic cathinones produces adverse risks ranging from local tissue injury to death following multi organ failure. In one of the first case reports related to synthetic cathinone use, Belhadj-Tahar and Sadeg (2005) describe the misfortune of a 29-year-old woman who was admitted to the emergency department following a toxicologically-verified coma following use of methcathinone—a methylated analog of cathinone—presented with mydriasis and hyperpnea. Abuse of MDPV was reported in a case report documenting a 25-year-old who was “markedly combative and foaming at the mouth” (Borek and Holstege 2012). The patient was hyperthermic, hypertensive and mydriatic upon arrival to the emergency department and subsequently developed renal failure and rhabdomyolysis indicative of multi organ failure although eventually recovered after a prolonged hospital stay. In addition to violent behaviors, MDPV has also been reported to lead to paranoid delusions and hallucinatory delirium which presents management obstacle as methods to control erratic behaviors including physical

restraints, tasers, and antipsychotics can worsen somatic toxicity (e.g., Penders et al. 2012).

While cocaine and cocaine-like synthetic psychostimulants including synthetic cathinones still detrimentally impact many communities, basic and preclinical research has worked to understand much about the circuits, behavioral effects and mechanisms of action underlying the progression from recreational psychostimulant use to dependence. In the following sections, the development of behavioral assays used to measure reward and reinforcement in laboratory animals is provided—these assays capture principal features of substance use disorders as can be seen in addicts. Moreover, important advances in our understanding of how psychostimulants including cocaine alter brain chemistry and physiology are discussed.

1.3) Behavioral features of reward and reinforcement: relevance to addiction

1.3.1) Delivery of electrical current to brain sites is reinforcing

Early studies designed to directly probe “brain reward function” in laboratory animals involved delivery of electrical currents via steel wires to target sites in brain contingent on behavioral responses from the implanted animal; this assay is henceforth termed intracranial self-stimulation (ICSS) and has been practiced since the mid-1950s (Olds and Milner 1954). Typically, the intensity of current needed to support self-stimulation is used as a metric to determine shifts in reward function, but the rate of responding at a fixed current can also be used. Upon current injection, it can be surmised that surrounding cells and fibers alike rapidly depolarize to permit cellular cation influx, action potential propagation and synaptic release. Numerous sites in the rodent central nervous system (CNS) support self-stimulation including hypothalamus, septal nucleus

and preoptic area (Corbett and Wise 1980). While this assay is not natural for laboratory animals, it has provided a consensus that acute administration of euphorogenic drugs which are abused by humans invariably “prime” reward function (Goodall and Carey 1975; Koob et al. 1975; Esposito et al. 1978). Remarkably, pairing a novel tastant with the subsequent opportunity to electrically self-stimulate imbues “reward” to the tastant and in turn produces conditioned taste place preference (Ettenberg 1979)—this assay is described in greater detail in [Section 1.3.2](#). Interestingly, the acquisition of positive reinforcement associated with ICSS is in part regulated by environmental factors including experimental handling and housing conditions (e.g., Schaefer and Michael 1991). In these experiments, for example, significantly higher self-stimulation rates were found from socially-housed rats compared to isolated rats.

Generally, deficits in reward function are thought to reflect an “anhedonic” (dysphoric) state that corresponds in part to negative mood/depressed states in humans although no such “self-report” from animal subjects exists to confirm this relationship. Accordingly, drug withdrawal states are characterized by deficient reward function as evidenced by elevated self-stimulation thresholds and/or reduced rates of self-stimulation at a fixed current (Schaefer and Michael 1983; Kokkinidis and McCarter 1990). Moreover, systemic injection of lithium chloride (LiCl), which induces behavioral suppression (for review, O’Donnell and Gould 2007), produces transient decrements in self-stimulation (Edelson et al. 1976). Taken together, these studies reveal ICSS as a flexible behavioral tool that probes neurobiological substrates of reward in a manner sensitive to environmental and pharmacological experimental treatments including drugs of abuse.

1.3.2) Place conditioning enables associative reward learning

Associative learning can imbue unique stimuli with “rewarding” or “aversive” value as interpreted from “approach-avoid” behaviors measured in laboratory animals. Often utilized in place conditioning is a shuttle-box apparatus composed of two contextually-distinguished compartments (“Contexts”) whereby constellations of visual, tactile, and olfactory cues enable the formation of two unique environments. Test subjects may be probed in a pre-conditioning screening trial to shuttle to one compartment (“Context A”) or the adjacent compartment (“Context B”). Time spent on each Context is recorded which may be used as a determinant of which Context an experimental treatment will be given versus the alternate Context in which a control treatment may be given—this is referred to as a biased design. Test subjects then undergo a series of conditioning trials during which experimental and control treatments become associated with each of two Contexts. Experimenters will pair an “experimental treatment” with confinement in Context A whereas, in different sessions during the conditioning phase, a “control treatment” will be paired with confinement in Context B. Following repeated conditioning sessions, test subjects are once again allowed to freely shuttle between two Contexts, and time on each Context is measured as the primary dependent measure. A place preference for the Context paired with experimental treatment (i.e. greater time spent on Context A compared to Context B during the post-conditioning test session) is interpreted as developing after the formation of a positive associative memory. Oppositely, a place aversion is formed when a test subject spends less time within the Context paired with experimental treatment and is thought to reflect the formation of a

negative associative memory. In utilizing place conditioning, researchers can assess both the rewarding and aversive properties of experimental treatments.

In initial assessment of the acute effects following injection of drugs of abuse, numerous teams captured an initial post-drug rewarding state as interpreted from the development of place preferences (Kumar 1972; Mucha et al. 1982). In other work, pairing an environment with precipitation of withdrawal in drug-dependent rats was shown to produce place aversion (Hand et al. 1988; Suzuki et al. 1996). Readers should note that, contrary to acute drug effects delineated from place conditioning studies, taste aversions frequently develop in test subjects receiving drug of abuse immediately following tastant access (for review, Goudie 1979). For cocaine, taste aversions develop when rats are injected with relatively high doses (20 and 40 mg/kg, IP) after tastant access (Heinrichs et al. 1998). Conditioned taste aversions are interpreted as reflecting “toxicity” and/or “sickness” induced in response to administration of experimental treatment, although temporal contiguity is inconsistent with place conditioning designs (i.e. drug is injected prior to place conditioning but after conditioning in tests of associative taste learning). As is detailed in [Section 5.2.1.3](#), the duration of conditioning trials—prior to or post-drug—could permit the experience of sequential epochs characterized by opposing subjective states such that a mixed associative memory develops. Place conditioning enables researchers to evaluate “passive” reward learning through Pavlovian-like associative memory formation.

1.3.3) Modeling volitional drug-taking using intravenous self-administration

In 1962, James Weeks announced his “experimental morphine addiction” model whereby unrestrained rats could perform an operant response, such as lever pressing, for

intravenous drug injection. Intravenous drug self-administration remains to-date a model with face and construct validity that measures abuse potential and captures the volitional aspect of drug-taking. Nearly all drugs that are abused by humans are reinforcing and self-administered intravenously by laboratory subjects including non-human primates and rats (Thompson and Schuster 1964). Important differences in the subjective effects associated with drug receipt have been determined between volitional as compared to experimental injection including toxicity and withdrawal symptom severity (Dworkin et al. 1995). As drugs of abuse are taken invariably by the user, self-administration better models the psychiatric conditions as they manifest in humans.

Several paradigmatic iterations of intravenous drug self-administration can be created by altering response-outcome contingencies. For example, a test subject's "motivational drive" may be assessed by measuring the number of operant responses (or frequency of operant responding) exerted to retrieve drug injection. Session length can be adjusted to model clinically-documented "binge" episodes and additionally appears to be a critical determinant in modeling the diagnostic feature of "escalation" of drug intake in self-administering rats when permitting extended-access availability (Ahmed and Koob 1998). Moreover, the rat's drug-taking environment as well as discrete cues are often imbued with behaviorally-activating salience following context- and cue-drug associative memory formation. Taking advantage of this, experimenters can model the extent to which contexts and cues drive drug-seeking in tests of relapse, which can be presented alongside "triggers" including stress and drug "priming" thought to behaviorally disinhibit test subjects. Collectively, drug self-administration presents a customizable model allowing researchers to probe how pharmacological and behavioral interventions

alter drug-taking and -seeking under controlled laboratory conditions. In improving how models reflect psychiatric states seen in humans, we are afforded improved accuracy in understanding how the brain and body become affected in such states.

1.4) Neurophysiology of reward prediction and receipt: role of mesolimbic dopamine transmission in the context of psychostimulant use

Of significance to the content of this thesis, the ventral tegmental area (VTA)—which houses neurons producing the catecholamine neurotransmitter dopamine (DA)—was revealed as a site readily supporting electrical self-stimulation as has the medial forebrain bundle (MFB) which encompasses DA fibers that project to ascending forebrain structures. A principal target of VTA DA output is the nucleus accumbens (NAcc) which itself is housed within the ventral striatum of rats. DA signals via two broad categories of G-protein coupled receptors (GPCRs) stratified based on net physiological effect—“D₁-like receptors” include D₁ and D₅ GPCRs, are G α_s -coupled and stimulate adenylyl cyclase to elevate intracellular cyclic adenosine monophosphate (cAMP) producing net excitation whereas “D₂-like receptors” include D₂, D₃ and D₄ GPCRs, are G α_i -coupled and produce an opposite, inhibitory effect when bound to (for review, Beaulieu et al. 2015). Electrical self-stimulation of MFB and VTA were found to evoke elevations in extracellular NAcc DA content and enhance firing rates of NAcc single units *in vivo* (Wolske et al. 1993; Young and Michael 1993). Years earlier, it was shown that local perfusion of DA and, to a lesser extent, noradrenaline (NA) into NAcc facilitates ICSS (Redgrave 1978). In agreement with abused drugs’ ability to facilitate reward function, neurochemical evidence shows that cocaine and amphetamine elevate NAcc DA content (Baird and Lewis 1964; Di Chiara and Imperato 1988; Pettit and

Justice Jr. 1989); these effects are attributed to pre-synaptic inhibition and release facilitation mechanisms (Ross and Renyi 1967). Decades of work have thereafter helped shape our understanding on how rewards including drugs of abuse and reward-predictive cues affect VTA DA physiology and neurochemistry.

For example, Schultz and team performed work in non-human primates to demonstrate that unexpected intra-oral delivery of a fruit juice reward robustly elevates phasic (“burst”) activity of VTA DA neurons *in vivo* (e.g., Mirenowicz and Schultz 1996; for review, Schultz et al. 2017). These same neurons became “tuned” to fire to a reward-predictive cue after an associative learning training period. In rats, “reward prediction” was expanded to find that initial receipt of a palatable, natural reward elevates extracellular DA content in NAcc, and that a similar effect can be observed following presentation of a reward-predictive cue in trained rats which is sensitive to VTA ablation (Yun et al. 2004). Complimentary elevations in NAcc DA were observed when rats were trained to associate a cue with the opportunity to electrically self-stimulate (Owesson-White et al. 2008). Notably, self-administration of cocaine evokes burst firing of NAcc single units which remain sensitive to drug-predictive cues weeks after abstinence (Peoples and West 1996; Ghitza et al. 2003). Neurochemical work shows that both cocaine and the synthetic cathinone MDPV significantly elevate extracellular NAcc DA content *in vivo*—the latter with approximately ten-fold greater potency (Baumann et al. 2013). Together, this work supported the integration of reward-predictive cues in behavioral studies including drug self-administration which has allowed researchers to probe the salience and persistence of maladaptive drug-cue memories.

Mixed results have circulated interrogating how cocaine affects cellular physiology of VTA DA and nearby cells. In the 1980s, intravenous cocaine was shown to inhibit firing of a majority of recorded VTA DA neurons *in vivo* (Pitts and Marwah 1987; Einhorn et al. 1988). In contrast, Cameron and Williams (1994) provided compelling evidence that cocaine may lead to VTA DA disinhibition by way of suppressed local γ -aminobutyric acid (GABA) release. Newer reports find that both excitatory and inhibitory acute actions of intravenous cocaine on VTA DA neurophysiology can be observed and that a likely explanation is heterogeneous neurochemical composition and input regulation (Shi et al. 2004; Mejias-Aponte et al. 2015; see [Section 4.1](#) of this thesis). In the nearby ventral pallidum (VP)—a structure positioned to integrate signals from both VTA and NAcc that outputs in large part to thalamus—self-administered cocaine evokes distinctive patterns of either excitation or inhibition from single units recorded *in vivo* (Root et al. 2010). Further, VP inputs from NAcc were found to critically regulate cocaine-seeking in relapse tests (Stefanik et al. 2013). Additional evidence finds that terminal release of DA within NAcc mediates cocaine reinforcement as local lesioning with 6-hydroxydopamine (6-OHDA) robustly blunts cocaine self-administration (Pettit et al. 1984). It seems clear, though, that cocaine withdrawal leads to disrupted VTA DA physiology, although the dosing regimen and means of injection appear to impact directionality of withdrawal-associated effects (e.g., Lee et al. 1999).

Mixed results acquired with other drug types (i.e., non-cocaine) further support “cell type” heterogeneity within VTA. Early *in vivo* electrophysiology work showed that acute amphetamine injection inhibits VTA DA activity likely via negative feedback from striatal inputs whereas neuroleptic agents such as haloperidol augment VTA DA

physiology (Bunney et al. 1973; Bunney and Achajanian 1976). Matthews and German (1984) showed that VTA DA neurons are strongly activated by morphine whereas cells in nearby substantia nigra (SN) fail to show morphine-elicited excitation. Contrarily, a subset of “tertiary” VTA DA cells hyperpolarized upon bath application of μ opioid receptor (MOR) agonists *ex vivo* (Cameron et al. 1997). Other lines of work show that systemic caffeine acutely depresses VTA DA activity (Stoner et al. 1988). The dissociative drug of abuse phencyclidine (PCP) elicits bimodal influence on VTA DA cell physiology—a rapid though transient depolarizing effect followed by relatively protracted inhibition (French et al. 1991). Cannabinoid receptor agonists transiently enhance firing rate in a majority of VTA DA cells in a CB₁-dependent manner (Cheer et al. 2003). Likewise, the commonly-abused inhalant toluene elevates firing rate of VTA DA neurons *in vivo* as well as terminal DA content in NAcc (Riegel et al. 2007). *Ex vivo*, ethanol depolarized VTA DA neurons but with appreciably reduced potency compared to toluene (Nimitvilai et al. 2016). Like cocaine, mixed effects measured from VTA DA neurons upon administration/application of other drug types support that differential neurophysiological effects are produced which are likely regulated by inputs to and neurochemical composition of recorded cells.

Collectively, seminal works described above provide foundation for how we understand rewards to be processed within mammalian brains. Basic and pre-clinical researchers have rightfully focused on how central catecholamine transmission is modulated, and a vast number of transmitter and peptide systems have been uncovered accordingly as important contributors. These systems at times provide pharmacological targeting opportunity which is performed in effort to artificially modulate catecholamine

transmission. Certainly, rewards (natural and drugs) can alter genetic landscapes within nuclei of certain cells, and this provides additional opportunity for targeting. While new technology focused on optically and/or chemogenetically targeting receptors (which largely require viral-mediated expression) has surged in the past decade and the first “optogenetics” clinical trial has started recruitment (NCT02556736 from <https://ClinicalTrials.gov>), pharmacological tools remain an effective way to normalize aberrant neural circuits known to underlie psychiatric disease states.

CHAPTER 2 – HYPOCRETIN/OREXIN ANATOMY, CONNECTIVITY AND PHYSIOLOGY

G-protein coupled receptors (GPCRs) present attractive targets for drug development to aide treatment regimens of nearly all known psychiatric disorders. In 1998, two independent research groups described their discoveries of a novel neuropeptide system produced exclusively within hypothalamic compartments with functions in appetite regulation and neuroexcitation. The following sections detail the discoveries of hypocretin/orexin (hcr/ox) peptides, their receptors and initial functions ascribed to their transmission. For explicit clarification, the terms hypocretin and orexin refer to identical mRNA/protein. Advances in our understanding of the physiology, connectivity and transmission capacity of hcr/ox cells will also be provided.

2.1) Discoveries of a novel neuropeptide system: hypocretin/orexin

While the primary discovery paper was published in 1998 by first-author Luis de Lecea, much of the work of this Scripps-based research team was provided by investigator Gautvik and team 2 years earlier (Gautvik et al. 1996). Using a directional tag polymerase chain reaction (PCR) subtraction identification strategy (for detail, see Usui et al. 1994) of RNA extracts from rat hypothalamus, 23 highly enriched novel mRNAs were detected. Compared to oxytocin (clone 2 from this report) which showed predominant signal within the hypothalamus, northern blot analysis of clone 35 also showed unique presence within hypothalamus with scant signal from cortical homogenates whereas all other novel sequences showed relatively promiscuous labeling in other brain regions including thalamus, hippocampus and pituitary. The authors concluded, “Preliminary nucleotide sequence data suggest that the clone 35 mRNA

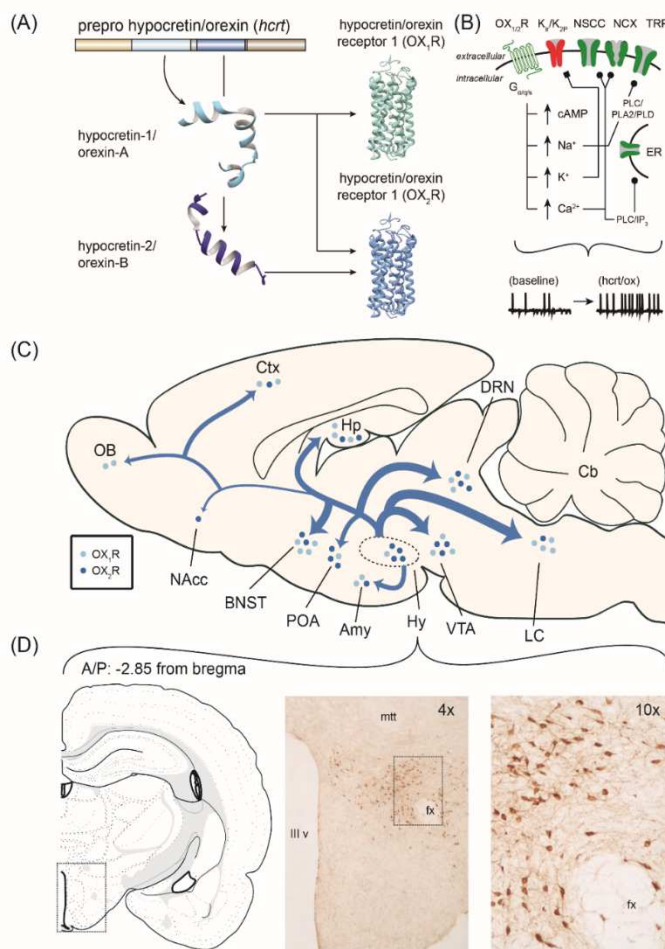


Figure 1. (A) Hcr/ox mRNA and two mature protein products (hcr1/OX-A and hcr2/OX-B). (B) Mechanisms through which hcr/ox exerts neuroexcitatory actions. (C) Origin and projections of hypothalamic orexin neurons with relative quantitative distribution of receptors derived from *in situ* hybridization study and pathway intensity from fiber innervation using studies immunofluorescence. Amy – amygdala, BNST – bed nucleus of stria terminalis, Cb – cerebellum, Ctx – cortex, DRN – dorsal raphe nucleus, Hp – hippocampus, Hy – hypothalamus, LC – locus coeruleus, NAcc – nucleus accumbens, OB – olfactory bulb, POA – preoptic area, VTA – ventral tegmental area. (D) Immunohistochemical characterization of the orexin field within rat hypothalamus. Atlas image adapted from Brain Maps: Structure of the Rat Brain, Third Edition (Swanson 2004). IIIv – third ventricle, fx – fornix, mtt – mammillothalamic tract. Figure originally contained within and adapted from Gentile et al. 2017a.

1a). Cleavage sites within the precursor sequence were identified for production of two mature peptides termed hypocretin-1 and -2. Notably, application of a synthetic hypocretin-2 peptide (corresponding to residues 69-96 of the precursor sequence; 1 μ M) to synaptically-coupled rat hypothalamic cells *in vitro* enhanced post-synaptic

encodes a novel small secretory protein that contains sites for proteolytic maturation, possibly the hallmarks of a new hypothalamic peptide hormone precursor” (Gautvik et al. 1996). Indeed, in a more comprehensive report (de Lecea et al. 1998), the hypocretin peptide (previously termed clone 35) was named for its localization within hypothalamus (hypo-) and sequential homology to the gut hormone secretin (-cretin) (**Figure**

depolarizations suggesting neuroexcitatory action. The authors suggested that hypocretin may be functionally involved in the promotion of food consumption or possess anorectic and/or satiety-promoting properties (e.g., as is known with cholecystokinin [CCK]).

A second research team, based in Japan, identified the same hypothalamic precursor mRNA sequence and mature peptides but advanced our understanding of its receptors. Using over 50 stable cell lines transfected with unique orphan GPCR cDNA constructs and measuring intracellular signaling readouts following exposure to tissue fractions resolved by high-performance liquid chromatography (HPLC), Sakurai and colleagues (1998) discovered the peptide orexin which was named after the Greek word *orexis* meaning “appetite”. Contained in this discovery report is the mRNA sequences for the orexin precursor peptide as well as its two excitatory cognate receptors presumed as G_q-linked (termed OX₁R and OX₂R). The orexin peptide precursor is cleaved at its Gln³³ site via N-terminal pyroglutamyl residue transamidation to match the purified orexin-A (OX-A) mature peptide mRNA sequence and is tailed by C-terminal amidated Gly⁶⁶. A second sequence of the precursor mRNA (Arg⁶⁹-Met⁹⁶) encodes the mature orexin-B peptide and is also C-terminal amidated. Northern blot tissue screening yielded a nearly exclusive compartmentalization of orexin peptide precursor and OX₁R/OX₂R mRNAs within the brain of rat subjects. Immunohistochemical and *in situ* hybridization supported that the orexin precursor mRNA and mature protein are localized to hypothalamus (namely within lateral, posterior and perifornical compartments). Orexin cell body structures were generally categorized as medium-sized yet varied from thin/fusiform to robust/multipolar. The authors took note that no signals were detected within paraventricular thalamus or arcuate nucleus—two structures critical for regulating food

consumption (e.g., Bernardis and Bellinger 1996). Behaviorally, central injection of OX-A or -B stimulated feeding above consumption measured from vehicle-injected control subjects at 1- to 4-h post-injection. Moreover, hypothalamic orexin precursor mRNA was found to be significantly upregulated (2.4-fold) in rats subjected to a 48-h fasting period relative to freely-fed control rats.

2.2) *Connectivity and physiology of the hypocretin/orexin neuropeptide system*

While the innervation of hypothalamic hcr/ox to target structures is still being explored, its first characterization was provided within a year following discovery. Peyron and colleagues (1998) used rabbit-raised antiserum to target the 17 amino acids of the hcr/ox precursor protein (sequence: CPTATATALAPRGGSRV) to profile cell bodies and fibers of the neuropeptide across rat brain (**Figure 1c**). To compliment, the team also utilized digoxigenin-amplified *in situ* hybridization to probe for the hcr/ox precursor mRNA to support initial findings that code for the precursor is restricted to hypothalamus. Double-labeling with a riboprobe targeting melanin-concentrating hormone (MCH), which is known to be synthesized predominantly within hypothalamus, revealed no overlap suggesting unique populations of cells produce the hcr/ox and MCH peptides. Ultrastructural analyses further revealed that hcr/ox peptide can be found proximal to cytoplasmic dense core granules as well as Golgi apparatus. Dense hcr/ox fiber immunoreactivity was observed in locus coeruleus (LC) followed by other structures including raphe nuclei, compacta division of substantia nigra (SN), bed nucleus of stria terminalis (BNST), central grey and VTA. This anatomical characterization paper is widely cited and provided a basis for possible functions across domains ranging from

energy homeostasis (i.e. feeding, thermoregulation), regulation of sleep/wakefulness via innervation to LC as well as motivated behaviors via innervation to midbrain.

Marcus and colleagues (2001) provided comprehensive profiling of hcr/ox receptor subtype (OX₁R and OX₂R) mRNA across rat brain using X-ray autoradiography (**Figure 1c**). Strong OX₁R mRNA signal was found within BNST, hypothalamus, A4/5/7 midbrain cell groups and LC. OX₂R mRNA was found in high density within septal/hippocampal regions, hypothalamus with relatively low labeling detected across midbrain and brainstem. A second report of comparable ambition using similar methodologies reported similar densities of OX₁R and OX₂R across rat brain (Trivedi et al. 1998).

Years later, Sakurai and colleagues (2005) developed a genetically-encoded retrograde tracing method to further map inputs to the hcr/ox cells. Primary input sites included the ventral striatum, amygdala and preoptic areas. A reciprocal connection between cholinergic neurons of ventral striatum and hcr/ox cells was uncovered, and slice physiology showed that one-third of hcr/ox are excited by carbachol, an acetylcholine receptor agonist. Other work using retrograde and anterograde tracers within hypothalamus and within sites suspected to provide input to hcr/ox cells, respectively, revealed dense local input to hcr/ox as approximately one-third of retrogradely-traced cells were found in hypothalamus (Yoshida et al. 2006). Dense inputs were also traced from cortex, ventral striatum and amygdala. To complicate topography further, España and colleagues (2005) found that single hcr/ox cells are capable of targeting multiple far-reaching targets throughout brainstem and forebrain. In addition to rodent work, comparative anatomical studies reveal similar patterns of hcr/ox cell

populations and projections across non-human primates, domestic pigs, certain avian species, and even fish (Singletary et al. 2006; Amiya et al. 2007; Chometton et al. 2014).

Physiology study finds that hcrt/ox peptide application enhances GABA- and glutamate-mediated spontaneous post-synaptic currents, suggesting influence of hcrt/ox transmission on excitatory and inhibitory cell types (van den Pol et al. 1998). Using *in vitro* Ca²⁺ imaging, hcrt/ox peptides significantly and dose-dependently elevated intracellular Ca²⁺ levels when bath-applied to cultured hypothalamic cells. Hcrt/ox was able to elevate intracellular Ca²⁺ levels in the presence of tetrodotoxin (TTX) suggesting a mechanism of neuroexcitation beyond synaptic release. Numerous direct and indirect intracellular signaling mechanisms have been proposed as contributors to net physiological excitation in neurons *ex vivo* following hcrt/ox ligand binding including: (i) G_{s/q}-mediated cyclic adenosine monophosphate (cAMP) production, (ii) G_{α/q}-mediated inhibition of two-pore-domain and inward rectifier K⁺ channels (K_{2P} and K_{ir}, respectively), (iii) activation of non-selective cation channels (NSCC) and/or Na⁺/Ca²⁺ exchanger (NCX), (iv) inositol-1,4,5-triphosphate (IP₃)-mediated release of Ca²⁺ from endoplasmic reticular stores following G_q-mediated activation of phospholipase C (PLC), (v) Na⁺ influx via PLC-/PLA₂/PLD-mediated activation of transient receptor potential (TRP) channels, (vi) voltage-gated L-type Ca²⁺ channel opening following PLC and protein kinase C (PKC) activation, and (vii) PLC-mediated 2-arachidonoyl glycerol (2-AG) production following lipase-mediated diacylglycerol (DAG) hydrolysis (for reviews, see Kukkonen 2014, 2016; **Figure 1b**).

With regard to regulation of hypothalamic output, it appears that hcrt/ox output is propagated following hcrt/ox-mediated excitation via local glutamate signaling (Li et al.

2002). Other work finds that hcrt/ox cells are sensitive to other peptides—glucagon-like peptide 1 depolarizes hcrt/ox cells whereas neuropeptide Y (NPY) produces hyperpolarization (Acuna-Goycolea and van den Pol 2004; Fu et al. 2004). Neuroexcitatory actions of hcrt/ox are shown *ex vivo* on putative VTA DA-producing cells (Korotkova et al. 2003; Muschamp et al. 2007) as well as noradrenaline (NA) producing neurons of LC (Hagan et al. 1999), histamine-producing cells of the tuberomammillary nucleus (Eriksson et al. 2001), GABA-producing cells of arcuate nucleus (Burdakov et al. 2003) and dorsal root ganglion (DRG) cells of spinal cord (Yan et al. 2008). Interestingly, neuroexcitation of VTA DA neurons following stimulation of putative glutamate-producing inputs from medial prefrontal cortex (mPFC) was augmented by local application of hcrt/ox (Moorman and Aston-Jones 2010) suggesting probable indirect modulatory actions of hcrt/ox in midbrain. Taken together, hcrt/ox produces excitation by cation transport regulation (enhancing Ca^{2+} influx, suppressing K^{+} efflux) and itself is promiscuously regulated by local peptides as well as by canonical neurotransmitters.

Of particular relevance to the experiments of this thesis, original tract tracing work using iontophoretically-injected FluroGold revealed ~20% of hcrt/ox cells target VTA, and that hcrt/ox terminals closely appose DA-producing cells of VTA (Fadel and Deutch 2002). Additional support for functional transmission of hcrt/ox in VTA was provided by Borgland and colleagues (2006) showing that hcrt/ox induces OX_1R -dependent NMDA-mediated plasticity of VTA DA synapses. Curiously, electron microscopy analysis revealed that the majority of hcrt/ox fibers within VTA pass to more caudal structures or signal within VTA non-synaptically, although a small proportion

were seen synaptically-linked to DA- and GABA-producing VTA neurons (Balcita-Pedicino and Sesack 2007). Even if synapsing onto a minority of VTA cells, OX₁R blockade significantly suppresses firing of VTA DA neurons during the active/wake phase in tested animals (Moorman and Aston-Jones 2010). Extensive reciprocal connectivity exists between hypothalamic hcr/ox cells and with NA-producing LC cells (Baldo et al. 2003; Carter et al. 2009) prompting functional interrogation of hcr/ox within arousal-promoting brainstem nuclei. In support of a functional hcr/ox → LC pathway, intra-LC administration of OX-A led to a robust yet transient elevation in extracellular NA within hippocampal targets (Walling et al. 2004). Separately, neurons producing serotonin (5-HT) arising from DRN can hyperpolarize hcr/ox cells via 5-HT_{1A}Rs (Muraki et al. 2004). Hcr/ox cells are additionally sensitive to purines by way of P2X₂Rs suggesting direct influence of local adenosine triphosphate (ATP) release (Florenzano et al. 2006). Whereas other hypothalamic cell populations are excited by cannabinoids, hcr/ox cells show a CB₁R-dependent reduction in spike frequency upon application of WIN55,212,2 (Huang et al. 2007). Strikingly, CB₁Rs and OX₁Rs have been shown to directly interact and form heteromultimers *in vitro* (Ward et al. 2011). Collectively, these studies support that hcr/ox synaptic inputs and outputs are diversely regulated, and that reciprocal connections are thus far known to exist between hcr/ox and monoaminergic nuclei of LC, DRN and VTA.

Like many neuronal cell types, hcr/ox populations are capable of packaging and releasing other peptide and canonical neurotransmitters. mRNA for the vesicular glutamate transporter type-2 (VGluT2) was detected in ~50% of hcr/ox-immunolabeled cells of rat brain (Rosin et al. 2003). As a testament to hcr/ox neuroexcitatory action, no

overlap with glutamic acid decarboxylase 67 (GAD67; marker of GABA-producing cell bodies) was found. Curiously, hcrt/ox cells were found to produce mRNA for the precursor peptide of dynorphin, the endogenous ligand of kappa opioid receptors (KORs) (Chou et al. 2001)—this finding was supported using both *in situ* hybridization to target mRNAs as well as co-immunolabeling methods against the mature peptides. Li and Van den pol (2006) then showed that hcrt/ox cells synapse onto other local hypothalamic hcrt/ox cells but that sensitivity of this pathway is biased towards dynorphin signaling. In an *ex vivo* preparation, dynorphin suppressed activity of hcrt/ox cells by altering spike frequency and calcium currents; opposingly, bath application of a KOR antagonist augmented hcrt/ox activity supporting the interpretation that hypothalamic-based dynorphin exerts suppressive tone over the hcrt/ox cell population. Williams and Behn (2011) modeled the dynamic interplay between hcrt/ox and dynorphin based on experimental slice physiology data and concluded that desensitization of hcrt/ox cells to KOR stimulation permits a rapid shift of these cells from KOR-mediated inhibition to hcrt/ox-driven excitation—in turn, hcrt/ox release is stimulated and excitatory activity is signaled to targets. Indeed, Muschamp and colleagues (2014) found that unique populations of putative DA-producing cells within VTA exhibit preferential sensitivity to excitatory actions of hcrt/ox, inhibitory actions of dynorphin, or mixed sensitivity whereby endogenous ligands of both transmitter types influence VTA DA cell activity. Follow-up retrograde tracing work revealed preferential excitatory actions of VTA DA cells targeting NAcc whereas VTA DA cells targeting amygdala were preferentially inhibited by dynorphin (Baimel et al. 2017). These papers set the stage for ongoing

studies interrogating functional interactions between hcrt/ox and dynorphin in behavioral states influenced by motivation and mood/affect which are mentioned in Chapter 3.

CHAPTER 3 – HYPOCRETIN/OREXIN FUNCTION AND ROLES IN PATHOLOGY

3.1) *Orexigenic and thirst-regulating properties of hypocretin/orexin*

Contained in their discovery paper, Sakurai and colleagues (1998) found that central injection of OX-A stimulates feeding in freely-fed rats—an effect that has since been replicated (e.g., Sweet et al. 1999) but clarified as diurnally linked to the inactive phase (Haynes et al. 1999; Yamanaka et al. 1999). Lubkin and Stricker-Krongrad (1998) used indirect calorimetry to reveal metabolism enhancing effects of central OX-A injection which would in part drive food-seeking and consumption. Indeed, central injection of OX-A was soon found to stimulate the cephalic phase of gastric acid secretion (i.e. prior to food consumption) (Takahashi et al. 1999) which aligns with the finding that fasting can enhance hypothalamic hcrt/ox content (Mondal et al. 1999). Relatedly, cues linked to palatable food consumption enhance Fos expression in hcrt/ox cells (Choi et al. 2010). In fact, hcrt/ox Fos expression following presentation of a food-paired cue persisted in rats that experienced punishment-induced suppression of food consumption (Campbell et al. 2017) suggesting resiliency of hcrt/ox to motivate food-seeking despite suspected adverse outcomes. Food-seeking is likely driven via OX₁R signaling as systemic OX₁R blockade suppresses hyperphagia induced by central injection of OX-A (Rodgers et al. 2001). In a stress-associated binge-eating paradigm, pharmacological blockade of OX₁Rs was effective in reducing palatable food consumption (Piccoli et al. 2012). Relatedly, central blockade of OX₁Rs disrupts sucrose taste preference (Mediavilla et al. 2011) and consumption (Cason and Aston-Jones 2014). Whereas hcrt/ox knockout mice are not impaired in responding for food reinforcement,

RNAi-mediated knockdown of *hcrt/ox* in adult mice impairs operant responses for food under progressive-ratio (PR) and variable-ratio (VR) schedules of reinforcement (Sharf et al. 2010)—these reinforcement schedules tax motivational drives of test subjects by employing high-effort reward contingencies. Taken together, amounting evidence supports a functional role of *hcrt/ox* in motivated food-seeking which is driven in large part via OX₁R transmission, although contributing target loci are not yet elucidated. These studies additionally demonstrate that *hcrt/ox* mediates energy homeostasis by engaging preparatory systems prior to food consumption, and that disorders characterized by pathological eating may be normalized in part by pharmacological intervention of *hcrt/ox* transmission.

In other lines of work delineating the conditions whereby *hcrt/ox* possesses orexigenic activity, Hagar and colleagues (2017) showed that *hcrt/ox* normalizes age-related deficits in feeding behaviors. The orexigenic property of *hcrt/ox* transmission is phylogenetically mixed—goldfish and bullfrog larvae consume more food upon central injection of OX-A (Volkoff et al. 1999; Shimizu et al. 2014) whereas neonatal chicks do not (Furuse et al. 1999). Further, activation of *hcrt/ox* cell populations in male voles was associated with feeding behavior (Zhang et al. 2011).

Orexigenic actions of *hcrt/ox* may in part be due to interactions with nearby peptide-sensitive hypothalamus cell populations including interactions with neuropeptide-Y, corticotropin-releasing factor and leptin (Funahashi et al. 2000; Ida et al. 2000; Jain et al. 2000). Moreover, OX-A can normalize hypophagia associated with injection of the satiety-signaling peptide cholecystokinin (CCK) (Asakawa et al. 2002). Hyperphagia induced by hypothalamic ghrelin injection was associated with increased

Fos activity of hcr/ox cells suggesting these peptides may interact within hypothalamus to stimulate feeding (Olszewski et al. 2003). shRNA-mediated knockdown of OX₁Rs within the paraventricular thalamic nucleus (PVT) reduced high-fat food consumption which the authors attribute in part to PVT OX₁R regulation of extracellular NAcc DA transmission (Choi et al. 2012). To compliment this work, Cole and colleagues (2015) showed that suppression of cue-induced food consumption following OX₁R blockade is met with concurrent increases in PVT Fos expression, suggesting that PVT may participate in inhibiting an otherwise motivated response to seek food. Within midbrain, OX₁Rs were shown to regulate palatable food consumption as intra-VTA OX-A injection stimulated feeding whereas bilateral intra-VTA OX₁R blockade reduced feeding (Terrill et al. 2016). Similarly, OX-A injection within the nucleus of the solitary tract (NTS) of brainstem was shown to increase high-fat food consumption (Kay et al. 2014). Hcr/ox-mediated food consumption seems to also recruit endocannabinoid signaling as pretreatment with the CB₁R antagonist rimonabant can block hyperphagia following central OX-A injection (Crespo et al. 2008). Collectively, whereas hcr/ox transmission in midbrain and brainstem seem to energize motivated food-seeking, select forebrain structures including PVT may participate in behavioral inhibition in part through regulating striatal DA content.

3.2) *Wakefulness and arousal promotion by hypocretin/orexin*

Two seminal *Cell* papers were published in 1999 that linked hcr/ox transmission to regulation of sleep. One study found that genetic mutation in the gene encoding hcr-r2 linked with narcolepsy—a debilitating condition characterized by unsignaled and uncontrollable bouts of sleep onset and dysregulated rapid eye movement (REM) sleep—

in canine subjects (Lin et al. 1999). A second paper by Chemelli and colleagues (1999) phenotyped the *hcr/ox* knockout mouse as displaying prominent narcolepsy-like behaviors and disrupted REM sleep which were later confirmed using an ataxin-3 genetic ablation strategy of *hcr/ox* cells. Clinical reports subsequently confirmed the regulatory role of *hcr/ox* transmission in arousal/sleep (Kubota et al. 2002; Kato et al. 2003) as did studies performed in non-human primates (Zeitzer et al. 2003). In direct support of a causal role, systemic treatment with OX-A was shown to rescue sleep deficits and cataplectic episodes in narcoleptic dogs (John et al. 2000). Similarly, expression of a *hcr/ox* transgene in *hcr/ox*-ablated mice can rescue sleep deficits and prevent cataplexy (Mieda et al. 2004).

Taheri and colleagues (1999) showed that *hcr/ox* peptide content fluctuates diurnally in rats with greatest release to arousal-regulating pontine structures (e.g., LC) captured in the dark phase of light cycle when rats are active, and that sleep deprivation can significantly elevate central *hcr/ox* content (Yoshida et al. 2001). Circadian regulation of plasma *hcr/ox* content has also been captured with greatest content observed in wake epochs (Kiyashchenko et al. 2002). Within the central nervous system, OX-A evokes Fos expression within structures known to regulate circadian rhythm including LC and suprachiasmatic nucleus (Date et al. 1999). Follow-up study confirmed that central OX-A injection concurrently suppresses REM sleep in part via depolarizing LC neurons *in vivo* (Bourgin et al. 2000). Subsequent studies reveal that optical as well as chemogenetic stimulation of the *hcr/ox* cell population can wake mice from sleep states and concurrently suppress REM sleep (Adamantidis et al. 2007; Sasaki et al. 2011). It appears *hcr/ox* projections to LC and tuberomammillary nucleus (TMN) are critical

regulators of wakefulness as wake states are promoted when hcr/ox innervation within these structures is restored (Mochizuki et al. 2011; Hasegawa et al. 2014). Similarly, forebrain cholinergic structures including ventral pallidum and preoptic area (collectively termed the “basal forebrain”) additionally promote wake states (España et al. 2001; Thakkar et al. 2001). Sleep deprivation leads to depleted hcr/ox cellular excitability which is in part mediated by altered local glutamate content and regulation of perisomatic hcr/ox inputs (Briggs et al. 2018). Thus, wake states are supported by hcr/ox transmission and deficits therein can lead to development of pathological sleep disorders, and a unique network of nuclei across the central nervous system participate in hcr/ox-mediated wake promotion.

3.2.1) Suvorexant (MK-4305) becomes first clinically-available hypocretin/orexin receptor antagonist

With the clear role of hcr/ox regulation of sleep, numerous major pharmaceutical companies raced to produce the first hcr/ox-based medication for treating sleep disorders. While earlier compounds were deemed “research only” agents for undesirable pharmacokinetics, toxicity profiles, and/or complications associated with Phase I-III clinical trials, Merck Research Laboratories synthesized and tested a hcr/ox receptor antagonist that eventually became the first-in-class medication approved by the Food and Drug Administration for management of primary insomnia—a disorder characterized by chronic sleep deficiencies (Whitman et al. 2009; Cox et al. 2010). Suvorexant (MK-4305) possesses a 7-methyl diazepam core, chlorobenzoxazole for improved lipophilicity for brain penetrability and boasts favorable potencies against OX₁R ($K_i = 0.55$ nM) and OX₂R ($K_i = 0.35$ nM) with clean ancillary profile against 170 screened off-target

enzymes, receptors and ion channels (Cox et al. 2010). Suvorexant produced significant enhancement in delta and REM sleep epochs with appreciable reductions in light sleep (no movement, predominantly consisting of theta waves, moderate electromyogram activity) from telemetry-implanted rats. In a multi-center trial of 522 patients diagnosed with primary insomnia, Michelson and colleagues (2014) reported significant improvement in subjective total sleep time and time to sleep onset in suvorexant-treated patients compared to the placebo-treated control population (NCT01021813 from <https://ClinicalTrials.gov>).

3.3) *Hypocretin/orexin and motivation for natural and drug rewards*

DiLeone and colleagues (2003) conjectured that hcrt/ox may participate in reward processing based on available anatomical and physiological data performed on midbrain cell populations. Published that year, Gulia and colleagues (2003) observed that local infusion of OX-A within the medial preoptic area (mPOA) can enhance sexual behaviors in male rats supporting a novel role for hcrt/ox in reward-seeking. Subsequent work revealed that hcrt/ox cells express significantly elevated Fos after male rats are given opportunities to copulate with sexually-receptive females (Muschamp et al. 2007). Curiously, castration led to a gradual deterioration of hcrt/ox cells commensurate with reductions in copulatory behavior—an effect in part normalized by gonadal hormone (estradiol) supplementation. Consistent with the hypothesis that hcrt/ox transmission mediates copulatory behavior, pharmacological OX₁R blockade increased intromission latency and decreased ejaculation frequency. Earlier work demonstrated sexually dimorphic expression of hcrt/ox receptor mRNAs in peripheral tissues including appreciably high steroid-sensitive OX₂R mRNA within adrenal glands of male rats

suggesting a controlling role of hcrt/ox in endocrine functions (Jöhren et al. 2001, 2003). Further still, OX-A can enhance testosterone secretion in a OX₁R-sensitive manner suggesting a role along the reproductive axis (Barreiro et al. 2004). These experiments set the stage for further interrogation of if/how hcrt/ox transmission regulates pathological states characterized by aberrant reward processing.

In the first documentation implicating hcrt/ox transmission in reward and reinforcement associated with drugs of abuse, Harris and colleagues (2005) showed that exposure to a morphine-paired context significantly elevates Fos expression within hcrt/ox cells. Moreover, morphine place preference was re-expressed following pharmacological activation of hcrt/ox cells. Finally, morphine place preference could additionally be re-expressed following bilateral injection of OX-A directed into VTA, suggesting that hcrt/ox transmission in VTA facilitates the retrieval of drug-paired associative memories. Not long thereafter, hcrt/ox genetically-deficient mice were found to have significantly attenuated morphine place preference relative to wildtype control subjects (Narita et al. 2006).

Numerous behavioral pharmacology reports followed to demonstrate that psychostimulant-associated reward and reinforcement is modulated by hcrt/ox transmission; these effects are summarized in **Table 1**. The development of locomotor sensitization from repeated cocaine injections is prevented in rats pre-treated systemically with an OX₁R antagonist (Borgland et al. 2006). OX₁R blockade was also shown to decrease motivated operant responding for intravenous cocaine (as well as for sucrose pellets) but failed to appreciably alter responding for normal food chow suggesting a selective role for hcrt/ox transmission via OX₁Rs in the retrieval of palatable reinforcers

(Borgland et al. 2009). These data supported prior work finding that central OX-A injection enhances the amount of work expended by rats for sucrose pellets (Thorpe et al. 2005) as well as intake of liquid saccharin (Furudono et al. 2006), and that OX₁R blockade suppresses responding for high-fat food pellets (Nair et al. 2008). A role for hcr/ox in the motivation required to self-administer cocaine was then made clear by España and colleagues (2010) showing that systemic OX₁R blockade has no effect when employing a low-effort, fixed-ratio 1 (FR-1) schedule of reinforcement but does reduce breakpoint in PR test sessions. OX₁R blockade additionally decreases cocaine infusions

| Paradigm | Cocaine Dose (mg/kg/inf) | Dependent Measure | Pre-Treatment | | | Effect Relative to Vehicle | Reference(s) | | |
|-------------------------------|--------------------------|-------------------------------|---------------------|-----------|-----------------------------------|----------------------------|--|-------------|----|
| | | | Target | Compound | Dose | | | | |
| Self-Administration | | | | | | | | | |
| PR | 0.50 | Breakpoint | OX ₁ R | SB-334867 | 10.0 mg/kg | ↓** | Borgland et al. 2009 | | |
| DT | 1.50 | Infusions (per 6 h) | OX ₁ R | SB-334867 | 7.5 mg/kg | ↓* | España et al. 2010 | | |
| | | | | | 15.0 mg/kg | ↓** | | | |
| | | | | | 30.0 mg/kg | ↓** | | | |
| PR | 0.75 | Breakpoint | | | 7.5 mg/kg | ↓* | | | |
| | | | | | 15.0 mg/kg | ↓** | | | |
| | | | | | 30.0 mg/kg | ↓** | | | |
| | | | | | 10.0 nmol (intra-VTA, unilateral) | - | | | |
| | | | | | 10.0 nmol (intra-VTA, bilateral) | ↓** | | | |
| FR 1 | 0.75 | Infusions (per 1 h) | OX _{1/2} R | Orexin-A | 0.5 nmol (icv, unilateral) | - | España et al. 2011 | | |
| | 1.50 | Infusions (per 6 h) | | | | ↑** ^a | | | |
| PR | 0.75 | Breakpoint | | | 0.5 nmol (intra-VTA, bilateral) | ↑* | | | |
| VR 1-3 | 0.50 | # of Infusions (Day 1) | OX ₁ R | SB-334867 | 10.0 mg/kg | - | Hutcheson et al. 2011 | | |
| | | | | | 30.0 mg/kg | ↓* | | | |
| | | | | | 10.0 mg/kg | ↓** | | | |
| | | # of Infusions (Day 4) | | | 30.0 mg/kg | ↓** | | | |
| FR 5 | 0.30 | # of Infusions | OX ₁ R | SB-334867 | 3.0 µg (intra-VTA, bilateral) | ↓**** | Muschamp et al. 2014 | | |
| PR | 0.75 | Breakpoint | OX ₁ R | SB-334867 | 7.5 mg/kg | - | Prince et al. 2014; Brodnik et al. 2015 | | |
| | | | | | 15.0 mg/kg | ↓* | | | |
| | | | | | 30.0 mg/kg | ↓* | | | |
| | | | | | OX ₂ R | 4PT | | 7.5 mg/kg | - |
| | | | | | | | | 15.0 mg/kg | - |
| | | | | | | | | 30.0 mg/kg | - |
| | | | | | OX _{1/2} R | Almorexant | | 25.0 mg/kg | - |
| | | | | | | | | 50.0 mg/kg | ↓* |
| | | | | | | | | 100.0 mg/kg | ↓* |
| Reinstatement | | | | | | | | | |
| Stress-induced Reinstatement | N/A | Lever Presses (per 2 h) | OX ₁ R | SB-334867 | 15 mg/kg | - | Boutrel et al. 2005 | | |
| | | | | | 30 mg/kg | ↓* | | | |
| Cued Reinstatement | N/A | Lever Presses (per 2 h) | OX ₁ R | SB-334867 | 10 mg/kg | - | Smith, See and Aston-Jones 2009; Smith et al. 2010 | | |
| | | | | | 20 mg/kg | ↓* | | | |
| | | | | | 30 mg/kg | ↓** | | | |
| | | | OX ₂ R | 4PT | 10 mg/kg | - | | | |
| | | | | | 30 mg/kg | - | | | |
| Cued Reinstatement | N/A | Lever Presses | OX ₁ R | SB-334867 | 10.0 mg/kg | - | Zhou et al. 2012 ^b | | |
| | | | | | 20.0 mg/kg | - | | | |
| | | | | | 30.0 mg/kg | ↓* | | | |
| Stress-induced Reinstatement | | | | | | ↓* | | | |
| Cocaine-induced Reinstatement | | | | | | - | | | |
| Cocaine + Cued Reinstatement | | | | | | ↓* | | | |
| Cued Reinstatement | N/A | Lever Presses (per 2 h) | OX ₁ R | SB-334867 | 1.0 mmol (intra-VTA, unilateral) | ↓*** | Mahler, Smith and Aston-Jones 2013 | | |
| Cued Reinstatement | N/A | Lever Presses (per 1 h) | OX ₁ R | SB-334867 | 1.0 mg/kg | - | Martin-Fardon and Weiss 2014 | | |
| | | | | | 3.0 mg/kg | ↓*** | | | |
| | | | | | 10.0 mg/kg | ↓*** | | | |
| Cued Reinstatement | N/A | Demand Elasticity (α) | OX ₁ R | SB-334867 | 30 mg/kg | ↑* | Bentzley and Aston-Jones 2015 | | |
| | | Consumption (Q ₁) | | | | - | | | |

^a Effect was observed when animals began self-administration at 3:00PM, a time at which animals normally reduce cocaine infusions.

^b Sex-dependent effects on cocaine seeking during reinstatement tests observed.

Table 1. Summary of select findings from studies utilizing pharmacological agents against hcrt/ox receptors on behaviors associated with cocaine self-administration and reinstatement of cocaine-seeking. Adapted from Gentile et al. 2017a.

when receipt is unpredictable under VR access conditions (Hutcheson et al. 2011).

Muschamp and colleagues (2014) soon revealed VTA OX₁Rs as direct contributors to

motivated cocaine taking—effects of which are counteracted by KOR activation. Complimentary work showed that direct infusion of OX-A into VTA elevated ventral striatal DA and facilitated operant responding for intravenous cocaine (España et al. 2011). Systemic pre-treatment with OX₁R as well as OX₂R antagonists decreased cocaine-evoked elevations in ventral striatal DA (Prince et al. 2014; Brodnik et al. 2015)—an effect additionally supported by the preliminary published studies summarized in Chapter 6 of this thesis.

Hcr/ox transmission appears to underlie seeking for other drug types as well. Moorman and Aston-Jones (2009) observed that OX₁R blockade suppresses alcohol consumption in a stratified subset of “highly motivated” rats which was later confirmed with alternative pharmacological tools (Lopez et al. 2016). Separately, systemic OX₂R blockade was shown to reduce escalation of self-administered heroin—a characteristic feature in the development of addiction (Ahmed and Koob 1998)—under extended access conditions (Schmeichel et al. 2015). Divergent from findings using cocaine self-administration, systemic OX₁R blockade suppresses low-effort (FR-1) responding for heroin (Smith and Aston-Jones 2012).

Hcr/ox receptor blockade additionally shows partial therapeutic potential in its ability to modulate drug-seeking in tests of relapse. Boutrel and team (2005) first showed that OX₁R antagonism reduces cocaine-seeking following footshock-evoked reinstatement. Other converging evidence finds that systemic or intra-VTA injection of hcr/ox receptor antagonists can reduce cocaine-seeking in reinstatement tests triggered by cues or contexts but not by priming injections (Smith et al. 2009; Zhou et al. 2012; Mahler et al. 2013; Martin-Fardon and Weiss 2014). OX₁R blockade is additionally

effective in reducing cued reinstatement of alcohol-seeking (Moorman et al. 2017)—prior topographical analyses suggested that hcrt/ox cells within lateral hypothalamus may promote cued drug-seeking as compared to hcrt/ox cells in dorsomedial hypothalamus (e.g., Moorman et al. 2016). Further still, OX₁R blockade was effective in reducing cue-elicited heroin seeking (Smith and Aston-Jones 2012). However, OX₁R blockade does not reduce seeking for high-fat food pellets when provoked by pellet consumption or central OX-A injection (Nair et al. 2008). Convincing new work reveals that hcrt/ox knockout mice show resilience in tests of cued reinstatement of cocaine-seeking compared to wildtype littermate control subjects (Steiner et al. 2018)—reductions in reward-seeking were additionally seen for saccharine. Taken together, these experiments support that hcrt/ox transmission underlies seeking behavior for multiple drug types under some but not all methods of relapse provocation.

CHAPTER 4 – VENTRAL MIDBRAIN: CELLULAR HETEROGENEITY, CONNECTIVITY AND ROLES IN REWARD-SEEKING

The recent two decades have been met with remarkable gain in our understanding of neural circuit complexities. Notably, circuit perturbation techniques that achieve cell-type and pathway-specific resolution including photostimulation/inhibition have introduced new contributors that participate in stimulus processing and reward-seeking. Contained in this section is a developing history on our understanding of midbrain anatomy in laboratory animals including new insights on how neurochemical heterogeneity underlies functions in the context of reward-seeking.

4.1) Cellular heterogeneity within ventral tegmental area confers divergent functions

The anatomical boundaries of VTA and cytoarchitectural features therein were introduced in the late 1950s as a structure containing one of twelve monoaminergic cell clusters (A10 of A1-12) (Montagu 1957; Nauta 1958). DA-producing cells can be immunohistochemically targeted upon application of a probe against tyrosine hydroxylase—the rate-limiting enzyme used in DA biosynthesis—and most of these cells were found to reside in the lateral parabrachial pigmented and paranigral compartments of VTA (Swanson 1982). Electrophysiological characteristics of immunohistochemically-confirmed VTA DA cells recorded *ex vivo* were provided in 1989 (Grace and Onn 1989) confirming slow depolarizing potentials and a relatively sustained spike threshold around -36 mV but distinguishing a regular “pacemaker” activity not identified in prior *in vivo* work. Kiyatkin and Rebec (1998) identified three subsets of VTA neurons in awake, behaving rats based on sensitivity to DA, GABA and glutamate, discharge stability, and relationship of firing to movement. Only one type (“Type I”) matched properties of

earlier-studied VTA DA neurons, whereas Type II and Type III neurons were speculated as non-DA. Steffensen and colleagues (1998) used *in vivo* electrophysiology and electron microscopy to characterize putative GABA-producing cells within VTA which demonstrated rapid-firing and non-bursting actions and were also found to become inhibited following NAcc stimulation.

Numerous transmitters and neuropeptides conveyed from unique circuits influence VTA cellular physiology and synaptic release in targets. Suaud-Chagny and colleagues (1992) showed bidirectional effects of intra-VTA pressure-injected GABA and glutamate—the former produced inhibition of DA cells along with decreased NAcc DA release whereas the latter produced physiological excitation and robustly elevated NAcc DA content. Mesolimbic DA pathway activity is additionally influenced by inputs from the medial prefrontal cortex (mPFC). Using a combination of *in vivo* electrophysiology, voltammetry and target-site pharmacology, Murase and colleagues (1993) showed that activation of mPFC via local glutamate application enhances VTA DA cellular activity and NAcc DA content concurrently. Using genetically-assisted retrograde tracing with Rabies viruses, Beier and colleagues (2015) mapped inputs to putative VTA DA and GABA neurons finding principal influence of neuropeptides hcrt/ox and neurotensin onto VTA DA cells whereas oxytocin and vasopressin more abundantly innervated VTA GABA cells. Together, a constellation of neurochemically-defined efferents target VTA DA cells and influence their net physiological activity.

Notably, electrophysiological properties of VTA DA neurons have undergone repeated refinement since their initial description and are still a topic of debate (e.g., Margolis et al. 2006; Ungless and Grace 2012). A general consensus is that VTA DA

neurons possess a low-firing “tonic” state which can be interrupted by occasional rapid-fire “bursts” that can be precipitated from salient stimuli and are thought to drive transmitter release for strongest influence post-synaptically. It was known for a couple preceding decades that some cells in VTA contained code for packaging and releasing both GABA and glutamate (Nagai et al. 1983; Bellocchio et al. 1998). Aligned with the notion of neurochemical heterogeneity within VTA, several teams provided evidence that VTA cells could co-transmit DA along with GABA and/or glutamate (e.g., Kosaka et al. 1987; Hnasko et al. 2010; Root et al. 2014a). It reasons that the physiological heterogeneity of VTA DA cells is likely influenced by neurochemical composition and, possibly, divergent synaptic inputs.

Our understanding of the functions associated with neurochemically-defined transmission from VTA afferents to various targets has widely expanded this recent decade. Stamatakis and colleagues (2013) observed that putative DA-producing cells within anterior VTA innervate the lateral habenula (LHb) and, upon photostimulation of terminals within LHb, encode reward as determined by real-time place preference. Using single-cell PCR of TH⁺ afferents projecting to LHb, many of the cells contained code for the vesicular GABA transporter (VGaT) suggesting possible GABA co-release in contrast to TH⁺ VTA afferents targeting NAcc which contained sparse VGaT mRNA. Taking advantage of the fact that LHb cells predominantly transmit glutamate to synaptic targets, it was then observed that stimulation of TH⁺ VTA→LHb terminals suppressed activity of recorded cells in the caudal division of VTA—a structure termed the rostromedial tegmentum (RMTg), described in detail in [Section 4.2](#)—which the LHb richly innervates. Thus, it was concluded that TH⁺ VTA→LHb transmission suppresses

glutamatergic output from LHb to targets including within RMTg, and that a likely mechanism of this circuit is by way of GABA co-release. Additional experiments revealed that terminal stimulation of the “mesohabenular” (i.e. VTA→LHb) pathway additionally produced reward and was reinforcing when optical channels were encoded under the glutamatic acid decarboxylase (GAD) promoter (Lammel et al. 2015). Conversely, stimulation of glutamatergic mesohabenular terminals (encoded under the VGluT2 promoter) produces real-time place aversion (Root et al. 2014b). Further still, single mesoaccumbens projections (VTA→NAcc) were shown to contain mRNA to package both glutamate and DA and were additionally shown to release both transmitters albeit at different synaptic output sites. Other lines of work found that photostimulation of local GABA neurons in VTA promotes place aversion (Creed et al. 2014), whereas stimulation of glutamatergic terminals arising from the dorsal raphe nucleus (DRN) participates in reward processing and concomitantly elevates NAcc DA release (Qi et al. 2014). Separately, both VTA DA and GABA neurons have been shown to contain mRNA for enzymes needed for endocannabinoid biosynthesis (Merrill et al. 2015). These few studies highlight the significance of neurochemical identification via mRNA and protein measurement when examining midbrain functional anatomy.

4.2) Rostromedial tegmental nucleus/tail of ventral tegmental area: anatomy and participation in aversive state processing

Atlases of Larry Swanson (2004) and of George Paxinos and Charles Watson (2013) superbly depict structural boundaries of the rat brain. Within a given anatomical structure, however, lie ever-changing sub-regions often populated by cells that produce or express receptors for a unique peptide, transmitter or signaling molecule. Serendipity and

breakthrough technologies permit sub-structure discovery, and the uncovering of a nucleus within the caudal division of VTA, which would later be termed RMTg, is no exception. In testing how modafinil (Provigil), a widely prescribed stimulant for treating narcolepsy and sleep apnea, alters cellular activity within the rat brain, Scammell and colleagues report, "...modafinil consistently produced large increases in Fos expression in a region that we refer to as the retro-ventral tegmental area; these Fos-immunoreactive neurons were clustered dorsolateral to the most caudal portion of the interpeduncular nucleus, along the lateral margin of the caudal linear and median raphe nuclei" (Scammell et al. 2000). At a Society for Neuroscience meeting in 2004, Chou presented data revealing the caudal division of VTA—an anatomically identical cluster of cells showing modafinil-evoked Fos—regulates fear-induced freezing behavior and targets the DA-producing cell population within relatively rostral compartments of VTA (Chou et al. 2004). Contained within a special issue entitled "The Anatomy of the Soul" published in the *Journal of Comparative Neurology*, Chou elaborates that this "retro-VTA" nucleus is predominantly comprised of GABA-producing cells and may participate as a final common pathway in the inhibition of DA-producing cells during receipt or prediction of aversive stimuli (Jhou 2005). Other work around this time showed that the GABA-producing nucleus within the "posterior tail of VTA" expresses the nuclear activation marker Δ FosB after systemic injection of cocaine as well as following cocaine self-administration (Perrotti et al. 2005).

The RMTg was anatomically detailed in 2009 as being situated dorsolateral to the caudal division of the interpeduncular nucleus (IPN) (Jhou et al. 2009a). Labeling strategies revealed a cluster of GABA-producing heterogeneous cells enriched in MOR

and somatostatin proteins. Across 3 cases of subjects injected with retrograde tracer within RMTg, dense inputs from lateral habenula (LHb), lateral hypothalamus, prelimbic cortex, lateral preoptic area (LPO), zona incerta and supramammillary nucleus were revealed which supported pioneering systematic neuroanatomy studies (e.g., Herkenham and Nauta 1979). Subsequent anterograde tracing with *Phaseolus vulgaris* leucoagglutinin (PHA-L) supported a robust LHb→RMTg connection which served as a strong basis for functional interrogation. RMTg densely innervated targets including substantia nigra (SN) and VTA with rostrally-extending targets spanning ventral pallidum (VP), LPO and lateral hypothalamus. In brainstem, periaqueductal grey (PAG), dorsal raphe nucleus (DRN), pedunculopontine tegmental nucleus (PPTg), laterodorsal tegmental nucleus (LDTg) and locus coeruleus (LC) were revealed as RMTg targets. Additional ultrastructural evidence supported that glutamatergic terminals of LHb terminate on GABA-producing cells of RMTg which provide inhibition to DA-producing cells of VTA (e.g., Brinschwitz et al. 2010, Balcita-Pedicino et al. 2011, Gonçalves et al. 2012).

Functionally, psychostimulant-responsive VTA-projecting RMTg cells were shown to robustly express Fos following footshock as well as following a footshock-predictive auditory cue (Jhou et al. 2009b). *In vivo* electrophysiology found that the majority of footshock-responsive single units resided within RMTg boundaries. Oppositely of putative DA-producing cells within VTA (e.g., Mirenowicz and Schultz 1996), RMTg cells transiently inhibit following presentation of a sucrose-predicting cue, and this “reward prediction” modulatory role of RMTg cells was recapitulated in non-human primates (Hong et al. 2011). Finally, RMTg lesions resulted in impaired

conditioned freezing following a footshock-predictive tone as well as impaired defensive behaviors following trimethylthiazoline (TMT) exposure—an ethologically-relevant fear-inducing predatory odorant found in fox urine. Stamatakis and Stuber (2012) provided the first direct evidence that RMTg cell activation (via optical stimulation of glutamatergic LHb terminals) produces real-time place aversion, complimenting an anti-reward (negative reward) function of excitatory LHb cells as shown in seminal work by Matsumoto and Hikosaka (2007). Stimulation of LHb terminals in RMTg additionally disrupted operant responding for sucrose rewards suggesting it negatively regulates positive reinforcement. Further, systemic injection of cocaine was shown to transiently inhibit LHb cell activity *in vivo*, and a proportion of these cells spiked in activity during the post-injection time epoch at which point cocaine is known to be aversive (Jhou et al. 2013). Indeed, the majority of activated LHb cells (i.e. during the ‘aversive’ post-cocaine time epoch) targeted the RMTg.

A new body of work has uncovered functional inputs from VP and LPO into ventral midbrain circuitry either directly or via LHb. Glutamate-producing ventral pallidal cells are suspected to produce aversion through an LHb→RMTg relay (Tooley et al. 2018). While pathway-specific cell targeting was not performed, general stimulation to the glutamate-producing VP population produced place aversion and concurrently enhanced a population of LHb and RMTg cell activity *in vivo*. These results are corroborated by a second report by Faget and colleagues (2018) who observe that stimulation of LHb-projecting glutamatergic VP cells produces place aversion while stimulation of VTA-projecting GABAergic VP cells drives preference thus conferring “opponent” actions of heterogenous VP populations recruited for encoding reward and

aversion. In a functional mapping study, Barker and colleagues (2017) reveal that GABA transmission from LPO—which itself lies just caudal to VP—to LHb promotes reward whereas glutamatergic projections along the same pathway promote aversion. Yet, *in vivo* Ca^{2+} imaging further showed that a footshock-predictive tone, as well as footshock itself, elicited concordant elevations from GABAergic and glutamatergic projection fibers from LPO to LHb which were suspected to influence DA-producing cell activity in VTA via the RMTg. To complicate circuitry further, diverse inhibitory inputs to VTA DA cells were captured in work by Polter and colleagues (2018) which additionally suggests that inhibitory RMTg inputs may co-transmit glycine in addition to GABA as inhibitory synaptic currents were normalized with bath-applied strychnine. Collectively, it was concluded that RMTg actively “brakes” reward function via inhibitory connections to DA-producing cells of VTA and in part via a LHb relay; in turn, this circuit is functionally recruited for aversive behaviors including freezing and behavioral avoidance (for comment, see Barrot et al. 2012).

Beyond the psychostimulants, RMTg cells were shown to be quieted by morphine as well as WIN55212-2 (cannabinoid receptor agonist) *in* and *ex vivo* (Lecca et al. 2011). In a subsequent report, VTA-projecting RMTg cells were uncovered as uniquely sensitive to MOR stimulation (evidenced via application of DAMGO) but insensitive to delta and kappa opioid receptor (DOR and KOR, respectively) stimulation (Matsui and Williams 2011; see also Matsui et al. 2014). Interestingly, enhancing cholinergic influence of MOR-responsive RMTg cells (via viral-mediated transfection of the M5 AChR) led to a pronounced decrease in morphine-induced locomotion whereas transfection into VTA more than doubled the morphine-induced locomotor response (Wasserman et al. 2013).

The collection of work implicating RMTg in effects from psychostimulants and other drugs of abuse was recently summarized by Fakhoury (2018).

RMTg cell activity is also implicated in motor execution via the nigrostriatal DA pathway. With tract tracing and electron microscopy, Bourdy and colleagues (2014) initially revealed a RMTg→SNc→dorsal striatum pathway. Interestingly, RMTg-lesioned rats showed improved performance in tests of motor coordination and motor skill learning. Further evidence found that direct bilateral injections of the GABA-A receptor agonist muscimol significantly elevated locomotor activity whereas bicuculline-mediated antagonism tended to inhibit locomotor responses (Lavezzi et al. 2015). Together, it can be concluded that the RMTg provides inhibitory control over SNc neurons that are critical for motor execution and coordination, and that this site should be considered a prominent contributor within the circuits of basal ganglia.

The ventral midbrain consists of VTA and RMTg structures that appear to exert

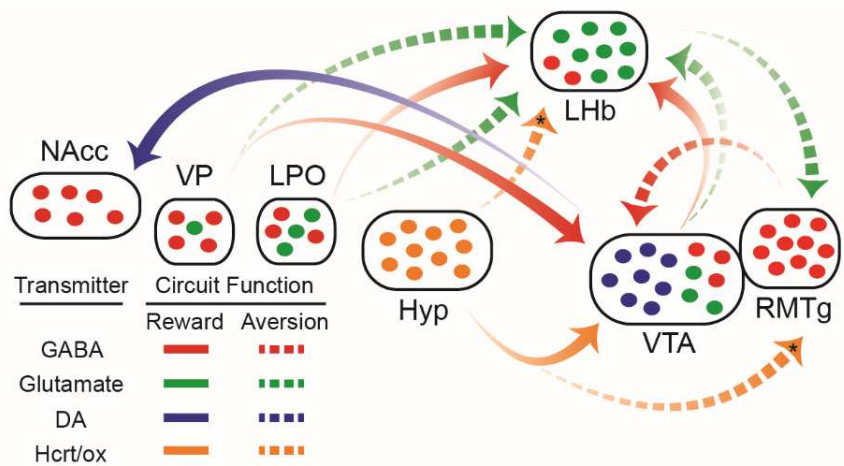


Figure 2. Circuit schematic of neurochemically-defined midbrain pathways participating in the encoding of reward and aversion. * indicates a hypothesized/presumed but untested circuit with corresponding function. Hyp – hypothalamus, Lhb – lateral habenula, LPO – lateral preoptic area, NAcc – nucleus accumbens, RMTg – rostromedial tegmentum, VP – ventral pallidum, VTA – ventral tegmental area.

opposing actions on reward processing—the former facilitates whereas the latter constrains reward processing by promoting the subjective experience of

aversion. Anatomy and select findings are described in **Figure 2**. Generally underlining the subjective experience of “rewarding” versus “aversive” are states of positive and negative affect, respectively. Ventral midbrain circuits are recruited in part to process an experience as rewarding or aversive, but quantifiably assessing whether positive or negative affect is concurrently experienced in laboratory subjects has remained relatively elusive. Ultimately, understanding circuits involved in affective processing can provide insight into the motivational “seeking” drives evident for natural and drug rewards.

CHAPTER 5 – AFFECT AND ADDICTION: ULTRASONIC VOCALIZATIONS
CAPTURE HIGHS AND LOWS ASSOCIATED WITH SUBSTANCE USE
DISORDERS

Affect can exert powerful influence over decisions and behavior in humans. Fluctuations in mood invariably manifest in psychiatric conditions including substance use disorders. Throughout its course, addiction is often conceived as a flux of positive and negative mood states related to the timing and frequency of drug administration. The opponent-process model of addiction derived from clinical observations captures these states by positing that, following drug administration, an initial positive mood state (“A-Phase”) is experienced followed by a consequential negative mood state (“B-Phase”) that tends to exacerbate in intensity after repeated drug use (Solomon and Corbit 1974). In studies probing affective states in humans, self-reports are employed whereby a user is asked to rank feelings of euphoria (or dysphoria) typically on a Likert scale. In rodents, no such self-report exists. Instead, four decades of research collectively support a role in measuring ultrasonic vocalizations (USVs) as analogs of mood/affect which can be non-invasively recorded for incorporation in behavioral studies. This chapter will detail initial studies that support the idea of USVs as sensitive, bivalent measures of positive and negative mood states. First, a brief introduction to our understanding of how USVs are produced as well as how they are detected will be provided.

5.1) Production and ethological relevance of rat ultrasonic vocalizations

Like other mammals, rats vocalize to convey information. Vocalizations within the human auditory range (0- to approximately 14-kHz) are emitted by rats typically in situations where predatory or conspecific contact is occurring or about to occur (*circa-*

strike – e.g., Litvin et al 2007). Rat vocalizations also include those within ultrasonic frequency ranges (>14-kHz) that can be detected using portable ultrasonic frequency detectors (“bat detectors”) or condenser microphone recording instruments. USVs are emitted by air flow through a constricted larynx and produce a “whistle-like” sound as determined *ex vivo* (Johnson et al. 2010). Acoustically, USVs are emitted within two broad categories based on frequency. USVs around 22-kHz can be relatively long in duration (up to 3.5 s) and are often monotonic following brief pitch fluctuation upon emission onset (Brudzynski et al. 1991, 1993). USVs with a mean frequency around 50-kHz are typically shorter in length (15-100 ms) and acoustically varied by way of pitch modulations (jumps/sweeps)—a contiguous emission characterized by rapid sinusoidal-like fluctuations between 40- and 90-kHz is termed “trill”. One research team categorized 14 USV sub-types within the 50-kHz category (Wright et al. 2010), but functional relevance is still not clear.

5.2) *Elicitation of 22- and 50-kHz ultrasonic vocalizations in laboratory settings*

The production of 22-kHz USVs has tightly linked to experimental conditions, either in isolation or in social settings, thought to evoke affective distress or alarm. Sales (1972) reported that long exhalations around 25-kHz are emitted by submissive male rats when pinned by a dominant conspecific. Similarly, when pairs of male rats were placed in an experimental setting encouraging aggressive behavior, “hissing” was observed with bouts of audible (12-kHz) and ultrasonic (20-kHz) calls (Berg and Baenninger 1973). It should be noted that Sales (1972) observed short bouts of frequency-modulated 50-kHz USVs prior to aggressive encounters which were perhaps emitted in effort to decrease intensity of imminent encounter. Separate work soon showed that male rats emit 22-kHz

USVs after copulating with sexually-responsive females, and that these USVs correspond to an absolute refractory period during which males do not re-initiate copulation suggesting an “avoidance” function (Barfield and Geyer 1972). 22-kHz USVs have additionally been observed following social isolation, electrical footshock, and upon exposure to the sight of a predator (Tonoue et al. 1986; Blanchard et al. 1991). More recently, playback of 22-kHz USVs was shown to promote general indices of behavioral avoidance (Wöhr and Schwarting 2007). Other work has positioned 22-kHz USV measurement as an indicator of subjective pain state in rats finding that pharmacological agents that alleviate behavioral measurements of pain intensity can additionally suppress USVs (e.g., Han et al. 2005).

Whereas 22-kHz USVs are elicited under experimental conditions associated with fear, alarm and/or distress, 50-kHz USVs have generally populated in appetitively-linked laboratory tests. Arguably the most consistent elicitation method of 50-kHz USVs in rats is manual “tickling” by an experimenter which was the subject of a recent sensationalized *Science* report (Ishiyama and Brecht 2016); this manipulation mimics rough-and-tumble play prominently exhibited by juvenile and adolescent rats. Initial work revealed that rats emit 50-kHz USVs (“chirps”) after this physical manipulation and will emit USVs in response to a tickling-predictive cue (Panksepp and Burgdorf 2000). Curiously, rats observed to elicit high rates of tickle-induced USVs were shown to have relatively blunted novelty-associated coping strategies as evaluated by open-field exploratory behavior and forced-swim test compared to low-calling comparator rats (Mällo et al. 2007). Perhaps the most comprehensive USV-related behavioral testing battery to-date and somewhat converse to prior work, Burgdorf and colleagues (2009) revealed that rats

bred for high rates of 50-kHz USVs exhibit increased exploratory behavior as well as preference for a sweetened solution and were relatively less aggressive compared to control subjects whereas rats bred for low rates of spontaneous 50-kHz USVs tended to show more anxiogenic/antisocial responses. Initial work demonstrated a robust communicative function of 50-kHz USVs from rat pups as playback would elicit an “orient-approach” response most abundantly in lactating adult female rats (but found to lesser extent in male and virgin female rats) (Allin and Banks 1972). Recorded from adult rats, 50-kHz USVs also induce approach behavior and in fact are responded for in operant tests (Wöhr and Schwarting 2007; Burgdorf et al. 2008).

5.2.1) Ultrasonic vocalizations in preclinical models of substance use disorders

The recent two decades have proven fruitful in expanding our understanding of how drugs of abuse alter USV calls in rats (for reviews, see Barker et al. 2015 and Simmons et al. in press). The following passages detail initial work utilizing systemic injection methods followed by a description of findings from intravenous self-administration experiments. Importantly, researchers have additionally captured how rats vocalize following chronic drug administration (i.e. during withdrawal) as well as in tests of drug relapse/reinstatement—these studies will then be described to collectively support the notion that drug-associated bivalent mood states, as are observed in humans, can be measured from rats in controlled laboratory settings. Findings from select studying capturing USVs at different stages of addiction in preclinical models are described in

Table 2.

| Treatment | Independent Variable(s) | Condition/Group | USV Frequency | Effect | Comparison Group | References |
|-------------------------------|--|---|---------------------|--------|---|--------------------------------------|
| <i>Anticipation</i> | | | | | | |
| Cocaine (i.v., S-A) | Cue incentive salience | Sign-Trackers | 50-kHz | ↑* | Goal-Trackers | Meyer <i>et al.</i> 2012 |
| Cocaine (i.v., S-A) | “Caller group” (mean across 10 sessions) | High USV Caller | 50-kHz ¹ | ↑* | Low USV Caller | Reno <i>et al.</i> 2013 |
| Amphetamine (1.5 mg/kg, i.p.) | “Caller group”; exposure history | High USV Caller, 2 d | 50-kHz ¹ | ↑** | Low USV Caller | Taracha <i>et al.</i> 2014 |
| | | High USV Caller, 9 d | | – | High USV Caller, 2 d | |
| | | High USV Caller, 10 d | | – | Low USV Caller | |
| Ethanol (10%, oral, S-A) | Exposure history | Dependent (via chronic intermittent vapor exposure, 14 h/d) | 50-kHz | – | Non-dependent | Buck <i>et al.</i> 2014 ³ |
| <i>Administration</i> | | | | | | |
| Cocaine (15 mg/kg, i.p.) | Pre-treatment (5 d) | Cocaine (following 2 d abstinence) | 50-kHz | ↑* | Saline pre-treated (following 2 d abstinence) | Mu <i>et al.</i> 2009 |
| Cocaine (i.v., S-A) | Dose | Low: 0.355 mg/kg/inf | 22-kHz | ↑* | High-dose group | Barker <i>et al.</i> 2010 |
| | | High: 0.710 mg/kg/inf | 50-kHz | ↑* | Low-dose group | |
| Cocaine (20 mg/kg, i.p.) | Drug pre-treatment, i.p. | SCH 23390 (D ₁ antagonist), 0.1 mg/kg | 50-kHz | ↓* | Vehicle pre-treated controls | Williams and Undieh 2010 |
| | | Raclopride (D ₂ antagonist), 0.1 mg/kg | | ↓* | | |
| Cocaine (i.v., S-A) | Drug availability | S-A Conditioning | 50-kHz | ↑ | Vehicle-treated controls | Maier <i>et al.</i> 2012 |
| | | Extinction | | – | | |
| Cocaine (i.v., S-A) | Dose (satiety level) | Sub-Satiety | 22-kHz | ↑* | Baseline USVs (respective frequencies) | Barker <i>et al.</i> 2014 |
| | | Circa-Satiety | | – | | |
| | | Supra-Satiety | | – | | |
| | Infusion Number | 0 ^b | 50-kHz | ↑* | | |
| | | 1 | | ↑* | | |
| | | 0 ^b | | ↑* | | |
| | | 1 | | ↑* | | |
| | | 2 | | ↑* | | |
| | | 3 | | ↑* | | |
| | | 4 | | ↑* | | |
| | | 5 | | ↑* | | |
| | | 6 | | ↑* | | |
| | | 7 | | ↑* | | |
| 8+ | – | | | | | |

| Treatment | Independent Variable(s) | Condition/Group | USV Frequency | Effect | Comparison Group | References |
|--|---|--|---------------|----------------|---------------------------------|---|
| <i>Administration</i> | | | | | | |
| Amphetamine (local infusion, NAcc, mixed doses) | Dose | 0.3 µg | 50-kHz | – | Vehicle-treated controls | Burgdorf <i>et al.</i> 2001 ^c |
| | | 1.0 µg | | ↑*** | | |
| | | 3.0 µg | | ↑*** | | |
| | | 10.0 µg | | ↑*** | | |
| Amphetamine (local infusion, mixed sites, 7.0 µg) | Infusion site | NAcc (shell) | 50-kHz | ↑* | NAcc (core) | Thompson <i>et al.</i> 2006 |
| | Drug pre-treatment, local infusion, NAcc | SKF-83566 (D ₁ antagonist), 7.0 µg | 50-kHz | ↓ | Vehicle pre-treated controls | |
| Raclopride (D ₂ antagonist), 7.0 µg | | ↓* | | | | |
| Amphetamine (2.0 mg/kg, i.p.) | “Caller group”; exposure history | High USV Caller, 7 d | 50-kHz | – | Low USV Caller | Taracha <i>et al.</i> 2012 |
| | | High USV Caller, 20 d | | – | High USV Caller, 7 d | |
| | | High USV Caller, 35 d | | ↑* | Low USV Caller | |
| | | | | ↑*** | High USV Caller, 7 d | |
| Amphetamine (0.1 mg/kg, i.p.) | Drug pre-treatment, i.p./s.c. | Clonidine (α ₂ agonist) | 50-kHz | ↓ [†] | Vehicle pre-treated controls | Wright <i>et al.</i> 2012 ^d |
| | | Prazosin (α ₁ antagonist) | | ↓ [†] | | |
| | | Atipamezole (α ₂ antagonist) | | – | | |
| | | Propranolol (β ₁ /β ₂ antagonist) | | – | | |
| | | Betaxolol (β ₁ antagonist) | | – | | |
| | | ICI 118,551 (β ₂ antagonist) | | – | | |
| Amphetamine (1.0 mg/kg, i.p.) | Drug pre-treatment, i.p. | SCH 23390 (D ₁ antagonist) | 50-kHz | ↓ [†] | Vehicle pre-treated controls | Wright <i>et al.</i> 2013 ^d |
| | | SCH 39166 (D ₁ /D ₅ antagonist) | | ↓ [†] | | |
| | | Haloperidol (D ₂ antagonist) | | ↓* | | |
| | | (-)Sulpiride (D ₂ /D ₃ antagonist) | | – | | |
| | | Raclopride (D ₂ antagonist) | | ↓* | | |
| | | Clozapine (D ₂ /5-HT _{2A} antagonist) | | ↓* | | |
| | | Risperidone (D ₁ /D ₅ antagonist) | | ↓* | | |
| | | Pimozide (D ₂ antagonist) | | ↓* | | |
| Caffeine (i.p.) | Dose | 3.0 mg/kg | 50-kHz | – | Vehicle-treated controls | Simola <i>et al.</i> 2010 |
| | | 10.0 mg/kg | | – | | |
| | | 30.0 mg/kg | | – | | |
| | | 50.0 mg/kg | | – | | |
| | | 2.0 mg/kg (amphetamine) | | ↑* | Caffeine groups (all doses) | |
| | | | | ↑* | | |

| Treatment | Independent Variable(s) | Condition/Group | USV Frequency | Effect | Comparison Group | References |
|------------------------------------|---------------------------|--|---------------|--------|-----------------------------------|--|
| <i>Administration</i> | | | | | | |
| Mixed drugs (i.p.) | Drug; dose | Amphetamine, 2.0 mg/kg | 50-kHz | ↑* | Vehicle-treated controls | Simola <i>et al.</i> 2012 |
| | | Methylphenidate, 2.5 mg/kg | | – | | |
| | | Methylphenidate, 5.0 mg/kg | | – | | |
| | | Methylphenidate, 10.0 mg/kg | | ↑* | | |
| | | MDMA (mixed doses) | | – | | |
| | | Morphine (mixed doses) | | – | | |
| | | Nicotine (mixed doses) | | – | | |
| Mixed drugs (i.p.) | Drug; exposure history | Amphetamine, 2.0 mg/kg, first exposure | 50-kHz | ↑* | Vehicle-treated controls | Simola <i>et al.</i> 2013 |
| | | MDMA, 7.5 mg/kg, first exposure | | – | | |
| | | Morphine, 7.5 mg/kg, first exposure | | – | | |
| | | Nicotine, 0.4 mg/kg, first exposure | | – | | |
| | | Amphetamine, 2.0 mg/kg, fifth exposure | | – | First exposure, respective groups | |
| | | MDMA, 7.5 mg/kg, fifth exposure | | – | | |
| | | Morphine, 7.5 mg/kg, fifth exposure | | ↑* | | |
| | | Nicotine, 0.4 mg/kg, fifth exposure | | – | | |
| <i>Withdrawal</i> | | | | | | |
| Cocaine (oral, S-A, 30 d) | Post-cessation time point | 1 d | 22-kHz | – | Vehicle-treated controls | Barros and Miczek 1996 |
| | | 3 d | | ↑ | | |
| | | 7 d | | – | | |
| | | 28 d | | – | | |
| Cocaine (i.v., S-A, 12 h binge) | Post-cessation time point | 6 h | 22-kHz | ↑* | Vehicle-treated controls | Mutschler and Miczek 1998a |
| | | 24 h (1 d) | | ↑* | | |
| | | 72 h (3 d) | | – | | |
| Cocaine (i.v., S-A, 16 h binge) | Administration control | Active S-A, 24 h post-binge | 22-kHz | ↑* | Vehicle-treated controls | Mutschler and Miczek 1998b; Mutschler <i>et al.</i> 2000 |
| | | Yoked, 24 h post-binge | | ↑* | Active S-A | |
| | | Yoked, 3 d post-binge | | – | Active S-A | |
| Cocaine (i.v., S-A, 16 h binge[s]) | Number of binge episodes | First binge | 22-kHz | ↑* | Vehicle-treated controls | Mutschler <i>et al.</i> 2001 |
| | | | | – | Second binge, 10-d interval | |
| | | | | – | Third binge, 10-d interval | |
| | | | | – | Fourth binge, 1-d interval | |
| Morphine (s.c., pellets, 72 h) | Post-cessation time point | 6 h | 22-kHz | ↑* | Vehicle-treated controls | Vivian and Miczek 1991 |
| | | 24 h (1 d) | | ↑* | | |
| | | 96 h (4 d) | | – | | |

| Treatment | Independent Variable(s) | Condition/Group | USV Frequency | Effect | Comparison Group | References |
|---|---|---|---------------|--------|--------------------------|--|
| <i>Withdrawal</i> | | | | | | |
| Morphine (s.c., osmotic minipump, 12 d) | Post-cessation time point (spontaneous) | 3 h | 22-kHz | ↓*** | Vehicle-treated controls | Kalinchev and Holtzman 2003 ^e |
| | | 6 h | | – | | |
| | | 24 h (1 d) | | – | | |
| | Naltrexone dose (precipitated) | 0.01 mg/kg, s.c. | | – | | |
| | | 0.10 mg/kg, s.c. | | ↓*** | | |
| | | 1.00 mg/kg, s.c. | | ↓*** | | |
| Heroin (s.c., pulsatile osmotic minipump, 14 h) | Dose (6-10 h post-cessation) | 0.75 mg | 22-kHz | – | Vehicle-treated controls | Williams <i>et al.</i> 2012 |
| | | 1.50 mg | | ↑*** | | |
| | | 3.00 mg | | ↑*** | | |
| Diazepam (i.p., 2x/d, 5 d) | Dose (24 h post-cessation) | 2.5 mg/kg/inj | 22-kHz | ↑* | Vehicle-treated controls | Miczek and Vivian 1993 |
| | | 5.0 mg/kg/inj | | ↑* | | |
| | | 7.5 mg/kg/inj | | ↑* | | |
| Ethanol (oral/intragastric) | Administration method (6-12 h post-cessation) | Oral (14 d, 7% ethanol ^f) | 22-kHz | ↑** | Vehicle-treated controls | Knapp <i>et al.</i> 1998 |
| | | Intragastric (4 d, 15% ethanol ^f) | | ↑** | | |
| Ethanol (oral, 14 d) | Drug (6-8 h post-cessation) | 7% ethanol ^g | 22-kHz | ↑* | Vehicle-treated controls | Moy <i>et al.</i> 2000 |

Table 2. Summary of select findings from studies measuring USVs from rats across several stages of addiction (anticipation for drug, administration, and withdrawal). Adapted from Barker *et al.* 2015.

5.2.1.1) 50-kHz ultrasonic vocalizations are elicited after psychostimulant use

Systemic cocaine and amphetamine elicit high rates of 50-kHz USVs which are sensitive to incubation/sensitization which crudely align with changes in locomotor activity (Ahrens *et al.* 2009; Mu *et al.* 2009). Indeed, individual differences in the rate of amphetamine-elicited USVs upon initial exposure related to locomotor sensitization (Mu *et al.* 2009) which posed an alluring hypothesis that USVs possess predictive utility in gauging abuse potential. In further support of this hypothesis, rats with high rates of 50-kHz USVs at baseline tend to show greater anticipatory responses for drug administration and develop stronger place preferences relative to low-calling comparator rats (Ahrens *et al.* 2013; Taracha *et al.* 2014). Rats selectively bred for high rates of 50-kHz USVs showed more robust amphetamine-evoked elevations in locomotor activity compared to low-calling control rats (Brudzynski *et al.* 2011). Psychostimulant-elicited 50-kHz USVs

are additionally sensitive to environmental factors including sociability and bedding availability—specifically, socially-housed rats elicit greater rates of 50-kHz USVs following amphetamine injection as do rats tested in chambers with bedding (Natusch and Schwarting 2010; Wright et al. 2010). Systemic administration of other drugs of abuse including morphine, nicotine, caffeine and MDMA do not readily elicit 50-kHz USVs (Simola et al. 2010, 2012, 2014; Sandananda et al. 2012; Wright et al. 2012; Manduca et al. 2013); however, returning rats to drug-paired testing environments can elicit greater rates of 50-kHz USVs compared to saline-injected control rats. These studies show that widely abused psychostimulant drugs, but not necessarily other drug types, elicit 50-kHz USVs after injection in laboratory rats.

Several studies utilizing intravenous drug self-administration demonstrate a robust presence of 50-kHz USVs following volitional drug receipt. In the first study of its kind, Barker and colleagues (2010) showed that self-administration of relatively high doses of cocaine (~0.71 mg/kg/inf) under variable-interval extended access conditions is characterized predominantly by emission of 50-kHz USVs whereas no such behavioral signature was observed in low-dose (~0.355 mg/kg/inf) cocaine self-administering rats. A second report soon followed restricting the euphorogenic effects of self-administered cocaine as interpreted by the presence of 50-kHz USVs to initial infusions only—referred to as drug “load-up” (Barker et al. 2014a). Thus, it was concluded that the transition from sobriety to intoxication produces 50-kHz USVs whereas maintenance of the drug “high” does not (for detail, see [Section 5.2.1.3](#)). Other studies determined that 50-kHz USVs escalate across initial training sessions as rats learn that an operant response yields drug infusion but decline thereafter suggesting a tolerance-like effect (Maier et al. 2012). Reno

and colleagues (2013) captured a strong positive anticipatory response to cocaine self-administration is observed in rats exhibiting high baseline USV activity. Interestingly, 50-kHz USVs emitted following the first self-administered cocaine infusion positively correlate with rates at which rats learn the lever-drug pairing (i.e., rate of acquisition; Browning et al. 2011).

Self-administration of other drugs including psychostimulants as well as heroin and alcohol can produce transient bouts of 50-kHz USVs similarly as cocaine self-administration. Intravenous self-administration of methamphetamine is met with robust emission of 50-kHz USVs most consistently in well-trained rats (Mahler et al. 2012). Our team recently showed that intravenous self-administration of cathinone-derived psychostimulant 4-methylmethcathinone (mephedrone) elicits strong rates of 50-kHz USVs in an enantiomer-sensitive manner (Philogene-Khalid et al. 2017) as does self-administration of 3,4-methylenedioxypropylamphetamine (MDPV; [Sections 6.2](#) and [6.3](#) of present thesis). Separate lines of work show that, in alcohol-dependent rats, anticipatory 50-kHz USVs are positively correlated with escalation of intake (Buck et al. 2014). Additionally, intravenous heroin self-administration leads to emission of 50-kHz USVs which are more pronounced when taking drug in a “resident” (home cage) setting unlike cocaine which was shown to strongly elicit 50-kHz USVs following self-administration in a non-resident setting (Avvisati et al. 2016). Taken together, these studies illustrate a consistent presence of 50-kHz USVs following self-administration across several drug types that vary based on numerous situational factors including self-administration context and prior drug exposure.

5.2.1.2) 22-kHz ultrasonic vocalizations align with negative affect experienced during drug withdrawal

The first studies capturing USVs by researchers studying addiction were designed in effort to capture dysphoria during drug withdrawal. Readers should note that withdrawal-associated USVs are most abundantly observed following induction from an otherwise ineffectual air-puff stimulus which is delivered to the nape of the rat by a trained experimenter (e.g., Browning et al. 2017). Barros and Miczek (1996) observed puff-elicited 22-kHz USVs following self-administration of an oral cocaine solution that emerged 72-h into withdrawal and ceased by 7-d. Mutschler and Miczek (1998a) soon found that puff-elicited 22-kHz USVs are produced following withdrawal from a 12- or 48-h intravenous cocaine binge—these post-cocaine USVs were detected 6-h after cessation but waned by 72-h. Soon thereafter, the same group reported that 22-kHz USVs following an intravenous cocaine binge intensify when cocaine is administered non-contingently (Mutschler and Miczek 1998b) which corroborated earlier evidence that post-cocaine aversion and toxicity are more pronounced following passive administration compared to volitional receipt (Dworkin et al. 1995). In the study introduced above by Barker and colleagues (2014), restricting rats' drug level to 50% of individually-assigned satiety points led to significant emission of spontaneous 22-kHz USVs as well as heightened operant responding despite inability to self-administer bolus infusions as rats had been trained to do throughout two weeks of acquisition. Beyond psychostimulants, 22-kHz USVs have been observed from rats experiencing withdrawal from opiates and alcohol (Vivian and Miczek 1991; Knapp et al. 1993, 1998; Moy et al. 2000; Williams et

al. 2012) but were not present during precipitated nicotine withdrawal (Simmons, unpublished observations).

5.2.1.3) Ultrasonic vocalizations as sensitive indicator of bivalent mood states in cocaine self-administering rats: revisiting the opponent-process model of addiction

In the mid-1970s, Solomon and Corbit (1974) synthesized numerous examples, empirical and anecdotal, within a cohesive theorem entitled “An Opponent-Process Theory of Motivation: Temporal Dynamics of Affect”. Here, the authors suggest that stimulus presentation, pleasurable or aversive, yields an affective state (“State A”; reaction) which wanes in intensity as the stimulus continues to be applied. Upon stimulus termination, a state of opposite affective valence is then experienced (“State B”; after-reaction) after which a normal baseline state resumes. A supporting example providing behavioral and physiological measurements conducted in laboratory dogs, after receiving numerous 10-s electrical shocks, is provided—shock onset yields an anxious/afraid behavioral state aligned with protracted tachycardia whereas shock termination yields a euphoric/social state aligned with abrupt bradycardia (Church et al. 1966). Appropriate to the content of this thesis, the authors surmise from earlier studies conducted in opiate users that an intense euphoric “rush” is evident after administration which wanes gradually and is dominated thereafter by a period in which “the user suffers aversive, painful, and frightening somatic withdrawal symptoms, together with a feeling of craving” (Solomon and Corbit 1974). Comparable subjective and physiological “opponent/after-reaction” effects are seen in psychostimulant addicts following drug use, and this provided a basis for Koob and colleagues (1997) to position psychostimulant addiction as being propagated by similar “opponent-process” motivational impetuses.

In efforts to capture the opponent processes of psychostimulants in an experimental setting, Ettenberg and colleagues (1999) intravenously injected rats with cocaine and incorporated a wait-period of 0-, 5- or 15-min before placing in one of two partitioned chambers of a shuttle-box apparatus. On alternate days of the conditioning period, all rats were injected with saline. In agreement with prior place conditioning work, a 0-min post-cocaine delay period conditioned a significant place preference in rats relative to pre-conditioning baseline. As rewarding stimuli including mating opportunity and electrical self-stimulation all produce place preference (e.g., Miller and Baum 1987; Duvauchelle et al. 1992), results suggest that acute cocaine produces “reward”. Incorporation of a 15-min post-cocaine delay period induced a significant place aversion as indicated by less time spent on the cocaine-paired context relative to baseline. Thus, it appears cocaine exerts “...dual or biphasic properties—an initial positive or rewarding action followed temporally by one that is negative or aversive in nature” (Ettenberg et al. 1999). Several years earlier, Ettenberg and Geist (1991) documented cocaine’s rewarding as well as its anxiogenic effects in an operant runway (“goal-box”) test whereby rats learned to traverse a runway for intravenous cocaine injection. The authors took note of episodic “retreat” behavior which were interpreted as internal conflict—that is, rats would traverse for cocaine but with hesitation. These early studies corroborate in support that cocaine produces time-locked positive and negative states which could be quantified by measuring preference and goal-box retreat behaviors.

In 2014, Barker and colleagues determined how drug level of intravenous cocaine influenced emission of 22- and 50-kHz USVs (Barker et al. 2014). The hypothesis that rising and sustained cocaine levels would facilitate emission of 50-kHz USVs was tested.

Interestingly, 50-kHz USVs were only detected during the initial few self-administered injections of cocaine (often termed “load-up” phase). A near absence of any USVs occurred when rats titrated cocaine level to a “satiety” point which is conceived as the point at which rats prefer to maintain drug level around during cocaine self-administration despite having access to escalate drug level further (Wise and Bozarth 1987; Tsibulsky and Norman 1999). In a series of experimental sessions whereby calculated drug level was manipulated at, below or above the aforementioned satiety point via a series of computer-controlled micro-infusions, the role of drug level on USVs was explicitly tested. Here, results found that keeping rats around the point of satiety yields a near absence of any USVs and is met with infrequent lever pressing—these data matched those from sessions at which point cocaine level was maintained slightly above each rat’s individually-defined satiety point. Curiously, maintaining cocaine levels below satiety (50%) led to a significant increase in 22-kHz USVs and lever pressing. The authors concluded that, whereas positive affect (euphoria) is experienced when rats titrate from sobriety to a state of intoxication but not thereafter, a negative affective state may emerge as drug levels decline but without necessity for drug to be completely removed from the animal’s system. This report, coupled with cocaine-associated place preference/aversion and “conflict behavior” by Ettenberg and colleagues (1991, 1999), supports that mixed positive and negative effects (“opponent processes”) can be observed after systemic and self-administration of cocaine in rats—these behavioral phenomena are depicted in **Figure 3**.

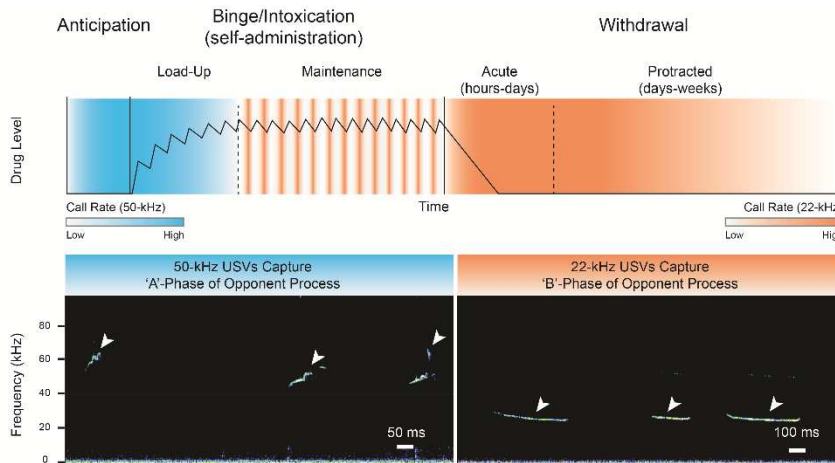


Figure 3. Ultrasonic vocalizations (USVs) capture affective opponent processes during cocaine self-administration in rats. Positive (bluish-cyan) and negative (reddish-orange) affective drives underlie drug-seeking behavior at different stages of cocaine self-administration. While anticipation and load-up of cocaine evoke positive affective 50-kHz USVs, decaying drug levels during the maintenance phase of self-administration as well as withdrawal from cocaine induce negative affective 22-kHz USVs. Representative 50- and 22-kHz USVs from rats are provided in lower panels. Figure originally contained within and adapted from Simmons et al. in press.

5.3) *Underlying neurobiology of ultrasonic vocalizations in rats: relevance to addiction*

As is

discussed in the Introduction, drugs of abuse elicit elevations in catecholamines by release

augmentation and/or pre-synaptic reuptake inhibition mechanisms. Accordingly, mesolimbic DA transmission has been implicated in the production of “appetitive” 50-kHz USVs in rats. Early studies revealed that direct injection of amphetamine into NAcc elicits a robust bout of 50-kHz USVs whereas no such response is elicited following injection into dorsal striatum, and that neuronal structures supporting electrical self-stimulation concomitantly yield 50-kHz USVs upon stimulation (Burgdorf et al. 2001). Thompson and team (2006) expanded this work to find that that central injection of amphetamine into the shell compartment of NAcc produces greater rates of 50-kHz USVs relative to injections into the core compartment of NAcc. Several investigators found that systemic pretreatment with D₁- or D₂-like receptor antagonists suppresses amphetamine-

and cocaine-elicited 50-kHz USVs (Thompson et al. 2006; Williams and Undieh 2010; Wright et al. 2013). Curiously, though, neither administration of D₁-/D₂-like receptor agonists nor DAT/NAT inhibitors appreciably elevate 50-kHz USVs compared to rates produced by psychostimulant injection (Williams and Undieh 2010; Wright et al. 2010). Additional evidence by Ciucci and colleagues (2009) found that central DA-depleting lesions reduce acoustic complexity of 50-kHz USVs but do not altogether eliminate their basal emission. Scardocho and colleagues (2015) observed that photostimulation of pre-synaptic DA-releasing terminals within NAcc elicited a transient bout of 50-kHz USVs following self-stimulation or non-contingent stimulation. Expanding on functional roles of noradrenergic transmission, α_1 antagonist as well as α_2 agonist pretreatment were shown to effectively suppress amphetamine-elicited 50-kHz USVs (Wright et al. 2012) whereas $\beta_{1/2}$ antagonists (“beta blockers”) altered the profile of 50-kHz USV sub-types but did not reduce net call rates. Taken together, the above-mentioned studies provide a firm association between catecholamine transmission—notably mesolimbic DA transmission—on the production of psychostimulant-associated 50-kHz USVs.

A handful of studies position the periaqueductal grey (PAG) as an active locus in producing 22-kHz USVs during drug withdrawal in rats. Initially, the inducible transcription factor *zif268*—used as a proxy for structural activation—was appreciably elevated in the PAG in rats withdrawn from a 16-h cocaine binge (Mutschler et al. 2000). Stemming from reports capturing 22-kHz USVs during cocaine withdrawal (Mutschler and Miczek 1998a, b) and that 22-kHz USVs can be elicited following pharmacological activation of PAG (Depaulis et al. 1992), the authors reasoned that the PAG may be a sensitive structure for the production of negative affect during drug withdrawal. The PAG

has long been known as a structure mediating anxiety- and pain-related behaviors/feelings in humans and rats (for review, see Brandão 1993). A complimentary study observed that intra-PAG pre-treatment with morphine, a selective μ opioid receptor (MOR) agonist, suppressed 22-kHz USVs elicited from socially defeated rats (Vivian and Miczek 1999). Ascending cholinergic transmission originating from the laterodorsal tegmental nucleus (LDTg)—housed within the ventral division of PAG—has repeatedly been shown as a critical modulator for the production of 22-kHz USVs (for review, see Brudzynski 2014). Thus, amounting evidence supports PAG transmission as participating in the generation of negative affect, perhaps also anxiety and pain, during drug withdrawal.

More recently, studies have implicated kappa opioid receptor (KOR) signaling in the elicitation of 22-kHz USVs during drug withdrawal. Using an intermittent alcohol vapor administration model to induce dependence in rats, Williams and colleagues (2012) captured a strong presence of 22-kHz USVs in alcohol-withdrawn rats. This group further observed that pre-treating alcohol-withdrawing rats with the long-acting KOR antagonist nor-binaltorphimine (nor-BNI) significantly reduces the emission rate of 22-kHz USVs (Berger et al. 2013). In alcohol-naïve rats, systemic administration of the selective KOR agonist U50,488 dose-dependently enhanced rates of 22-kHz USVs. Additionally, pre-treatment with nor-BNI decreased alcohol consumption following presentation of an alcohol-predictive cue. Coupled with seminal work finding that KOR antagonism normalizes suppressed brain reward function in drug-withdrawn rats (Chartoff et al. 2012), these studies highlight an important role of KOR transmission in the production of a negative affective state—measured in part by rates of 22-kHz USVs—during drug

withdrawal, and ultimately that suppressing KOR transmission may therapeutically benefit humans attempting to maintain drug abstinence.

The LHB→RMTg→VTA circuit could bidirectionally modulate positive and negative affective states and, in doing so, the production of 50- and 22-kHz USVs, respectively. In a Fos mapping study, Sadananda and colleagues (2008) observed that playback of 50-kHz USVs significantly reduces Fos activation within LHB. A possible explanation is that VTA DA cells become disinhibited because of reduced GABAergic RMTg input which itself is quieted via suppressed glutamatergic LHB input. This notion is supported by neurochemical evidence showing that playback of 50-kHz USVs transiently elevates DA efflux within NAcc (Willuhn et al. 2014). Separate evidence finds that glutamate-producing LHB neurons become transiently suppressed immediately following a cocaine injection but spike in firing ~15 min post-injection—an epoch at which cocaine is empirically evidenced as aversive (Jhou et al. 2013). Additional support for bidirectional control of affect from the LHB→RMTg→VTA circuit includes photostimulation experiments showing that excitation of glutamate-producing LHB afferents is aversive whereas LHB inhibition is rewarding as demonstrated by real-time place preference (Root et al. 2014a; Lammel et al. 2015). Taken together, several studies support that the LHB→RMTg→VTA circuit may provide bidirectional control of affective states which can be behaviorally demonstrated via USVs as well as assays probing reward and aversion. Still, other circuit mediators including from hypothalamus may be involved in the regulation of VTA/RMTg actions and ultimately on regulation of the encoding of stimulus valence.

CHAPTER 6 – HYPOCRETIN/OREXIN ROLE IN PSYCHOSTIMULANT-ASSOCIATED REINFORCEMENT AND AFFECTIVE CHANGES

So far, we recognize that psychostimulant abuse remains a pervasive societal problem, and that no medications are available to manage psychostimulant use disorders. Hypothalamic hcr/ox, in large part via connections to ventral midbrain, regulates motivated, effortful states including those needed for procurement of drugs. In reaching for high translational value, [Section 6.1](#) describes our work evaluating the first-in-class clinically-available hcr/ox receptor antagonist suvorexant in behavioral models of cocaine addiction. In response to an emerging crisis concerning synthetic psychostimulant drug manufacture and distribution, work in [Section 6.2](#) is designed to characterize patterns of responding and “affective responses” to self-administered cocaine compared to the potent synthetic psychostimulant MDPV which acts at central sites by comparable mechanisms as cocaine. We profile emission of USVs as a non-invasive supplemental measure as rats actively self-administer their assigned psychostimulant drug. In continuing our work with MDPV, [Section 6.3](#) details a study that merges the prior two reports—that is, we test how systemic suvorexant influences drug-taking and USVs associated with MDPV self-administration. Collectively, these three published preliminary reports provide encouraging results as to the potential efficacy of hcr/ox receptor antagonists for the management of psychostimulant use disorders in humans.

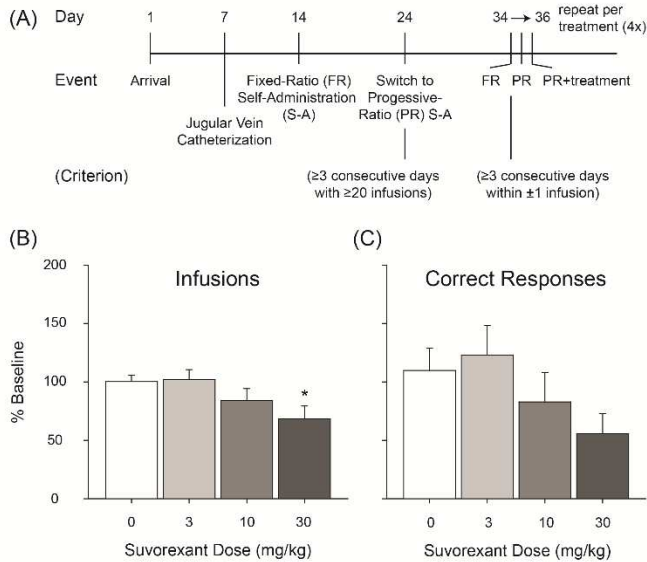


Figure 4. (A) Timeline of events to train rats in cocaine self-administration. Effects of suvorexant on the number of (B) infusions and (C) correct responses relative to prior-day baseline performance. * $p < 0.05$ relative to vehicle-pretreated control data (Bonferroni-corrected contrasts against vehicle group). Data are presented as mean \pm S.E.M. $n=12$. Figure originally contained within and adapted from Gentile et al. 2018.

6.1) Suvorexant, a hypocretin/orexin receptor antagonist, attenuates motivational and hedonic properties of cocaine (Gentile et al. 2018)

Our team first sought to assess effects of the clinically-available hcrt/ox receptor

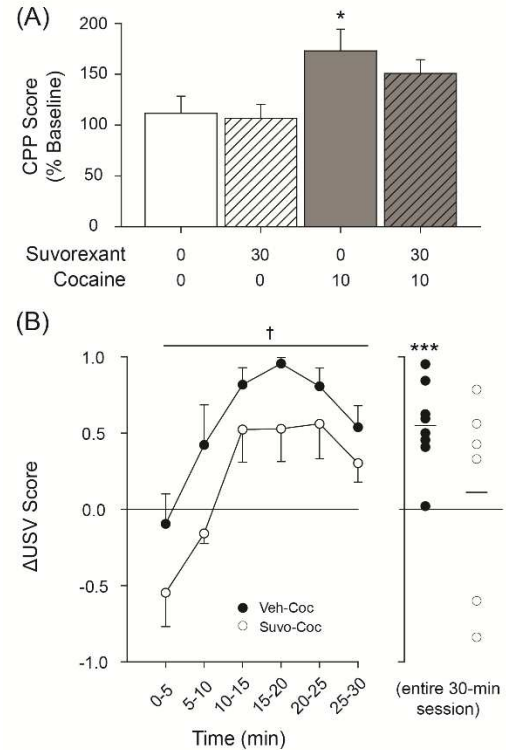
antagonist suvorexant in models of psychostimulant addiction. In this work, we test how suvorexant influences passive reward learning using cocaine place conditioning and how suvorexant impacts

motivated cocaine-taking using intravenous self-administration. Additionally, we examine how suvorexant influences “hedonic reactivity” to cocaine by analyzing cocaine-elicited USVs in rats pre-treated either with or without suvorexant. In effort to control for suvorexant-associated somnolence, we test how suvorexant influences cocaine-elicited locomotor activity. Finally, we use electrochemical measurement of ventral striatal DA to test if suvorexant can suppress cocaine-elicited mesolimbic DA activation. For description of specific metrics and analyses, readers are referred to supplemental [Section S1.6](#).

In this study, we show for the first time that the clinically-available hcr/ox receptor antagonist suvorexant is effective in suppressing some measures of cocaine-associated reward and reinforcement. Suvorexant did not significantly alter place preference for a cocaine-paired chamber but significantly reduced cocaine infusions earned when self-administering rats were taxed under high-effort access conditions (**Figures 4 and 5A**). One reason suvorexant may not have significantly reduced place preference for cocaine is because of the seemingly selective role for hcr/ox transmission in providing motivational drive for reward receipt. For example, pre-treatment with hcr/ox receptor antagonists generally fails to alter cocaine infusions earned under low-effort

fixed-ratio 1 (FR-1) access conditions (e.g., España et al. 2010).

It does not appear that high-dose suvorexant (30 mg/kg, IP) interferes with motor initiation to the point where task performance would be obfuscated. In a locomotor ambulation assay, we observed that rats were comparably active following systemic cocaine injection irrespective of pre-treatment (i.e. no difference between Suvo-Coc and Veh-Coc groups; **Figure 6**). We also observed that suvorexant-pretreated rats still self-



*Figure 5. Effects of suvorexant on (A) CPP Score and (B) Δ USV Score. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to Veh-Sal in (A; Bonferroni-corrected contrasts against Veh-Sal control group) or to “0” in (B; independent samples t-test). All data are presented as mean \pm S.E.M. n=6-8/group. Figure originally contained within and adapted from Gentile et al. 2018.*

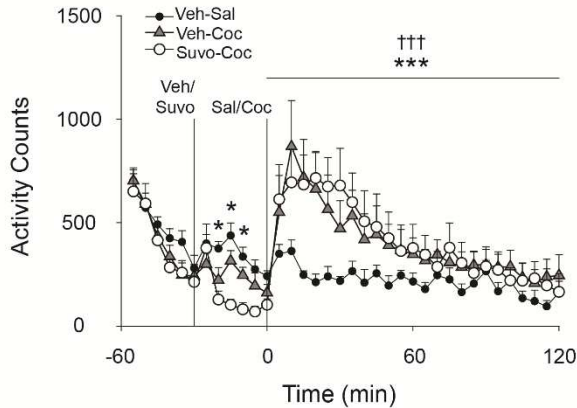


Figure 6. Effects of suvorexant on cocaine-induced locomotor activity. Solid vertical line indicates pretreatment with either suvorexant (30 mg/kg) or vehicle at -30 minutes. Solid vertical line indicates acute treatment with either cocaine (10 mg/kg) or vehicle at 0 minutes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicates activity count difference between Veh-Sal and Suvo-Coc groups (ANOVA main effect). † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ indicates activity count difference between Veh-Sal and Veh-Coc groups. Data are presented as mean \pm S.E.M. n=8/group. Figure originally contained within and adapted from Gentile et al. 2018.

administered cocaine albeit significantly fewer infusions as compared to infusions earned following a vehicle pre-treated baseline session. We surmise that a salient environment, such as one paired with opportunity to perform for rewards, accommodates a behaviorally active state that negates somnolence associated with hcr/ox receptor blockade. During place conditioning in which rats are confined to one chamber without employment of an operant task, suvorexant-treated rats tended to exhibit signs of behavioral suppression (Simmons and Gentile, unpublished observations). However, in

an earlier study from our team, we observed that suvorexant (30 mg/kg, IP) failed to significantly alter measures in the 5-choice serial reaction time task (5-CSRTT) used to probe for possible confounding locomotor effects (e.g., latency to retrieve reward, trials omitted, % accuracy) (Gentile et al. 2017b). Thus, it appears that suvorexant may induce somnolence when rats are confined in an environment removed of stimuli to interact with, but “spikes” in hcr/ox transmission during operant reward-based tests promote a state of wakefulness and vigilance such that sleep is not promoted.

Notably, suvorexant significantly reduced cocaine-elicited elevations in NAcc extracellular DA (**Figure 7**) levels within one hour post-cocaine injection, suggesting that hcr/ox positively regulates mesolimbic DA transmission. Many reports lead to the suggestion that inhibiting mesolimbic DA transmission may be able to reduce drug-associated reward, and prior work has shown that both OX₁R and OX_{1/2}R antagonists can suppress cocaine-elicited NAcc DA content (Prince et al. 2014; Brodnik et al. 2015). In support of this, we additionally observed that suvorexant pre-treatment blunts hedonic reactivity to systemically-injected cocaine as interpreted by reductions in a normalized

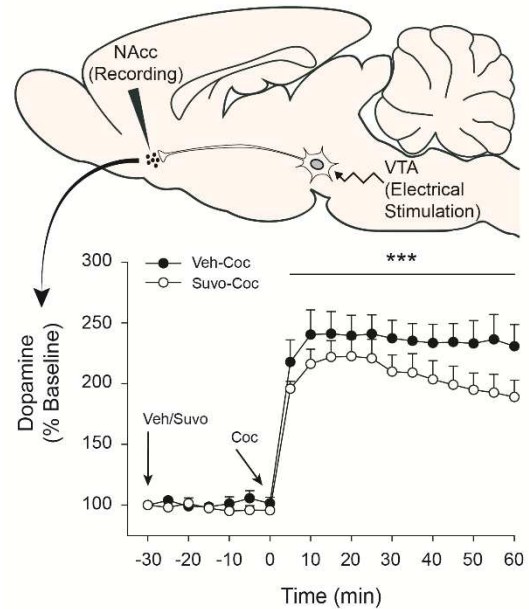


Figure 7. Dopamine levels (% Baseline) in ventral striatum between Veh-Coc and Suvo-Coc groups. Arrows indicate points of vehicle/suvorexant injection (-30 min; 30 mg/kg, i.p.) and cocaine injection (0 min; 10 mg/kg, i.p.). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ relative to Veh-Coc group (ANOVA main effect). Data are presented as mean \pm S.E.M. $n=5$ /group. Figure originally contained within and adapted from Gentile et al. 2018.

measure of 50-kHz USVs (**Figure 4B**). The emission of 50-kHz USVs has been extensively linked to normal mesolimbic DA functioning and is associated with reward receipt including from psychostimulant injections. Thus, we interpret the concomitant reductions in cocaine-associated 50-kHz USVs and NAcc DA content following suvorexant as data supporting the agent's therapeutic potential to aide in the management of reward-related psychiatric disorders. Of great excitement, readers should note that

suvorexant is currently in clinical testing for the management of cocaine use disorder (NCT02785406 from <https://ClinicalTrials.gov>).

6.2) Comparing rewarding and reinforcing properties between 'bath salt' 3,4-methylenedioxypyrovalerone and cocaine using ultrasonic vocalizations in rats (Simmons et al. 2018)

Several studies show that rats elicit “positive affective” 50-kHz USVs during receipt of initial infusions of self-administered cocaine as well as upon systemic injection. The rise in cocaine-like synthetic stimulants, including the synthetic cathinone MDPV, warrant characterization of behavioral effects used to measure abuse potential. The purpose of this study was to compare the reinforcing efficacy and subjective state associated with anticipation and self-administration of cocaine compared MDPV. We additionally conducted studies to compare the extent to which non-contingent administration of MDPV altered 50-kHz USVs. Experimental procedures can be found in supplemental [Section S2.1](#), and metrics/statistics in supplemental [Section S2.3](#).

In this study, we utilized intravenous self-administration and trained rats across daily 2-h sessions for 14 d to self-administer cocaine (0.56 mg/kg/inf) or MDPV (0.056 mg/kg/inf) according to an approximate ten-fold greater potency attributed to MDPV compared to cocaine (Baumann et al. 2013). We replicated earlier work demonstrating that 50-kHz USVs associated with cocaine self-administration wane following a “load-up” period (Barker et al. 2014; for methodological detail, readers are referred to supplemental [Section S2.2](#)) which was additionally observed from MDPV self-administering rats. These results support that euphoria is experienced during the transition from sobriety to psychostimulant intoxication but not necessarily thereafter

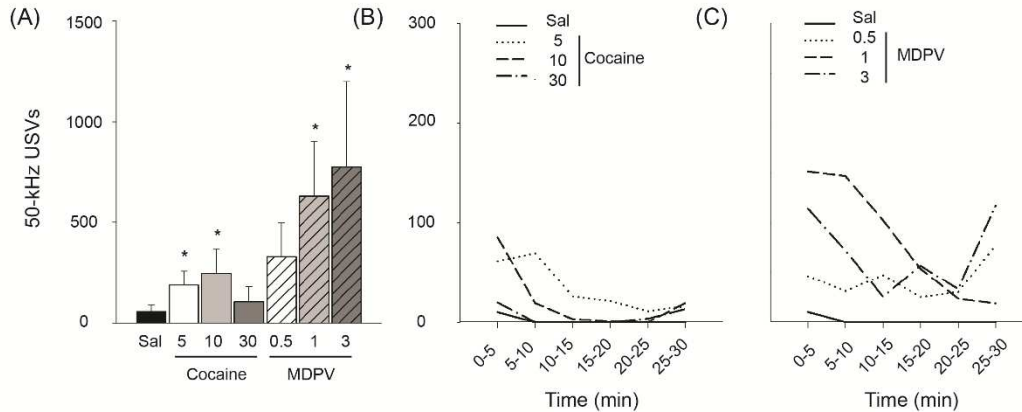


Figure 8. Systemic injection dose-response. (A) 50-kHz USVs across 30-minute recording session on day 7 of cocaine (solid bars) or MDPV (hashed bars) administration. (B-C) Time course of cocaine- or MDPV-elicited 50-kHz USVs in 10-minute time bins. * $p < 0.05$ against saline control group, Mann Whitney tests. Data for (A) are means \pm *S.E.M.*, and data for (B-C) are medians. $n=5-8$ /group. Figure originally contained within and adapted from Simmons et al. 2018.

despite high drug levels. These data contribute to an ongoing hypothesis that the underlying drive to self-administer psychostimulants shifts from positive to negative reinforcement upon titrating to a point of preferred drug level (satiety). Barker and colleagues (2014) additionally observed that the “maintenance” phase of cocaine self-administration (i.e. at drug levels after titration to satiety is achieved) is characterized by periodic bouts of 22-kHz USVs that predominate when calculated cocaine levels are in the lowest quartile, suggesting these “negative affective” USVs dissipate following bolus injections. The underlying neural circuits that are recruited as drug levels wane and negative affect emerges during cocaine self-administration may correspond to stress-associated circuits that are known to activate during withdrawal. Transmitters including dynorphin and corticotropin releasing factor (CRF), notably in extended amygdala structures, dynamically activate and underlie withdrawal-associated reward hypofunction (for review, see Koob 2015). Real-time measurements of circuit-specific transmitter and peptide release using voltammetry and fiber photometry can better determine systems

underlying the shift from positive to negative reinforcement during psychostimulant self-administration.

This is the first study to measure USVs following MDPV injection (experimenter- and self-administration). As MDPV is readily self-administered and induces place preference in laboratory animals (e.g., Karlsson et al. 2014), we anticipated that MDPV injection would elicit 50-kHz USVs and, indeed, observed such behavioral effects. Moreover, MDPV facilitates brain reward function as determined by electrical self-stimulation—a measure explicitly measuring “hedonic state” of the rat (Watterson et al. 2014). Experimenter-delivered MDPV injection elicited dose-related elevations in 50-kHz USVs similarly to cocaine but with high variability (**Figure 8**). While we did not stratify subjects into “high” and “low” responders to better accommodate individual differences, this strategy has been applied in prior work and is suspected to capture individual vulnerabilities to drug abuse (e.g., Tripi et al. 2017). Self-administered MDPV

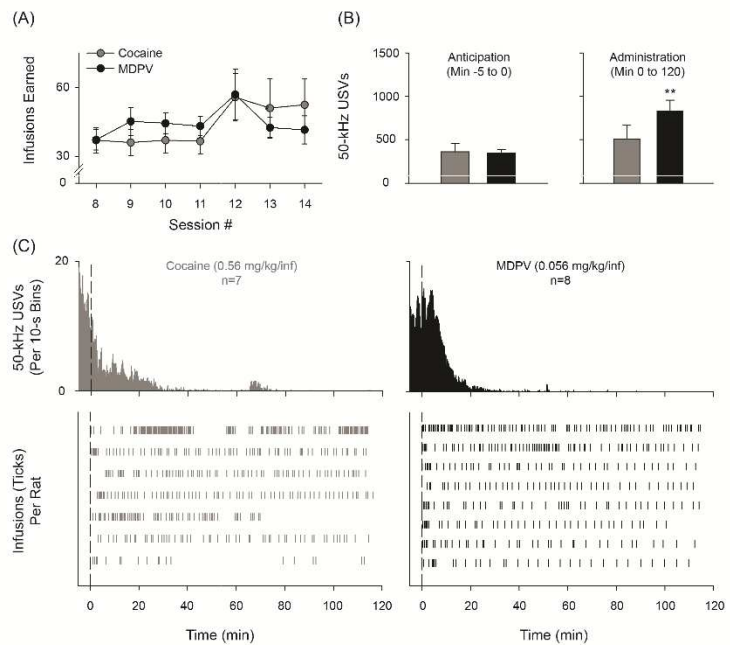
elicited stronger rates of 50-kHz USVs with relatively lower variation and greater persistence through “load-up” compared to cocaine (**Figure 9 and 10**). MDPV

additionally appeared to be intravenously self-

administered more quickly than cocaine, although a within-subjects comparison would have allowed for better assessment. We surmise that MDPV, like cocaine, elicits high rates of 50-kHz USVs and can be measured during intravenous self-

administration which may be useful in evaluating subjective euphorogenesis.

Figure 9. Self-administration of cocaine or MDPV, whole session. (A) Number of infusions earned during second week of self-administration. Mean number of 50-kHz USVs during anticipation (B) or administration (C) of either cocaine (grey bars) or MDPV (black bars). (D, upper) 50-kHz USVs throughout 2-hours of drug self-administration in 10-s time bins. (D, lower) Drug infusion vertical line plots for individual rats. Vertical dashed line represents lever extension. ** $p < 0.01$ against respective anticipation data (independent samples t-test). Data in A, B and C are mean \pm S.E.M. $n=7-8$ /group. Figure originally contained within and adapted from Simmons et al. 2018.



Self-administration of MDPV showed a profile of USV emissions like self-administered cocaine but at one-tenth the dose. MDPV blocks catecholamine transporters and elevates NAcc DA content with ~10-fold greater potency compared to intravenous cocaine (Baumann et al. 2013). Indeed, several teams have published that MDPV is intravenously self-administered at doses as low as 0.03 to 0.05 mg/kg/inf (Watterson et al. 2014; Schindler et al. 2016). Qualitative analysis on the patterns of responding for MDPV versus cocaine across subjects revealed relatively “regulated” injection intervals, suggesting perhaps greater titration efficiency with intravenous MDPV which may relate to its ability to move quickly across the blood-brain barrier and exert central effects (Simmler et al. 2013). Collectively, these results confirm the alarming potency of MDPV with respect to the amount of drug needed to facilitate self-administration.

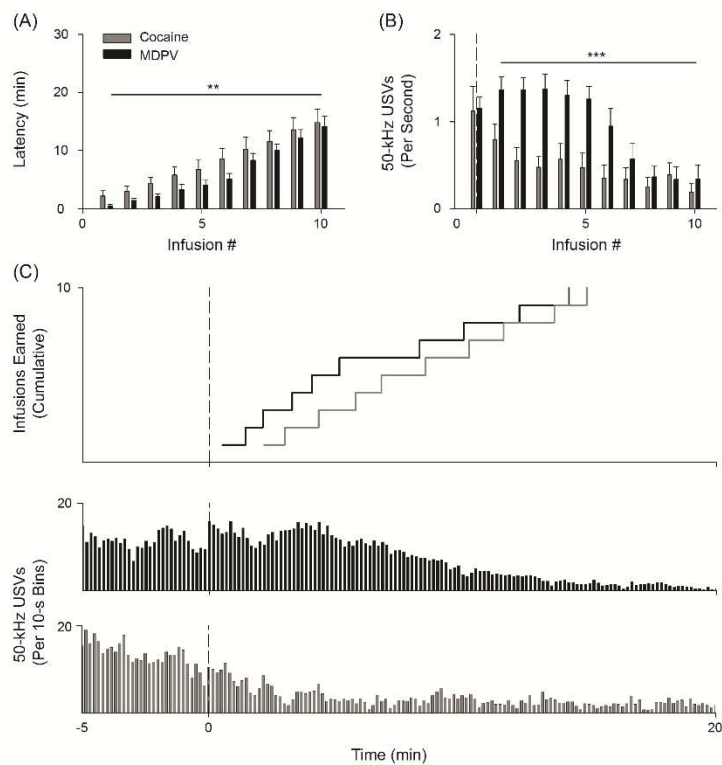


Figure 10. Self-administration of cocaine or MDPV, load-up. (A) Latency to receive infusions 1-10 of cocaine (grey bars) or MDPV (black bars) relative to lever extension. (B) 50-kHz USVs per infusion where infusion "0" represents USV rate prior to infusion 1. (C, upper) Cumulative time to earn infusions 1-10 plotted using horizontal lines with corresponding 50-kHz USVs across anticipation and load-up (C, lower). Vertical dashed line represents lever extension. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ main effect of Drug Type. Data in A and B are mean \pm S.E.M. $n=7-8$ /group. Figure originally contained within and adapted from Simmons et al. 2018.

Finally, this study was the first to capture robust rates of 50-kHz USVs from exposure to a chamber paired with psychostimulant (MDPV and cocaine) self-administration. Earlier work showed that rats emit 50-kHz USVs upon exposure to an environment associated with experimenter-delivered cocaine injections (Ma et al. 2010), and thus it is not altogether surprising to observe strong USV rates from a salient, drug-paired context in which rats engage in an operant task to volitionally take drugs. In some subjects, the rate of 50-kHz USVs from placement into the drug-paired operant chamber surpassed that which followed intravenous drug self-administration. Notably, in the study performed by Barker and colleagues (2014), USVs prior to the first self-administered cocaine injection were measured and found to be approaching near-zero levels. Aligned with the idea that a salient context drives pre-drug USV responses, rats in the study described by Barker and colleagues (2014) resided permanently within their operant chamber after catheterization, whereas rats in our study lived in separate home-cages. This finding contributes to a well-documented phenomenon that salient contextual cues can lead to behavioral disinhibition and drive motivational/mood states.

6.3) Role of hypocretin/orexin receptor blockade on drug-taking and ultrasonic vocalizations associated with self-administration of 'bath salt' 3,4-methylenedioxypyrovalerone in rats (Simmons et al. 2017)

The following study was the first to capture effects of suvorexant pre-treatment on infusions earned and USVs associated with MDPV self-administration. In prior work (Simmons et al. 2018), we identified that MDPV, comparably as cocaine but with greater persistence, elicits 50-kHz USVs following systemic and self-administration injection methods. We additionally examined the influence of suvorexant of acoustically-defined

USV call types as briefly described in [Chapter 5](#). For additional detail on this study, experimental procedures are provided in supplemental [Section S3.1](#) as is detail on USV recording and analysis (supplemental [Section S3.2](#)) and metric/statistics (supplemental [Section S3.3](#)).

Briefly, rats were trained to self-administer MDPV (0.03 mg/kg/inf) across daily 2-h sessions for 14 d. A principal finding from this report is that, while suvorexant failed to significantly decrease infusions earned under a low-effort FR-1 self-administration task (**Figure 11**), pre-treatment with a moderate dose (10 mg/kg, IP) did, indeed, significantly suppress 50-kHz USVs during “anticipation” and “post-lever” time epochs (**Figure 12**). Non-significant suppression of 50-kHz USVs during each of the above-mentioned time epochs was additionally seen upon high-dose (30 mg/kg, IP) suvorexant pre-treatment. Finally, the degrees to which suvorexant altered 50-kHz USV emissions and infusions earned trended to positively correlate (**Figure 13**), promoting potential utility in measuring USVs to predict subsequent drug-taking.

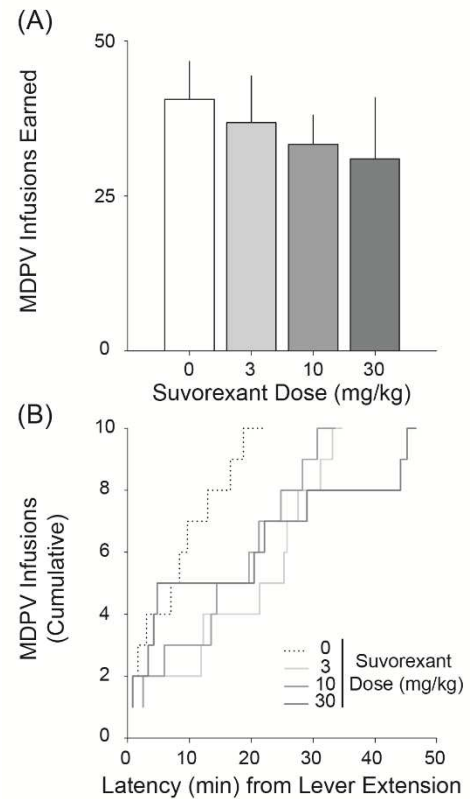


Figure 11. Effect of suvorexant on MDPV self-administration. (A) Change in infusions relative to each rat's vehicle pre-treated control data. (B) Latency to self-administer the first 10 infusions of MDPV. Data in (A-B) are means + *S.E.M.* * $p < 0.05$ relative to "100"—the point of no-change from vehicle pre-treatment. $n=7-14$ /group. Figure originally contained within and adapted from Simmons et al. 2017.

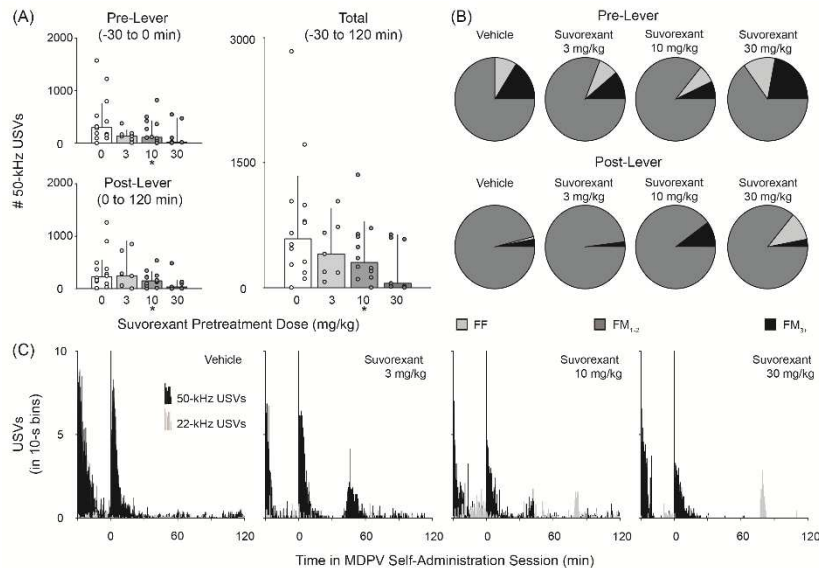


Figure 12. Effect of suvorexant on ultrasonic vocalizations (USVs) associated with MDPV self-administration. (A) Vertical bar charts depicting change in 50-kHz USVs relative to vehicle pre-treatment control data during "anticipation" (upper panel) and "post-drug" (lower panel) time-points. Plotted using right y-axis are individual data points showing change in number of 50-kHz USVs from vehicle during "anticipation" and "post-drug" time-points. (B) Pie charts showing USV type composition of 5-min samples during "anticipation" and "post-drug" time-points during pre-treatment sessions. (C) 50- and 22-kHz USVs plotted across session with black vertical line showing lever extension and access to MDPV. Data in (A) are means + *S.E.M.* * $p < 0.05$, ** $p < 0.01$ relative vehicle pre-treated control data (non-parametric corrected pairwise comparisons). $n=7-14$ /group. Figure originally contained within and adapted from Simmons et al. 2017.

Similar to our previously published work (Simmons et al. 2018), we observed that trained rats elicit robust rates of 50-kHz USVs from exposure to a context paired with MDPV self-administration (i.e. during "Anticipation"). Whereas Simmons and colleagues (2018) incorporated a 5-min "Anticipation" recording, the present

study used a 30-min recording window in effort to better resolve bouts of 50-kHz USVs occurring prior to and after operant lever extension. Indeed, in our prior study the rate of 50-kHz USVs prior to lever extension often overshadowed 50-kHz USVs emerging following intravenous receipt of MDPV. Incorporation of an "Anticipation" time window longer than 5 min, but not necessarily as long as 30 min, is useful in capturing distinct signatures of USVs associated with "Anticipation" and self-administration of MDPV.

This study replicated the finding that, under FR-1 access conditions, 50-kHz USVs wane following initial drug infusions (“load-up”) (Barker et al. 2014; Simmons et al. 2018).

Consistent with prior interpretations, we surmise that the motivational drive to self-administer psychostimulants shifts

from positive to negative reinforcement such that rats propagate binge-like self-administration to mitigate feelings of distress/negative affect that would otherwise emerge. Interestingly, suvorexant pre-treatment at moderate and high doses tended to precipitate sporadic bouts of 22-kHz USVs which may be due to constrained reward function despite high drug levels during the “maintenance” phase of MDPV self-administration. Nevertheless, under low-effort conditions, suvorexant failed to appreciably suppress drug-taking similar to work shown in cocaine self-administering rats (España et al. 2010). In sum, this study found that suvorexant suppresses 50-kHz USVs associated with anticipation and self-administration of MDPV without significantly affecting drug-taking under low-effort access conditions. Increasing

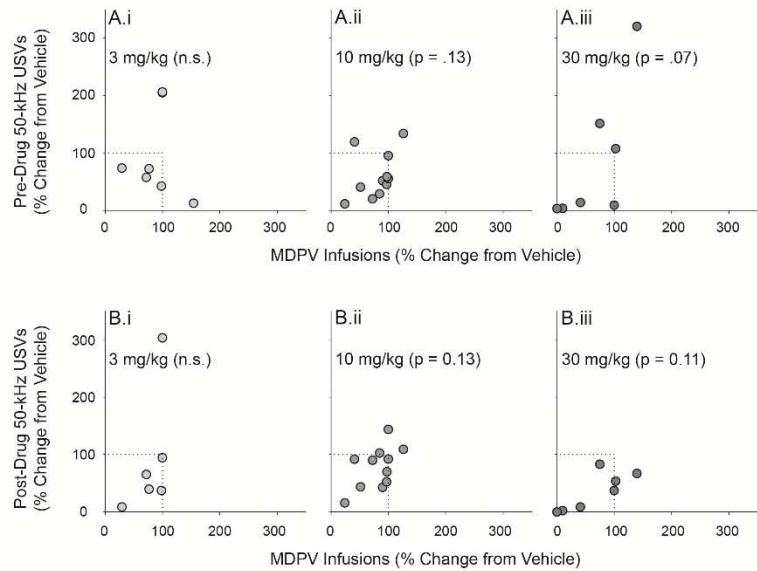


Figure 13. Associating efficacy of suvorexant pre-treatment on MDPV infusions (x-axes) and on pre- or post-lever 50-kHz USVs (A.i-iii, B.i-iii, respectively). Dotted lines enclose “100”—the points of no-change of each measure relative to respective vehicle pre-treatment scores. * $p < 0.05$ using Pearson’s correlation coefficient. $N=7-14$ /group. Figure originally contained within and adapted from Simmons et al. 2017.

response requirements, such as through employment of PR reinforcement, may capture suvorexant's reward-attenuating properties and may aid in understanding possible relationships between USVs and drug-taking.

SPECIFIC AIMS

Addictions to psychostimulants collectively form a crippling epidemic facing many communities on an international scale. Drugs of abuse augment extracellular monoamine content—notably, DA—in structures associated with reward and emotion processing. Transmitters and peptides that regulate the ability of drugs to perturb central neurochemical transmission present attractive targets in effort to normalize motivation and affective changes associated with their abuse. Among contributors, hypothalamic hcrt/ox innervates midbrain and forebrain structures implicated in reward- and mood-related pathologies. Pharmacological studies support that hcrt/ox receptor blockade, systemically and directly within ventral midbrain, attenuates pathological motivation including for drugs of abuse. Our understanding of ventral midbrain structural heterogeneity, however, is continually evolving—within the past decade, the caudal division of VTA (now termed RMTg) was highlighted for its ability to negatively regulate VTA DA activity. Curiously, anatomy work depicts the hypothalamus as a principal input to RMTg, though the cellular phenotypes and functions of this connection have not been fully resolved. Thus, the continuum residing within ventral midbrain is composed of rostral and caudal divisions that lie adjacently yet function in opposition.

Aim 1. Identify topographic organization of hcrt/ox afferents to ventral tegmental area (VTA) and rostromedial tegmentum (RMTg).

For Aim 1, rats will be intracranially cannulated for injections of fluorescent microspheres within VTA and RMTg targets. Immunolabeling will be used to label and map putative hcrt/ox afferents to each structure. Mapping hcrt/ox afferents within ventral

midbrain will provide support for potential functional roles in the domains of reward and emotion regulation.

Aim 2. Determine functional involvement of hcrt/ox transmission within VTA and RMTg on affect and motivated drug-taking.

For Aim 2, rats will receive bilateral cannulation and jugular vein catheterization prior to cocaine self-administration training. Rats will train under fixed-ratio 1 access conditions followed by progressive-ratio during which one of two self-administered cocaine doses (0.375 and 0.750 mg/kg/inf) will be available. During experimental sessions, intra-VTA pre-treatment with suvorexant (0.0, 0.3, 1.0, 3.0 µg/hemi) or intra-RMTg pre-treatment with hcrt/ox (0.0, 0.3, 1.0, 3.0 nmol/hemi) will precede placement in operant chambers and initiation of the self-administration session. Ultrasonic vocalizations (USVs) will be recorded throughout as a proxy for shifts in affective state. Aim 2 will test the hypothesis that intra-VTA suvorexant will attenuate positively-valenced 50-kHz USVs and decrease motivated drug-taking whereas augmenting intra-RMTg hcrt/ox transmission will enhance negatively-valenced 22-kHz USVs and also reduce motivated drug-taking.

Collectively, these experiments are designed to determine topographical arrangement of hcrt/ox afferents to VTA and RMTg and will test the central hypothesis that, while hcrt/ox signaling in VTA contributes to reward/positive affect, hcrt/ox signaling in RMTg contributes to aversion/negative affect (depicted in Figure 14).

| Condition | Cocaine Infusions | Ultrasonic Vocalizations | |
|----------------------------------|-------------------|--------------------------|--------|
| | | 22-kHz | 50-kHz |
| Hcrt/ox in RMTg | ↓ | ↑ | --- |
| Hcrt/ox receptor blockade in VTA | ↓ | --- | ↓ |

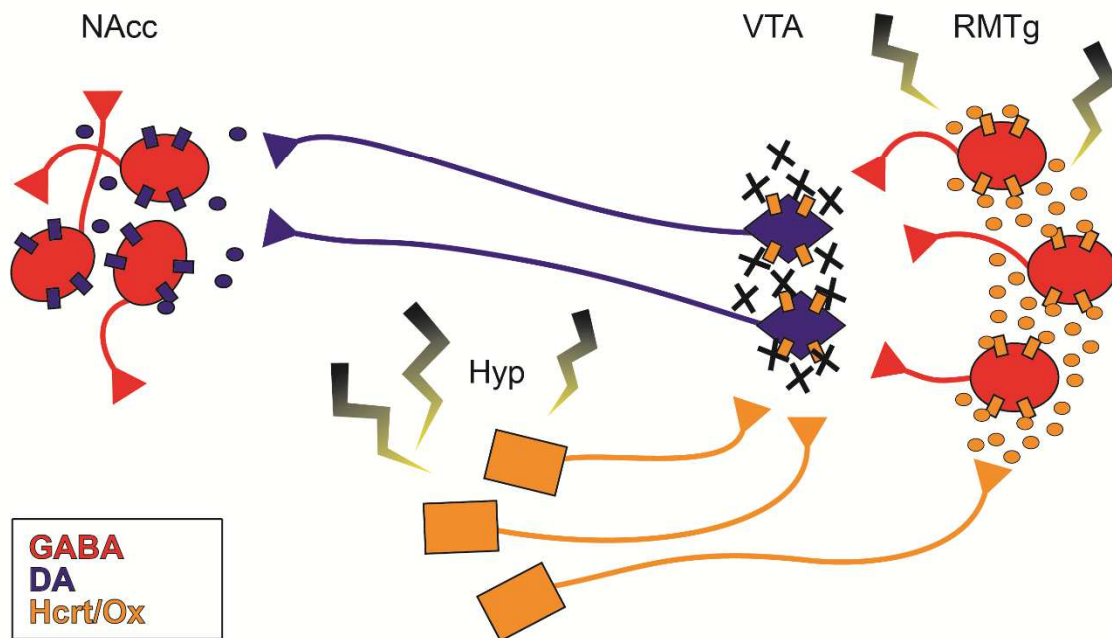


Figure 14. Hypothesis of predicted effects of hcr/ox receptor perturbation within VTA and RMTg on motivated cocaine-taking and USVs. It is predicted that hcr/ox transmission within RMTg and/or receptor blockade within VTA will diminish mesolimbic DA transmission to reduce the number of cocaine infusions earned under high-effort access conditions. Additionally, blockade of hcr/ox receptor in VTA is hypothesized to reduce the rate of ‘positive affective’ 50-kHz USVs while hcr/ox transmission augmentation in RMTg is hypothesized to promote aversion and, in turn, enhance the rate of ‘negative affective’ 22-kHz USVs.

CHAPTER 7 – HYPOCRETIN/OREXIN INNERVATION TO VENTRAL MIDBRAIN

Several studies have supported a critical role of VTA hcrt/ox in motivated responding for salient rewards including palatable food and drugs of abuse. Early work revealed that neurons containing hcrt/ox project to midbrain and indeed influence VTA DA cellular physiology (Fadel and Deutch 2001; Korotokova et al. 2003; Borgland et al. 2009). Since 2009, several research teams worked to uncover a distinct structure within caudal division of ventral midbrain named RMTg (“tail of VTA”). GABA-producing cells within RMTg negatively regulated VTA DA neurons and participate in encoding aversive stimuli (Jhou et al. 2009a, b). The hypothalamus is a principal input to RMTg (Jhou et al. 2009a), but the cellular phenotypes of these afferents have not been uncovered. To the best of our knowledge, explicit study of hcrt/ox innervation to VTA and RMTg of ventral midbrain has not been done. Thus, this study utilized retrograde tract tracing and immunolabeling methods to map hcrt/ox afferents projecting to VTA and RMTg.

7.1) Materials and methods

7.1.1) Animals

Adult male Sprague-Dawley rats (Charles River Laboratories; Horsham, PA, USA) weighing ~300 g at experimental onset were used for the present experiment. Rats were provided food and water freely upon arrival to vivarium, were pair-housed until cannulation surgery after which rats were individually-housed and maintained on a reverse 12-h : 12-h light cycle (lights off at 9:00 AM) in a temperature- and humidity-

controlled vivarium. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Temple University.

7.1.2) Experimental procedures

Rats underwent unilateral stereotaxic cannulation surgery as specified below followed by retrograde tracer injections. After allowing time for retrograde transport (10-14 d), tissue was harvested and processed to label hcrt/ox afferents to target structures in ventral midbrain (**Figure 15A.i**).

7.1.3) Stereotaxic cannulation

Rats received cannulation with 26-gauge guide cannulae directed 1 mm above target structure. Unilateral cannulations were performed and were implanted without angling the cannula (VTA: A/P -5.6 mm, M/L \pm 0.6 mm, D/V -6.6; RMTg: A/P -6.8 mm, M/L \pm 0.4 mm, D/V -6.4 mm from dura rupture). Rats were anesthetized using isoflurane gas anesthesia (5% induction, 3-4% maintenance) at a flow rate of 1.2 – 1.5 L/min mixed with O₂, and hair on head region was shaved using electric clippers. Rats were administered ketoprofen (5 mg/kg, SC) as pre-operative analgesia and secured in ear bars of stereotaxic frame atop water-circulating heating pad. Shaved head region was wiped intermittently with betadine and alcohol, and a midline incision was thereafter performed using a stainless steel scalpel blade (#10). Connective tissues were gently cleared to reveal skull, blood was wiped using cotton-tipped applicators, and clamps were used to keep skin pulled laterally to facilitate visualization. Epinephrine-soaked cotton was thereafter applied to minimize bleeding, and a sickle scaler was used to etch skull surface to improve skull-cap adhesion. If needed, Z-plane was calibrated to allow for even anterior-posterior positioning. Burr holes were drilled using a 106 engraving cutter (1/8”

shank) of drill—two incomplete holes for jeweler screw installations (each per hemisphere approximately 2 mm caudolateral from bregma) and holes according to atlas coordinates to affix guide cannulae above target structure as detailed above. Guide cannulae were lowered, and dura rupture was observed; if not observed, guide tip would be lowered 0.8 – 1.0 mm beneath outer skull surface prior to lowering per D/V coordinate. Guide cannulae were lowered at a rate of ~1.5 mm/min. A resin-ionomer dental paste was applied directly proximal to lower threads of guide and allowed 3-5 min to dry. Fast-curing acrylic resin powder was sprayed to form outer skull-cap and hardened following liquid initiator application. Rats were supplemented with 4 mL of 0.9% saline prior to waking from anesthesia, and lidocaine gel analgesic was topically applied around skull-cap. A 33-gauge dummy cannula (0-mm projection) beyond the end of the guide cannula was tightened in place to prevent backflow and clogging of cannula track.

7.1.4) Retrograde tracing

Rats were anesthetized with isoflurane gas for retrograde tracer injections, and dummy cannulae were thereafter removed. Fluorophore-conjugated latex microspheres (Lumafluor [Red RetroBeads IX]; Durham, NC, USA) with 530-/590-nm maximum excitation/emission spectra, diluted 1:1 with aCSF, were deposited into target structure using a 33-gauge locking injector extending 1-mm beyond pedestal of guide cannulae. A series of 3 150-nL injections per day across three days was performed on each rat. Injections were separated by 1-2 h, and each day of injections was separated by ~24 h. PE-50 tubing connected the locking injector to a 22-gauge microliter syringe which itself was secured on an automated syringe pump. Injection parameters were established at 50

nL/min flow rate, and locking injectors were kept in place for an additional 1 min to allow for diffusion from injector tip. Patency/flow of injector was checked in between each injection. Dummy cannulae were then re-tightened onto guides, and rats were placed in homecage and back into vivarium upon waking from anesthesia.

7.1.5) Tissue collection and analysis

After allowing 10-14 d for retrograde transport of tracers, rats were deeply anesthetized with isoflurane anesthesia for intracardiac perfusion. Anteriorly-extending lateral incisions from mid-section of rat were made using large sharp scissors. Diaphragm was snipped and rib-cage clamped against anterior aspect to reveal beating heart. Connective tissues were carefully separated, and a 22-gauge needle connected to peristaltic perfusion pump (LongerPump; Boonton, NJ, USA) was inserted into left ventricle. Perfusion with ice-cold phosphate buffer (30-34 rpm) was then initiated, and the right atrium was snipped with small sharp scissors to permit exsanguination. About 7-8 min later (until drained content was pale in color), the perfusion pump was briefly halted to switch uptake solution to ice-cold paraformaldehyde (PFA; 4% mixed in phosphate buffer, pH = 7.40). Rats were perfused with PFA for 10 min, and heads were then decapitated to allow for whole brain dissection through skull removal using rangeurs and small sharp scissors. Brains were kept at 4° C in PFA for 24-72 h followed by 30% sucrose in phosphate buffer for 5-7 d. Dehydrated brains were then sectioned on a cryostat (Leica) at after blocking cerebellum for even mounting position. For some brains, a marking was made using a 22-gauge needle on one hemisphere of lateral midbrain (i.e. outside of hypothalamus) to demarcate ipsilateral versus contralateral hemisphere relative to tracer deposit. Tissue containing guide cannula track was

sectioned at 60 μm , and hypothalamus was sectioned at 30 μm . Tissue was stored in cryoprotectant (30% ethylene glycol and 30% sucrose in 0.1M phosphate-buffered saline [PBS]) and kept at -20°C until processed for immunolabeling.

Hypothalamus-containing tissue (constituting a 1-in-3 series of entire hypothalamus) was processed throughout under conditions minimizing exposure to white light. Tissue was first washed in PBS 6 times (5-min each on rocker) to clear away residual cryoprotectant. Tissue was then blocked with 5% donkey serum in PBS containing 0.3% Triton X-100 (hereafter termed "PBS+") for 60 min at room temperature. Tissue was then transferred to 1.5-mL microcentrifuge tubes containing goat anti-orexinA primary antibody (1:1,000; SC8070, Santa Cruz) in 1.5% donkey serum in PBS+ and gently rocked for 24-48 h at 4°C . Thereafter, tissue was washed with PBS 6x (5-min each on rocker) and then transferred to 1.5-mL microcentrifuge tubes containing donkey anti-goat 488 (AlexaFluor) secondary antibody (1:750) in 1.5% donkey serum in PBS+ and gently rocked for 3-4 h at room temperature. Tissue containing cannula track and tracer deposit site was rinsed in phosphate buffer and gently rocked in fluorescent Nissl (NeuroTrace 500/525) at 1:500 concentration in PBS+. Finally, all tissue was again washed in PBS (3x, 5-min each on rocker) and kept at 4°C until mounted on charged microscope slides using a petri dish containing ddH₂O. Tissue was allowed to dry on slides before coverslipping with medium that protects against fluorophore decay (ProLong Gold).

Sections from confirmed "hits" to target structure (based on histology of sections containing cannula track) were imaged on an Eclipse 80i upright fluorescent microscope (Nikon; Minato, Tokyo, Japan). Hypothalamic sections containing orexinA-

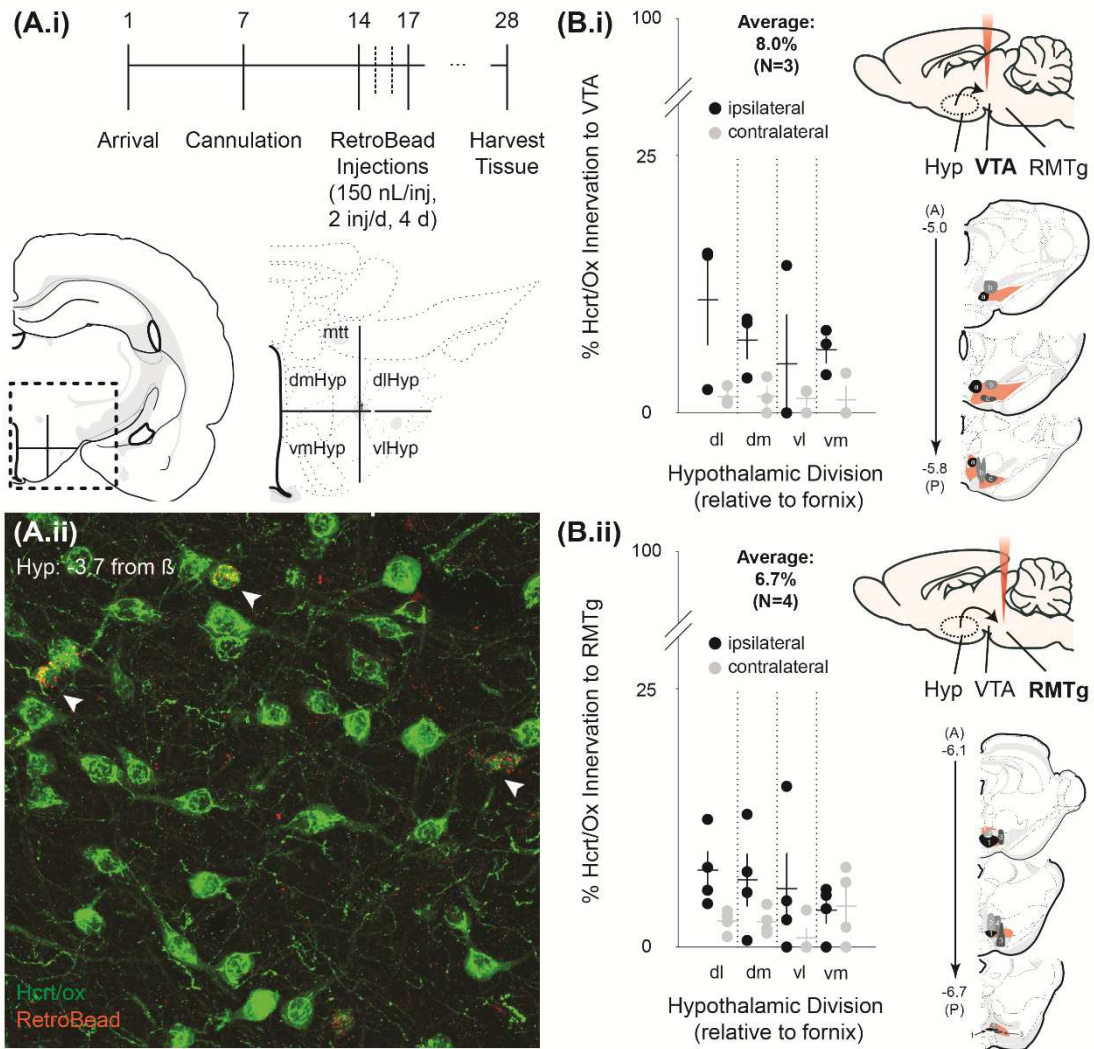


Figure 15. Hcrt/ox innervates VTA and RMTg at comparable densities with no clear topographical arrangement. Timeline of retrograde tracing experiment is depicted in (A.i) and photomicrograph captured at 10x objective magnification (A.ii) shows hcr/ox cell populations containing retrograde tracer. Innervation densities per case, hemisphere and hypothalamic afferent sub-region relative to fornical tract for projections to VTA (n=3) (B.i) and to RMTg (n=4) (B.ii).

immunolabeled cells (4-8 sections per subject) were imaged at 10x objective magnification. For brains not receiving hemispheric demarcation via 22-gauge needle during sectioning as described above, laterality was determined by fluorescence intensity of tracer signal at 4x objective magnification, and hemisphere with discernibly greater fluorescent signal was classified as ipsilateral relative to tracer deposit. Analyses proceeded by manually counting cells containing only orexinA, cells containing both

orexinA and tracer,
and tracer-
containing cells
without orexinA.

Each hypothalamus
section used for
anterior-posterior
topographical
analysis was

assigned to atlas
levels 26 through
32 (Swanson 2004)
based on gross
morphological

features including extent of hippocampus across medial-lateral and dorsal-ventral axes,
positions of fornical and mammillothalamic tracts, and shape of ventral division of third
ventricle.

7.1.6) Statistical analyses

Independent samples t-tests were conducted to compare total % innervation of
hcr/ox-producing cells targeting VTA and RMTg as well as comparisons between each
sub-region (i.e., dorsolateral, dorsomedial, ventrolateral, ventromedial). Separate t-tests
were conducted for % innervation comparisons of contralateral versus ipsilateral
afferents.

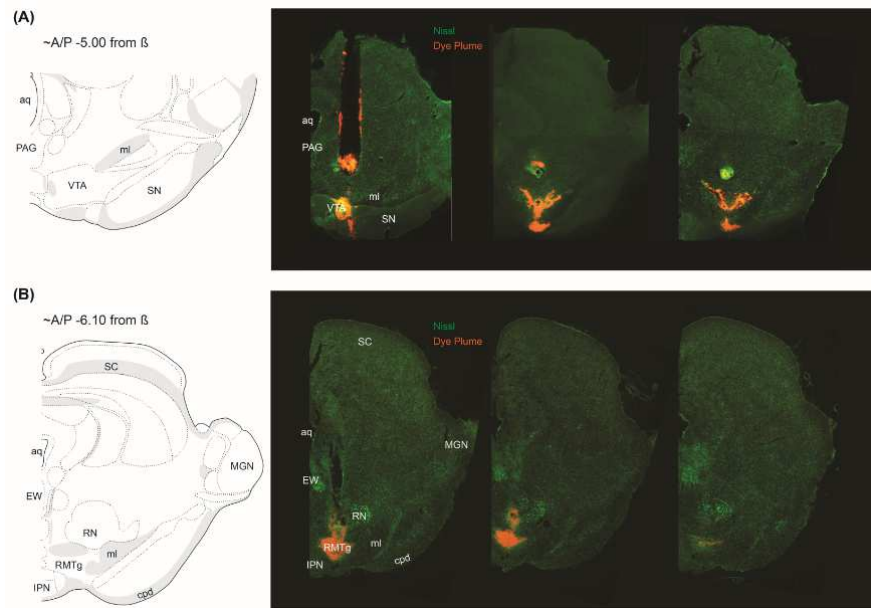


Figure 16. Representative retrograde tracer deposit histology. Dye plumes are shown for retrograde tracer deposits to VTA (A) and to RMTg (B). aq – central aqueduct, cpd – cerebellar peduncle, EW – Eddinger-Westphal nucleus, IPN – interpeduncular nucleus, MGN – medial geniculate nucleus, ml – medial lemniscus, PAG – periaqueductal grey area, RMTg – rostromedial tegmental nucleus, RN – red nucleus, SC – superior colliculus, SN – substantia nigra, VTA – ventral tegmental area.

7.2) Results

Histological analysis confirmed retrograde tracer deposit within VTA (n=3) and RMTg (n=4) with minimal tracer spillover into adjacent structures (**Figure 16**). Analysis of putative hcr/ox-producing afferent cells indicated comparable targeting density to each of VTA (8.0%) and RMTg (6.7%) targets from entire hcr/ox-producing cell population of ipsilaterally-injected hemisphere (**Figure 15B.i-ii**). No significant differences were found when examining % innervation of total hcr/ox cells of ipsilateral or contralateral hemisphere relative to retrograde tracer deposit between putative hcr/ox-producing VTA- and RMTg-projecting afferents [all $|t(5)| \leq 0.40$, n.s.]. Similarly, no significant differences between VTA- and RMTg-projecting hcr/ox afferents were found when examining afferent cells arising from ipsilaterally- or contralaterally-injected dorsolateral, dorsomedial, ventrolateral or ventrolmedial hypothalamus. Sparse labeling was found on hemisphere contralateral to tracer deposit (VTA: 1.7%; RMTg: 2.5%). The majority of hcr/ox-producing cells were found within dorsolateral and dorsomedial hypothalamus relative to fornical tract, and these sub-regions contained the greatest proportion of retrogradely-traced hcr/ox cells compared to the field of hcr/ox-producing cells within sub-region (VTA: 11.6% and 7.8%, respectively; RMTg: 7.4% and 7.9%, respectively). Data per each analyzed case are reported in **Table 3**. Topographical analysis across anterior-posterior extent of hypothalamus revealed that sections containing the densest quantity of hcr/ox cells (i.e. at atlas levels 28-30) tended to most robustly target VTA or RMTg relative to anterior or posterior extent of hypothalamus (i.e. at atlas levels 26 and 32, respectively) (**Table 3**). Innervation densities can be found in **Table 4**. Overall, these data show that hcr/ox afferents target both VTA and RMTg of

| Target | Hemisphere | Case # | # tracer+ hcrt/ox cell count | | | | total hcrt/ox cell count | | | | % tracer+ hcrt/ox cells | | | |
|--------|---------------|------------------|------------------------------|----|----|----|--------------------------|-----|----|-----|-------------------------|------------|------------|------------|
| | | | dl | dm | vl | vm | dl | dm | vl | vm | dl | dm | vl | vm |
| VTA | Ipsilateral | 01.003 | 48 | 35 | 0 | 1 | 310 | 385 | 28 | 27 | 15.5 | 9.1 | 0.0 | 3.7 |
| | | 01.004 | 20 | 27 | 2 | 8 | 131 | 309 | 14 | 100 | 15.3 | 8.7 | 14.3 | 8.0 |
| | | 01.005 | 4 | 6 | 0 | 1 | 179 | 178 | 50 | 15 | 2.2 | 3.4 | 0.0 | 6.7 |
| | | (average) | | | | | | | | | 11.0 | 7.1 | 4.8 | 6.1 |
| | | (average) | | | | | | | | | 11.0 | 7.1 | 4.8 | 6.1 |
| | Contralateral | 01.003 | 2 | 4 | 1 | 1 | 212 | 289 | 47 | 26 | 0.9 | 1.4 | 2.1 | 3.9 |
| | | 01.004 | 6 | 10 | 0 | 0 | 229 | 285 | 16 | 0 | 2.6 | 3.5 | 0.0 | 0.0 |
| | | 01.005 | 2 | 0 | 1 | 0 | 165 | 153 | 48 | 20 | 1.2 | 0.0 | 2.1 | 0.0 |
| | | (average) | | | | | | | | | 1.6 | 1.6 | 1.4 | 1.3 |
| | | (average) | | | | | | | | | 1.6 | 1.6 | 1.4 | 1.3 |
| RMTg | Ipsilateral | 02.003 | 6 | 1 | 0 | 0 | 143 | 156 | 10 | 2 | 4.2 | 0.6 | 0.0 | 0.0 |
| | | 02.008 | 28 | 43 | 9 | 1 | 227 | 335 | 58 | 20 | 12.3 | 12.8 | 15.5 | 5.0 |
| | | 02.014 | 21 | 25 | 4 | 5 | 382 | 343 | 90 | 90 | 5.5 | 7.3 | 4.4 | 5.6 |
| | | 02.015 | 9 | 7 | 1 | 1 | 117 | 133 | 38 | 27 | 7.7 | 5.3 | 2.6 | 3.7 |
| | | (average) | | | | | | | | | 7.4 | 6.5 | 5.7 | 3.6 |
| | Contralateral | 02.003 | 3 | 2 | 0 | 1 | 122 | 150 | 14 | 16 | 2.5 | 1.3 | 0.0 | 6.3 |
| | | 02.008 | 5 | 5 | 0 | 0 | 143 | 121 | 12 | 18 | 3.5 | 4.1 | 0.0 | 0.0 |
| | | 02.014 | 10 | 7 | 1 | 4 | 335 | 270 | 28 | 52 | 3.0 | 2.6 | 3.6 | 7.7 |
| | | 02.015 | 1 | 2 | 0 | 1 | 98 | 114 | 28 | 52 | 1.0 | 1.8 | 0.0 | 1.9 |
| | | (average) | | | | | | | | | 2.5 | 2.5 | 0.9 | 4.0 |

Table 3. Retrograde tract tracing data expanded; cell count data per case are included as are proportions of afferent innervation from hypothalamic hcrt/ox (by sub-region) to VTA and RMTg.

ventral midbrain with comparable densities and suggest possible opponent functions of hcrt/ox in RMTg to known reward-mediating functions in VTA. Specifically, hcrt/ox may provide excitatory transmission to GABA-producing cells of the RMTg which in turn may inhibit the DA-producing cells of VTA to “brake” reward signals and/or participate in the encoding of aversive stimuli.

7.3) Interim discussion

Outputs from hypothalamic hcrt/ox-producing neurons are of interest in determining the loci underlying behavioral effects of hcrt/ox transmission modulation (i.e., with pharmacological agents). Specifically, numerous teams reveal that hcrt/ox receptor antagonism normalizes pathological motivation in the context of drug-seeking (e.g., Borgland et al. 2009; Zhou et al. 2012). Prior studies additionally find that hcrt/ox

| Target Structure | Hypothalamus | | Hcrt/Ox Innervation Densities | | | | | | | |
|------------------|--------------|-----------------|-------------------------------|------|------|------|---------------|-----|------|------|
| | Atlas Level | mm from β | Ipsilateral | | | | Contralateral | | | |
| | | | dl | dm | vl | vm | dl | dm | vl | vm |
| VTA (n=3) | 26 (A) | -1.78 | ++++ | ++++ | --- | --- | + | + | --- | --- |
| | 27 | -2.00 | ++++ | ++++ | 0 | ++++ | + | ++ | + | ++ |
| | 28 | -2.45 | ++++ | +++ | ++ | ++ | + | + | 0 | 0 |
| | 29 | -2.85 | ++++ | ++ | 0 | +++ | 0 | +++ | 0 | --- |
| | 30 | -3.25 | +++ | +++ | 0 | --- | + | 0 | 0 | 0 |
| | 31 | -3.70 | ++++ | ++ | 0 | 0 | 0 | + | ++++ | --- |
| | 32 (P) | -3.90 | --- | --- | --- | --- | --- | --- | --- | --- |
| | Total | | | ++++ | +++ | + | +++ | + | + | + |
| RMTg (n=4) | 26 (A) | -1.78 | +++ | +++ | ++++ | +++ | ++ | +++ | 0 | ++++ |
| | 27 | -2.00 | + | + | 0 | 0 | + | + | 0 | ++ |
| | 28 | -2.45 | ++++ | +++ | ++ | 0 | 0 | + | 0 | 0 |
| | 29 | -2.85 | ++ | +++ | + | ++++ | + | + | ++ | ++ |
| | 30 | -3.25 | ++ | +++ | 0 | ++++ | + | + | 0 | 0 |
| | 31 | -3.70 | ++++ | ++++ | + | ++ | + | + | 0 | +++ |
| | 32 (P) | -3.90 | +++ | +++ | 0 | 0 | ++ | + | 0 | 0 |
| | Total | | | +++ | +++ | +++ | ++ | + | + | + |

--- not detected 0 0% + 0-3% ++ 3-6% +++ 6-9% ++++ 9+%

Table 4. Retrograde tract tracing data expanded; relative innervation densities of putative hypothalamic hcr/ox afferents to VTA and RMTg are included and divided by sub-region of hypothalamus based on position of fornix.

innervates VTA (Peyron et al. 1998; Fadel and Deutch 2001) and direct-site pharmacology finds that blockade of OX_1 Rs within VTA reduces motivated cocaine-taking (Muschamp et al. 2014). In 2009, the caudal division of VTA—a structure termed “tail of VTA” and RMTg—was anatomically and functionally characterized (Jhou et al. 2009a, b). Interestingly, this GABAergic nuclei inhibits the rostrally-positioned DA-producing cell population in VTA to inhibit mesolimbic DA transmission, and the RMTg is activated following presentation of aversive stimuli. Our tract-tracing study was conducted to reveal if hcr/ox additionally transmits to RMTg which would support an omnibus modulatory role in positively and negatively regulating motivated reward-seeking. Indeed, we observed comparable innervation of putative hcr/ox afferents to VTA and RMTg and, thereafter, begged to question potential functions of these connections.

CHAPTER 8 – ROLE OF HYPOCRETIN/OREXIN TRANSMISSION WITHIN VENTRAL MIDBRAIN ON PSYHOSTIMULANT-ASSOCIATED REINFORCEMENT AND AFFECTIVE CHANGES

To-date, no studies have examined possible functions of hcr/ox transmission within RMTg. Numerous behavioral pharmacological reports demonstrate therapeutic utility of hcr/ox receptor blockade within VTA in the context of attenuating pathological motivation, and work contained within Chapter 7 of this thesis supports that hcr/ox additionally innervates RMTg. Critically, the RMTg negatively regulates DA-producing cells housed within VTA and thus hcr/ox within RMTg may participate in the physiological encoding of aversive states. In this study, we tested the functional impact of hcr/ox perturbation within ventral midbrain structures using local injections of hcr/ox peptide (bilaterally into RMTg) or of the clinically-available hcr/ox receptor antagonist suvorexant (bilaterally into VTA). Specifically, we measured the impact of site-directed hcr/ox receptor agonist and antagonist administration on USVs and drug-taking associated with cocaine self-administration in a high-effort task. Moreover, we used two doses of self-administered cocaine (0.375 and 0.75 mg/kg/inf) to evaluate potential dose-related differences as has been shown in prior pharmacological studies using hcr/ox receptor antagonists (España et al. 2010).

8.1) Materials and methods

8.1.1) Animals

Rats used for the present study were adult male Sprague-Dawley rats (Charles River Laboratories; Horsham, PA, USA) weighing ~250 g from experimental onset. Rats were pair-housed until cannulation surgery after which rats were singly-housed and

maintained on a 12-h : 12-h reverse light cycle in a humidity- and temperature-controlled vivarium. Rats were provided food and water *ad libitum* except during experimental sessions (drug self-administration). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Temple University.

8.1.2) Drugs

Cocaine hydrochloride (Sigma-Aldrich; St. Louis, MO, USA) and suvorexant (AstaTech; Bristol, PA, USA) were commercially purchased. OrexinA peptide was generously provided by the Drug Supply Program of the National Institute on Drug Abuse (PolyPeptide Group; San Diego, CA, USA). Cocaine hydrochloride was stored at 4° C and was dissolved in 0.9% saline, vortexed, and filtered through 0.45 µm cellulose acetate prior to being loaded in syringes for self-administration—the concentration was adjusted for target infusion dose as well as average bodyweight of cohort every 5-7 d. OrexinA was prepared for intracerebral injections by dissolving in artificial cerebrospinal fluid (aCSF, ion concentrations in mM: Na⁺ 150, K⁺ 3.0, Ca²⁺ 1.4, Mg²⁺ 0.8, P³⁻ 1.0, Cl⁻ 155) and was kept desiccated at -20° C prior to loading in injectors. Suvorexant was stored at 4° C and was prepared for intracerebral injections immediately prior to each treatment session by dissolving in dimethyl sulfoxide (DMSO) through vortexing and ultrasonication.

8.1.3) Experimental procedures

Rats underwent bilateral cannulation and jugular vein catheterization surgeries prior to cocaine self-administration. Using a within-subjects design, effects of intra-VTA suvorexant (0.0, 0.3, 1.0, 3.0 µg/hemisphere) were assessed on motivated responding for and ultrasonic vocalizations (USVs) associated with either low-dose (~0.375 mg/kg/inf)

or moderate-dose (~0.75 mg/kg/inf) of self-administered cocaine. In separate cohorts, effects of intra-RMTg orexinA (0.0, 0.3, 1.0, 3.0 nmol/hemisphere) were assessed on identical dependent measures. Following pharmacological agent pretreatment, rats were immediately placed in operant chamber, and USVs were recorded for 15 min prior to lever extension and onset of 2-h self-administration session. Following all experimental sessions, tissue was harvested to histologically verify cannula placements. Experimental timelines and designs are depicted in **Figure 17**.

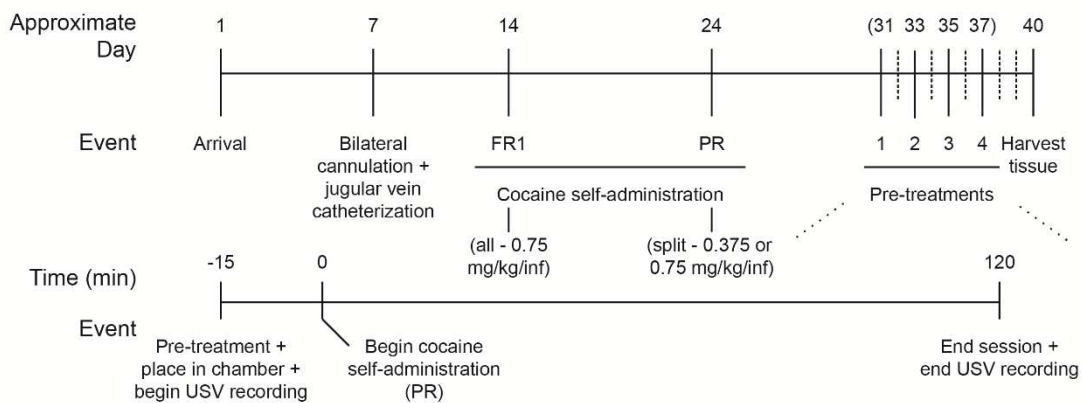


Figure 17. Experimental designs for target site pharmacology probing hcrt/ox function in VTA and RMTg on motivated cocaine-taking. FR1 – fixed ratio 1, PR – progressive ratio, USV – ultrasonic vocalization.

8.1.4) Stereotaxic cannulation

For detail of stereotaxic cannulation surgery, readers are referred to [Section 7.1.3](#). Briefly, bilateral cannulations were performed, and each of two individual guides were angled at 10° and lowered to reach depth of target structure (VTA: -5.4 mm, M/L ± 2.1, D/V -6.6; RMTg: A/P -6.8 mm, M/L ±0.4 mm, D/V -6.6 mm from dura rupture). Dummy cannulae were tightened onto guide cannulae to prevent backflow and clogging.

8.1.5) Intravenous drug self-administration: jugular vein catheterization, apparatus and behavioral procedures

For full description, readers are referred to supplemental [Section S1.3](#). Briefly, rats were trained to self-administer cocaine (~0.75 mg/kg/inf) under fixed-ratio 1 (FR-1) access conditions during 2-h sessions for 10-14 d, 5-7 d/wk. Thereafter, rats were shifted to a progressive-ratio (PR) schedule of reinforcement for at least 5 d before receiving pre-treatments. At least 1 d of PR cocaine self-administration without pre-treatment proceeded between each of 4 pre-treatment sessions as depicted in **Figure 17**.

8.1.6) Ultrasonic vocalization recording and analysis

USVs were recorded and analyzed as described in supplemental [Section S1.4](#). Briefly, USV recordings began 15-min prior to the onset of self-administration sessions (i.e. before triggering MED-PC programs). Recordings proceeded throughout the 2-h PR sessions.

8.1.7) Statistical analyses

For cocaine self-administering training sessions, mixed model ANOVAs were used to compare the number of infusions earned examining session (10 sessions of FR-1 or 7 sessions of PR) by cocaine dose (0.375 or 0.750 mg/kg/inf) for each target-site (VTA or RMTg). For experimental test sessions, analyses proceeded on the % of infusions earned, active and inactive lever presses performed, and breakpoints relative to prior-day baseline performance using one-way ANOVAs with pre-treatment dose (vehicle, low, moderate, high) as the repeated-measures factor. Separate ANOVAs were performed dependent on cannulated target site (VTA and RMTg) which influenced levels of the “pre-treatment dose” variable (for VTA, suvorexant doses were: 0.0, 0.3, 1.0, 3.0 µg; for

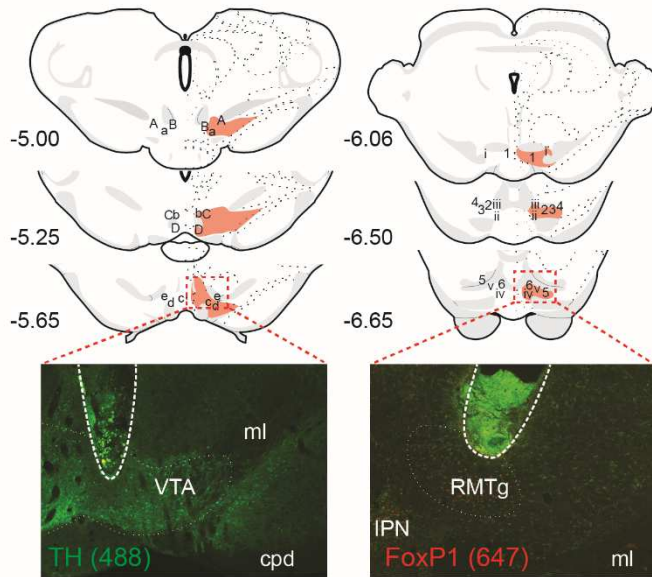


Figure 18. Cannula placement histology for rats used for target-site behavioral pharmacology experiments. Histologically-confirmed “hits” for intra-VTA injected rats are indicated by lower-case (a, b, c) and upper-case (A, B, C) letters for low-dose (0.375 mg/kg/inf; n=5) and high-dose (0.750 mg/kg/inf; n=4) cocaine self-administering rats, respectively. Similarly, Roman numerals (i, ii, iii) and Arabic numerals (1, 2, 3) are used to depict cannula placement for intra-RMTg injected rats self-administering low-dose (n=5) versus high-dose (n=6) cocaine, respectively. Red highlight indicates intended target structure (VTA in left panels, RMTg in right panels). Representative histology sections with cannula track outlines are shown in lower panels. cpd – cerebellar peduncle, IPN – interpeduncular nucleus, ml – medial lemniscus, RMTg – rostromedial tegmental nucleus, VTA – ventral tegmental area.

RMTg, hert/ox peptide doses were: 0.0, 0.3, 1.0, 3.0 nmol) as well as on cocaine dose (0.375 and 0.750 mg/kg/inf); thus, four one-way repeated-measures ANOVAs were conducted in total for each analyzed %-change metric (VTA-0.375, VTA-0.750, RMTg-0.375, RMTg-0.750). Greenhouse Geisser adjustments were implemented when sphericity violations were detected. Contrast analyses proceeded to compare each “% change” from pre-treatment metric (low, moderate, high) against vehicle pre-treatment. 22- and 50-kHz USV count data during “Anticipation” and “Post-

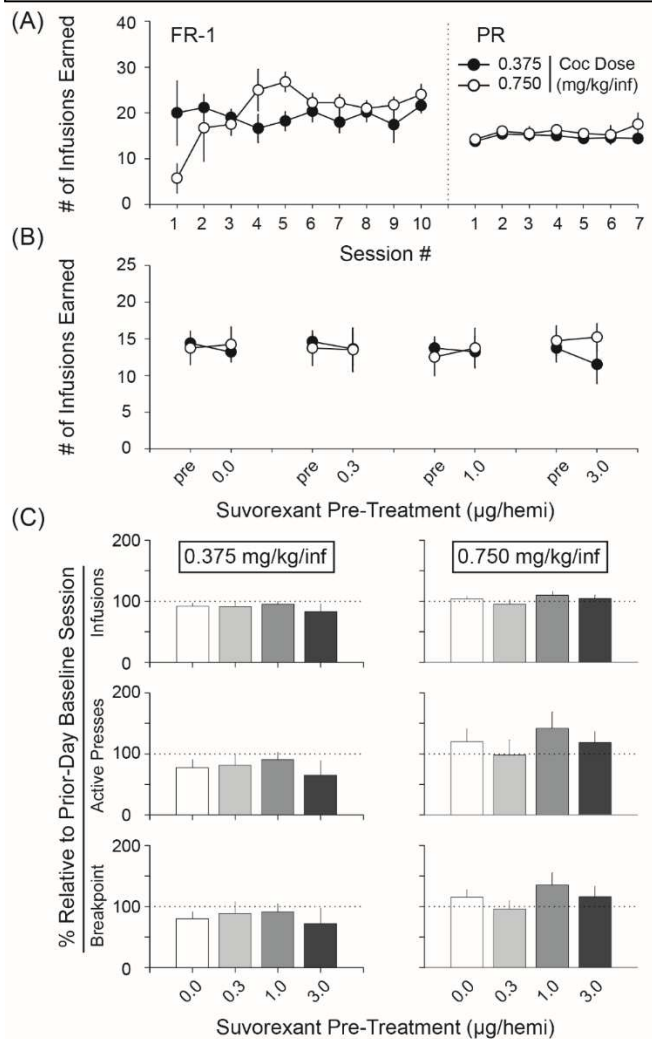
Lever” time epochs were analyzed using mixed ANOVAs as described above for lever press data¹. Correlations between breakpoint and USVs proceeded using Pearson coefficients¹. Statistical significance was defined as Type I error rate (α) below 0.05.

¹ Readers should note that these data are still undergoing collection and thus will not be statistically analyzed until fully completed.

8.2) Results

Results described below are for rats with histologically-confirmed cannula placements (**Figure 18**). For intra-VTA cannulated rats, a significant interaction was found when examining infusions earned across 10 FR-1 training sessions by cocaine dose [$F(9, 63) = 2.365, p < 0.05$] (**Figure 19A**). A trending main effect of training session was observed [$F(9, 63) = 1.667, p = 0.12$] with more infusions earned in later sessions but not of cocaine dose [$F(1, 7) = 0.202, n.s.$]. When examining infusions earned during 7 PR training sessions, no significant interaction was found [$F(2.083, 10.415) = 0.480, n.s.$], and no main effects of session number [$F(2.083, 10.415) = 0.614, n.s.$] or of cocaine dose [$F(1, 5) = 0.770, n.s.$] were

Figure 19. Effects of intra-VTA suvorexant on motivated cocaine-taking. (A) Number of infusions earned across 10 FR-1 and 7 PR training sessions. (B) Number of infusions earned following each pre-treatment dose and prior-day baseline performance. (C) Effects of intra-VTA suvorexant on % infusions earned, % active presses and % breakpoint metrics separated by self-administered cocaine dose. n=4-6/group.



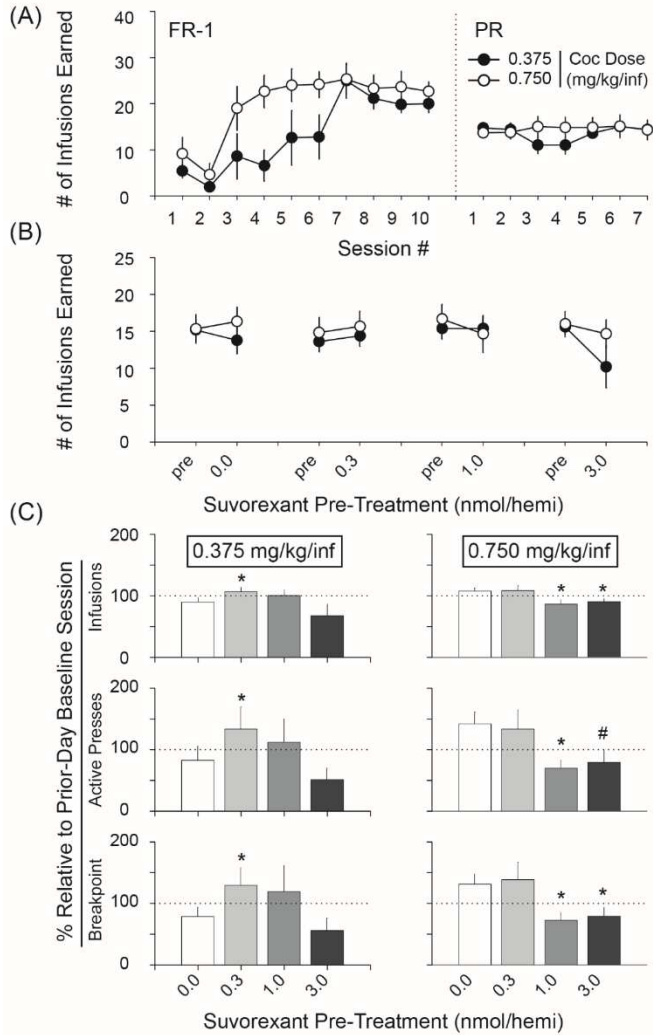


Figure 20. Effects of intra-RMTg hcrt/ox on motivated cocaine-taking. (A) Number of infusions earned across 10 FR-1 and 7 PR training sessions. (B) Number of infusions earned following each pre-treatment dose and prior-day baseline performance. (C) Effects of intra-RMTg hcrt/ox on % infusions earned, % active presses and % breakpoint metrics separated by self-administered cocaine dose. * $p < 0.05$ and # $p = 0.06$ relative to vehicle pre-treatment comparator scores (Bonferroni-corrected contrasts). $n=4-6$ /group.

reinforcement.

observed. When examining infusions earned during 10 FR-1 training sessions of intra-RMTg cannulated rats, no significant interaction was found [$F(9, 81) = 1.565, n.s.$] (**Figure 20A**), but main effects of training session [$F(9, 81) = 10.669, p < 0.001$] and, of lesser power, cocaine dose [$F(1, 9) = 6.010, p < 0.05$] were detected. When examining infusions earned during 7 PR sessions of intra-RMTg cannulated rats, no significant interaction [$F(2.344, 21.098) = 2.088, n.s.$] or main effects of training session [$F(2.344, 21.098) = 1.160, n.s.$] or cocaine dose [$F(1, 9) = 0.224, n.s.$] were observed. These data show that rats across groups can train to self-administer cocaine under FR-1 and PR schedules of

For experimental sessions, intra-VTA suvorexant in rats self-administering low-dose cocaine (0.375 mg/kg/inf) had no effect on % infusions earned [$F(3, 9) = 0.303$, n.s.], % active presses [$F(3, 9) = 0.332$, n.s.], % inactive presses [$F(3, 9) = 0.321$, n.s.], or % breakpoint [$F(3, 9) = 0.214$, n.s.] (**Figure 19B-C**). Similarly, no effect of intra-VTA suvorexant was found in high-dose cocaine (0.750 mg/kg/inf) self-administering rats when examining % infusions earned [$F(3, 9) = 0.895$, n.s.], % active presses [$F(3, 9) = 0.551$, n.s.], % inactive presses [$F(3, 9) = 0.264$, n.s.], and % breakpoint [$F(3, 9) = 0.804$, n.s.]. Raw behavioral metrics for intra-VTA cannulated rats subjected to suvorexant pre-

Table 5. Behavioral metrics per subject for intra-VTA cannulated rats receiving suvorexant pre-treatment prior to cocaine self-administration. Data in parentheses indicates prior-day baseline performance from which % change metrics were computed throughout analyses.

| Intra-VTA suvorexant on low-dose cocaine self-administration (0.375 mg/kg/inf) (n=5) | | | | | | Intra-VTA suvorexant on high-dose cocaine self-administration (0.750 mg/kg/inf) (n=4) | | | | | |
|--|------------------|----------------|------------------|-------------|---------|---|------------------|----------------|------------------|--------------|---------|
| Dose | Infusions Earned | Active Presses | Inactive Presses | Breakpoint | Subject | Dose | Infusions Earned | Active Presses | Inactive Presses | Breakpoint | Subject |
| Veh | 13 (15) | 264 (392) | 15 (25) | 50 (77) | C3B1 | Veh | 20 (19) | 1339 (929) | 23 (26) | 219 (178) | C4B10 |
| 0.3 µg | 15 (16) | 405 (566) | 25 (21) | 77 (95) | | 0.3 µg | 20 (19) | 1328 (892) | 12 (9) | 219 (178) | |
| 1.0 µg | 13 (14) | 264 (314) | 7 (10) | 50 (62) | | 1.0 µg | 18 (17) | 714 (569) | 1 (6) | 145 (118) | |
| 3.0 µg | 8 (13) | 63 (241) | 8 (10) | 15 (50) | | 3.0 µg | 20 (19) | 1336 (896) | 4 (2) | 219 (178) | |
| Veh | 10 (12) | 96 (203) | 4 (4) | 25 (40) | C3B4 | Veh | 12 (13) | 167 (275) | 11 (20) | 40 (50) | C4B15 |
| 0.3 µg | 7 (12) | 39 (167) | 18 (22) | 12 (40) | | 0.3 µg | 12 (12) | 201 (167) | 3 (6) | 40 (40) | |
| 1.0 µg | 9 (11) | 82 (128) | 5 (14) | 20 (32) | | 1.0 µg | 14 (11) | 279 (127) | 49 (12) | 62 (32) | |
| 3.0 µg | 11 (10) | 127 (114) | 15 (3) | 32 (25) | | 3.0 µg | 13 (13) | 217 (217) | 18 (18) | 50 (50) | |
| Veh | 17 (18) | 631 (755) | 6 (7) | 118 (145) | C3B7 | Veh | 16 (15) | 451 (372) | 0 (0) | 95 (77) | M1B14 |
| 0.3 µg | 19 (17) | 927 (668) | 10 (9) | 178 (118) | | 0.3 µg | 16 (16) | 451 (520) | 0 (0) | 95 (95) | |
| 1.0 µg | 19 (18) | 1066 (870) | 10 (7) | 178 (145) | | 1.0 µg | 17 (16) | 569 (451) | 0 (0) | 118 (95) | |
| 3.0 µg | 19 (19) | 1081 (1099) | 7 (10) | 178 (178) | | 3.0 µg | 16 (17) | 520 (673) | 0 (0) | 95 (118) | |
| Veh | 13 (15) | 263 (428) | 3 (29) | 50 (77) | M1B9 | Veh | 9 (8) | 89 (58) | 63 (19) | 20 (15) | M1B15 |
| 0.3 µg | 11 (12) | 148 (183) | 0 (10) | 32 (40) | | 0.3 µg | 6 (8) | 23 (65) | 7 (22) | 9 (15) | |
| 1.0 µg | --- | --- | --- | --- | | 1.0 µg | 6 (6) | 27 (28) | 11 (14) | 9 (9) | |
| 3.0 µg | --- | --- | --- | --- | | 3.0 µg | 12 (10) | 173 (116) | 6 (31) | 40 (25) | |
| Veh | 13 (12) | 245 (194) | 6 (2) | 50 (40) | M1B11 | Veh | 14.3 (13.8) | 511.5 (408.5) | 24.3 (16.3) | 93.5 (80.0) | AVG |
| 0.3 µg | 16 (16) | 453 (490) | 7 (14) | 95 (95) | | 0.3 µg | 13.5 (13.8) | 500.8 (411.0) | 5.5 (9.3) | 90.8 (82.0) | |
| 1.0 µg | 12 (12) | 170 (187) | 4 (7) | 40 (40) | | 1.0 µg | 13.8 (12.5) | 397.3 (293.8) | 15.3 (8.0) | 83.5 (63.5) | |
| 3.0 µg | 8 (13) | 54 (217) | 0 (9) | 15 (50) | | 3.0 µg | 15.3 (14.8) | 561.5 (475.5) | 7.0 (12.8) | 101.0 (92.8) | |
| Veh | 13.2 (14.4) | 299.8 (394.4) | 6.8 (13.4) | 58.6 (75.8) | AVG | | | | | | |
| 0.3 µg | 13.6 (14.6) | 394.4 (414.8) | 12.0 (15.2) | 78.8 (77.6) | | | | | | | |
| 1.0 µg | 13.3 (13.8) | 395.5 (374.8) | 6.5 (9.5) | 72.0 (69.8) | | | | | | | |
| 3.0 µg | 11.5 (13.8) | 331.3 (417.8) | 7.5 (8.0) | 60.0 (75.8) | | | | | | | |

treatment prior to cocaine self-administration are reported in **Table 5**. These results show that intra-VTA suvorexant has negligible effects on motivated cocaine-taking.

For rats self-administering low-dose cocaine, the effect of intra-RMTg hcr/ox on % infusions earned trended towards significance [$F(1,090, 4.359) = 3.256, p = 0.14$] (**Figure 20B-C**). Contrast analyses revealed that rats self-administered significantly greater infusions when pre-treated with 0.3 nmol hcr/ox relative to vehicle pre-treatment [$p < 0.05$]. Similarly, a main effect of hcr/ox pre-treatment was found for % active presses [$F(3, 12) = 4.653, p < 0.05$] and % breakpoint [$F(3, 12) = 4.373, p < 0.05$] but not for inactive presses [$F(1,063, 3.189) = 0.802, n.s.$]. Consistent with % infusion contrast analyses,

0.3 nmol

Table 6. Behavioral metrics per subject for intra-RMTg cannulated rats receiving hcr/ox pre-treatment prior to cocaine self-administration. Data in parentheses indicates prior-day baseline performance from which % change metrics were computed throughout analyses.

| Intra-RMTg hcr/ox on low-dose cocaine self-administration (0.375 mg/kg/inf) (n=5) | | | | | | Intra-RMTg hcr/ox on high-dose cocaine self-administration (0.750 mg/kg/inf) (n=6) | | | | | |
|---|------------------|----------------|------------------|---------------|---------|--|------------------|----------------|------------------|---------------|---------|
| Dose | Infusions Earned | Active Presses | Inactive Presses | Breakpoint | Subject | Dose | Infusions Earned | Active Presses | Inactive Presses | Breakpoint | Subject |
| Veh | 14 (13) | 426 (267) | 52 (53) | 62 (50) | C2B2 | Veh | 20 (17) | 1121 (686) | 13 (8) | 219 (118) | C3B10 |
| 0.3 nmol | 16 (12) | 550 (209) | 29 (51) | 95 (40) | | 0.3 nmol | 16 (12) | 521 (203) | 67 (3) | 95 (40) | |
| 1.0 nmol | 19 (14) | 1084 (414) | 10 (17) | 178 (62) | | 1.0 nmol | 14 (17) | 355 (597) | 25 (56) | 62 (118) | |
| 3.0 nmol | 17 (16) | 608 (550) | 51 (26) | 118 (95) | | 3.0 nmol | 15 (16) | 427 (554) | 1 (14) | 77 (95) | |
| Veh | 14 (16) | 283 (551) | 20 (24) | 62 (95) | C2B4 | Veh | 17 (15) | 604 (378) | 3 (1) | 118 (77) | C3B11 |
| 0.3 nmol | 16 (15) | 503 (402) | 78 (77) | 95 (77) | | 0.3 nmol | 19 (16) | 893 (500) | 8 (15) | 178 (95) | |
| 1.0 nmol | 16 (16) | 477 (523) | 30 (45) | 95 (95) | | 1.0 nmol | 17 (18) | 619 (861) | 40 (4) | 118 (145) | |
| 3.0 nmol | 14 (16) | 317 (539) | 26 (57) | 62 (95) | | 3.0 nmol | 19 (17) | 1084 (619) | 10 (40) | 178 (118) | |
| Veh | 7 (11) | 35 (127) | 1 (3) | 12 (32) | M1B1 | Veh | 7 (6) | 47 (24) | 7 (14) | 12 (9) | C3B12 |
| 0.3 nmol | 9 (10) | 70 (113) | 0 (1) | 20 (25) | | 0.3 nmol | 7 (6) | 38 (34) | 1 (7) | 12 (9) | |
| 1.0 nmol | 10 (11) | 113 (127) | 8 (1) | 25 (32) | | 1.0 nmol | 7 (8) | 46 (63) | 16 (32) | 12 (15) | |
| 3.0 nmol | 10 (12) | 105 (168) | 4 (10) | 25 (40) | | 3.0 nmol | 6 (8) | 24 (64) | 3 (6) | 9 (15) | |
| Veh | 18 (20) | 714 (1111) | 17 (33) | 145 (219) | M1B3 | Veh | 19 (17) | 1084 (648) | 22 (4) | 178 (118) | C3B15 |
| 0.3 nmol | 17 (18) | 625 (833) | 11 (30) | 118 (145) | | 0.3 nmol | 18 (19) | 846 (1094) | 4 (15) | 145 (178) | |
| 1.0 nmol | 14 (16) | 290 (472) | 9 (18) | 62 (95) | | 1.0 nmol | 20 (20) | 1111 (1111) | 19 (1) | 219 (219) | |
| 3.0 nmol | 10 (16) | 108 (477) | 9 (33) | 25 (95) | | 3.0 nmol | 16 (18) | 451 (714) | 12 (23) | 95 (145) | |
| Veh | 16 (16) | 558 (513) | 2 (0) | 95 (95) | M1B4 | Veh | 18 (19) | 836 (966) | 3 (1) | 145 (178) | M1B5 |
| 0.3 nmol | 14 (13) | 339 (238) | 8 (4) | 62 (50) | | 0.3 nmol | 21 (19) | 1379 (1010) | 2 (5) | 268 (178) | |
| 1.0 nmol | 18 (20) | 720 (1295) | 2 (5) | 145 (219) | | 1.0 nmol | 22 (22) | 1707 (1707) | 9 (7) | 328 (328) | |
| 3.0 nmol | 0 (18) | 0 (778) | 0 (5) | 0 (145) | | 3.0 nmol | 18 (20) | 714 (1111) | 2 (12) | 145 (219) | |
| Veh | 13.8 (15.2) | 403.2 (513.8) | 18.4 (22.6) | 75.2 (98.2) | AVG | Veh | 17 (18) | 629 (809) | 6 (4) | 118 (145) | M1B8 |
| 0.3 nmol | 14.4 (13.6) | 417.4 (359.0) | 25.2 (32.6) | 78.0 (67.4) | | 0.3 nmol | 13 (17) | 278 (699) | 34 (46) | 50 (118) | |
| 1.0 nmol | 15.4 (15.4) | 536.8 (566.2) | 11.8 (17.2) | 101.0 (100.6) | | 1.0 nmol | 8 (15) | 63 (390) | 0 (8) | 15 (77) | |
| 3.0 nmol | 10.2 (15.6) | 227.6 (502.4) | 18.0 (26.2) | 46.0 (94.0) | | 3.0 nmol | 14 (17) | 353 (632) | 1 (7) | 62 (118) | |
| Veh | 16.3 (15.3) | 720.2 (584.4) | 9.0 (5.3) | 131.7 (107.5) | AVG | 0.3 nmol | 15.7 (14.8) | 659.2 (590.0) | 19.3 (15.2) | 124.7 (103.0) | AVG |
| 0.3 nmol | 14.7 (16.7) | 650.2 (788.2) | 18.2 (18.0) | 125.7 (150.3) | | 1.0 nmol | 14.7 (16.0) | 508.8 (615.7) | 4.8 (17.0) | 94.3 (118.3) | |
| 1.0 nmol | 14.7 (16.0) | 508.8 (615.7) | 4.8 (17.0) | 94.3 (118.3) | | 3.0 nmol | 14.7 (16.0) | 508.8 (615.7) | 4.8 (17.0) | 94.3 (118.3) | |
| 3.0 nmol | 14.7 (16.0) | 508.8 (615.7) | 4.8 (17.0) | 94.3 (118.3) | | | | | | | |

significantly increased % active presses [$p < 0.05$] and % breakpoint [$p < 0.05$] relative to vehicle pre-treatment comparator data. Finally, for intra-RMTg cannulated high-dose cocaine self-administering rats, a significant main effect of hcr/ox pre-treatment was found for % infusions [$F(3, 15) = 5.499, p < 0.01$], % active presses [$F(3, 15) = 3.708, p < 0.05$], and % breakpoint [$F(3, 15) = 4.899, p < 0.05$] but not for % inactive presses [$F(1.403, 7.015) = 0.719, n.s.$]. Contrast analyses revealed that 1.0 and 3.0 nmol hcr/ox pre-treatment significantly suppressed % infusions [both $p < 0.05$], % active presses [1.0 nmol: $p < 0.05$; 3.0 nmol: $p = 0.06$], and % breakpoint [both $p < 0.05$]. A significant reduction inactive presses was found for rats pre-treated with 3.0 nmol hcr/ox [$p < 0.05$] but not 1.0 nmol. Raw behavioral metrics for intra-RMTg cannulated rats subjected to hcr/ox pre-treatment prior to cocaine self-administration are reported in **Table 6**. Interestingly, these data show that hcr/ox peptide injection in RMTg significantly influence motivated cocaine-taking, but direction of effect is based in part on self-administered cocaine dose. Insufficient data have been generated for missed cannula placements which generally tended to target dorsal to intended structure. For low-dose cocaine self-administering rats receiving intra-VTA suvorexant with confirmed “missed” cannula placement, mean % infusions ranged from 75.2 to 122.2, but only one to three data points per pre-treatment condition have been collected so far. Similarly, intra-VTA cannulated rats self-administering high-dose cocaine, means for % infusions earned relative to prior-day baseline ranged from 81.3 to 104.0 with only two data points per pre-treatment condition. One subject was a confirmed “miss” for intra-RMTg cannulated rats self-administering low-dose cocaine, and, likewise, two subjects were confirmed “misses” for intra-RMTg cannulated high-dose cocaine self-administering rats.

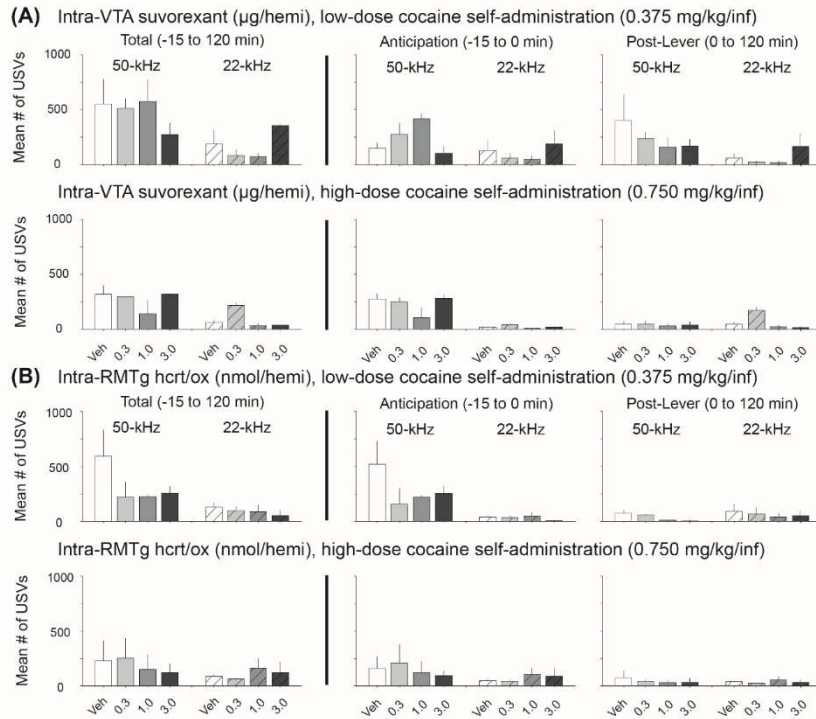


Figure 21. Effects of intra-VTA suvorexant and intra-RMTg hcr/ox on USVs associated with anticipation and self-administration of low-dose (0.375 mg/kg/inf) and high-dose (0.750 mg/kg/inf) cocaine. Data are mean \pm S.E.M. Solid bars indicate 50-kHz USVs whereas hatched bars indicate 22-kHz USVs. n=2-4/group.

confirmed rats as described for above-mentioned infusion data. However, trending effects which can be found on **Figures 21** and **22** will herein be reported. In terms of anticipatory USVs, intra-VTA suvorexant tended to enhance 50-kHz USVs at 0.3 and 1.0 μ g/hemi doses whereas this effect destabilized with high-dose (3.0 μ g/hemi) pre-treatment in rats trained to self-administer low-dose (0.375 mg/kg/inf) cocaine. No appreciable effects of either intra-VTA suvorexant or intra-RMTg hcr/ox on 22-kHz USVs across groups was observed. In the low-dose cocaine self-administering, intra-RMTg hcr/ox suppressed anticipatory 50-kHz USVs across pre-treatment doses, however no effects were observed in rats trained to self-administer high-dose (0.750

Readers should note that studies examining intra-VTA suvorexant and intra-RMTg hcr/ox on USV emissions is still being collected, and thus sample sizes (n=2-4/group) are inadequate for statistical analyses and do not reflect all histologically-

mg/kg/inf) cocaine.

Secondly, nearly all rats

(excepting intra-VTA

cannulated rats self-

administering low-dose

cocaine) emitted a

paucity of 50-kHz

USVs following time

“0” at which point self-

administered cocaine

was available. Bouts of

post-lever 22-kHz

USVs were present

across groups, notably

in intra-VTA

cannulated low-dose

cocaine self-administering rats. Collectively, these data suggest that hcr/ox transmission

in RMTg influences motivated cocaine-taking, but effects are dependent on reward

magnitude (i.e., dose of self-administered cocaine). It remains possible that histories of

cocaine self-administration influenced the extent to which RMTg cells are impacted by

hcr/ox transmission.

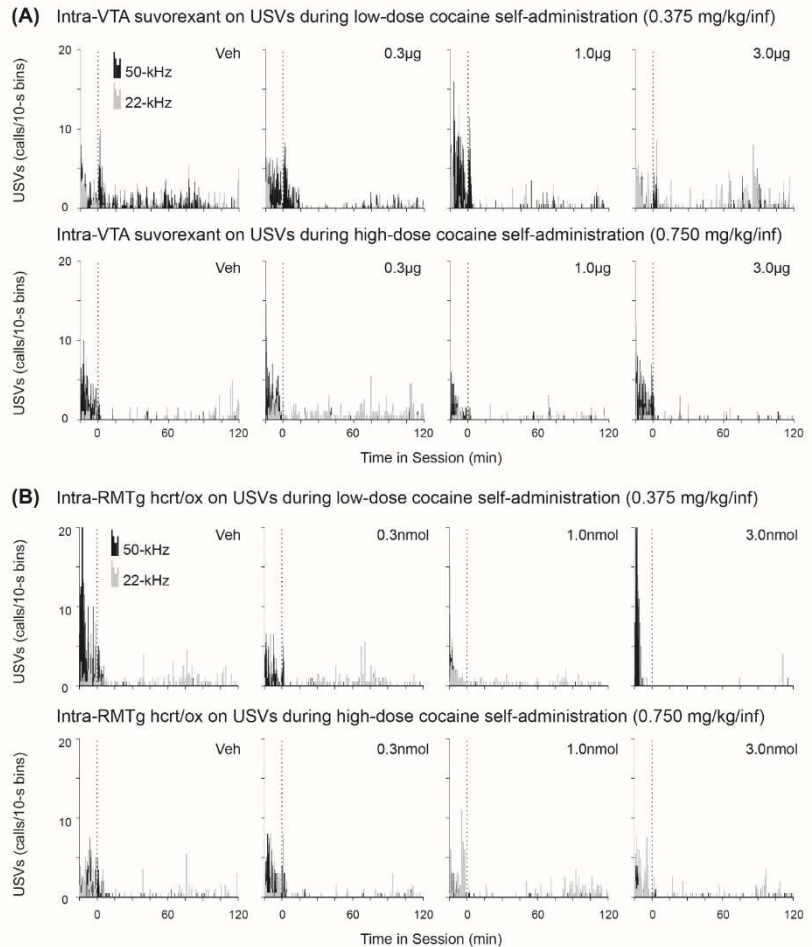


Figure 22. Effects of pharmacological pre-treatments (intra-VTA suvorexant, intra-RMTg hcr/ox) on profile of 22- and 50-kHz USVs across cocaine self-administration sessions.

8.3) *Interim discussion*

In target-site pharmacology experiments, we failed to observe appreciable effects of intra-VTA hcrt/ox receptor blockade (via suvorexant) on motivated cocaine-taking. Previous work finds that OX1R blockade with VTA-directed SB334867 significantly reduces the number of cocaine infusions earned under progressive-ratio access conditions (Muschamp et al. 2014). Systemic injection of almorexant or suvorexant dose-dependently decreases motivated cocaine-taking (Prince et al. 2015; Gentile et al. 2018). *In situ* hybridization data supports that both OX₁R and OX₂R exist in comparable densities within VTA (Marcus et al. 2001), but we lack appreciation of which cell populations hcrt/ox receptors are produced within. As intra-VTA suvorexant had negligible effects on cocaine self-administration, we suspect that OX₂Rs may be on GABA-producing interneuron populations which may negate motivation-suppressing effects normally observed following OX₁R blockade. Interestingly, hcrt/ox peptide injection within RMTg produced mixed effects on motivated cocaine-taking dependent on self-administered cocaine dose (i.e., 0.375 vs 0.750 mg/kg/inf). This preliminary data supports that hcrt/ox functionally transmits to RMTg, however we lack an understanding of how hcrt/ox influences physiology of cell populations within RMTg. Interestingly, prior work shows that putative GABA-producing cells within RMTg are insensitive to KOR stimulation (Matsui and Williams 2011) whereas a DA-producing VTA cell sub-population possesses robust sensitivity to KOR stimulation (determined via bath application of dynorphin—the endogenous ligand of KORs) (Muschamp et al. 2014; Baimel et al. 2017). Unique sensitivity of neurochemically-defined midbrain cells to KOR and hcrt/ox receptor stimulation suggests selective influence of hcrt/ox versus

dynorphin—peptides that are vastly co-localized (Chou et al. 2001; Muschamp et al. 2014) and are suspected to co-release. The balance between hcr/ox and dynorphin transmission in midbrain warrants further attention, and preferential sensitivity of VTA and RMTg to these peptides may provide a second layer through which hcr/ox-producing cells bivalently regulates motivated reward-seeking.

DISCUSSION

Psychiatric disorders characterized by aberrantly motivated states including those seen with substance use disorders can significantly deteriorate lives of the afflicted as well as whole families and communities. Medications indicated to manage substance use disorders prove effective by suppressing drug-associated reward and/or by reducing the intensity of withdrawal-related negative affective symptoms. For psychostimulants including cocaine, amphetamine and novel synthetic psychostimulants, no FDA-approved medications are available to manage their abuse. An underlying theme throughout work in this thesis is to better understand the transmitters and circuits that support subjective experiences of positive and negative affect—in the context of addiction, these “drives” propagate binge-like drug consumption and facilitate the escalation to a state of dependence. Seminal work focused on reward neurobiology promotes studying the ventral midbrain—notably, the DA-producing afferent population residing in VTA—as a key locus in reward processing.

As stated in [Chapter 4](#), the ventral midbrain is richly heterogeneous and possesses unique, adjacently-lying structures that seem to function in opposition. Groups have demonstrated that the RMTg—resting caudally in midbrain, dorsolateral to IPN, populated by GABA-producing cells—negatively regulates the DA-producing cells of rostrally-positioned VTA. These two structures, then, may work to provide net output that encodes a stimulus as rewarding or aversive. It was tantalizing to consider a “master controller” that innervates these structures to regulate affective valence and drive motivated behavioral states. We placed our attention on a hypothalamic neuropeptide—hcr/ox—which has a ~12-year history of being implicated pre-clinically in motivated

states associated with consumption and seeking of drugs. Interestingly, a single nucleotide polymorphism in the *hcrtr1* gene is associated with greater risk of developing either major depressive disorder or bipolar disorder, suggesting a possible of function of *hcrtr/ox* transmission in the regulation of negative affective states (Rainero et al. 2011). *Hcrtr/ox* regulates an array of behaviors concerning homeostasis and vigilance, and its discovery positioned *hcrtr/ox* as orexigenic and physiologically excitatory. Within a year, teams showed critical involvement of *hcrtr/ox* as a sleep regulator noting circadian-linked fluctuations in systemic peptide content. Of note, the FDA approved the first-in-class *hcrtr/ox* receptor antagonist—suvorexant—for the management of primary insomnia in late 2014.

Studying clinically-available pharmacotherapeutic agents affords rapid translational power, and thus in preliminary assessment our team explored the efficacy of suvorexant in normalizing motivated drug-taking. We additionally phenotyped affective responses in rats trained to self-administer cocaine and the synthetic psychostimulant MDPV. While not as widely studied, MDPV mechanistically inhibits clearance of DA via blockade of DATs with approximately ten-fold greater potency compared to cocaine (Baumann et al. 2013; Kolanos et al. 2013). MDPV is readily self-administered by rodents and acutely facilitates brain reward function (Watterson et al. 2014). Our work showed that MDPV, like cocaine, elicits predominantly positive affective 50-kHz USVs. Further, we provided additional evidence that the “positive affective” 50-kHz USV response observed early in sessions corresponds with the transition from sobriety to intoxication, but that 50-kHz USVs diminish to near-zero rates thereafter (Barker et al. 2014; Simmons et al. 2018). In a follow-up study, we determined that systemic

suvorexant could attenuate USVs associated with anticipation and self-administration of MDPV. Although, as mentioned below, no effects of suvorexant were observed on the number of MDPV infusions earned (Simmons et al. 2017). Together, these lines of work promoted further interrogation of possible circuits whereby hcr/ox could regulate affective states and, in doing so, pathological motivation.

Suvorexant decreases rewarding and reinforcing properties of psychostimulants

Consistent with prior studies demonstrating therapeutic value of hcr/ox receptor antagonists to normalize pathological motivation (e.g., Borgland et al. 2009; Smith et al. 2009), we observed that systemic suvorexant decreases infusions earned when rats are taxed under high-effort access conditions for intravenous cocaine. From our study, it appeared that a means through which hcr/ox receptor blockade decreased motivated cocaine-taking was through attenuating cocaine-associated positive affect commensurate with suppression of mesolimbic DA transmission. Prior studies observed that hcr/ox innervates VTA and modulates cocaine-elicited DA efflux in NAcc targets (Fadel and Deutch 2002; España et al. 2011; Bernstein et al. 2017). Additionally, the production of 50-kHz USVs is supported by mesolimbic DA transmission as DA receptor antagonists suppress psychostimulant-elicited 50-kHz USVs, and electrical/optical excitation of DA release in NAcc robustly, yet transiently, elicits 50-kHz USVs (Ciucci et al. 2009; Williams and Undieh 2010; Scardocho and Clarke 2013; Scardocho et al. 2015). Thus, the ability of suvorexant to reduce cocaine-elicited 50-kHz USVs aligns with its ability to suppress cocaine-stimulated NAcc DA release (Gentile et al. 2018). However, consistent with prior work, we failed to find significant effects of systemic hcr/ox receptor blockade on low-effort (FR-1) psychostimulant self-administration (Simmons et al.

2017). These results collectively support that hcrt/ox is selectively recruited for motivated action directed on seeking/consuming positive reinforcers, and that hcrt/ox influence on VTA DA transmission supports reward-seeking.

When measuring motivated behaviors, blockade of hcrt/ox receptors has been shown to decrease reproductive behaviors in male rats as well as high-effort responding for “natural” rewards typically associated with an obesogenic diet (i.e. high in fats and sugar) and for drugs of abuse (Muschamp et al. 2007; Borgland et al. 2009). Indeed, we observed that suvorexant (30 mg/kg, IP) pre-treatment significantly decreased the effort expended for intravenous cocaine (Gentile et al. 2018). There is clear evidence that suvorexant can induce somnolence as its indication is for managing primary insomnia (Winrow et al. 2011). However, in our test, we observed that systemic cocaine injection elicited comparable elevations in ambulation irrespective of pre-treatment (i.e., suvorexant vs. vehicle). Moreover, in a separate assessment, we observed significant effects of suvorexant on cocaine-elicited elevations in premature responding in the 5-CSRTT, but no apparent differences were found for “control” measures including trial accuracy and latency to retrieve sugar reward (Gentile et al. 2017b). Finally, in a series of unpublished observations, we observed that suvorexant does, indeed, promote sleep when rats are placed in e.g., a place conditioning or homecage test setting. However, we consistently observe an absence of somnolence when rats are placed in settings demanding behavioral performance (i.e., operant responses) such as in 5-CSRTT or during intravenous drug self-administration. We interpret from these observations that sleep-promoting effects of suvorexant will manifest when subjects are returned to an uninteresting environment where interactions (with conspecifics and environment) are

limited or altogether absent. Indeed, suvorexant in humans is suggested to be taken before bed. It does not appear that suvorexant significantly impacted the ability for rats to perform the self-administration task, and so we interpret suvorexant-mediated reductions in cocaine-taking as an effect on the animal's motivated state.

An evaluation of 50-kHz ultrasonic vocalizations as representing a “positively-valenced” subjective state

The observed results that suvorexant suppresses cocaine-elicited 50-kHz USVs (Gentile et al. 2018) and 50-kHz USVs occurring prior to and during MDPV self-administration (Simmons et al. 2018) is captivating based on the interpretation that this behavioral signature indexes a shift in positive affect. While drugs of abuse including psychostimulants generally produce 50-kHz USVs above baseline rates (Ahrens et al. 2009; Ma et al. 2010), other work finds that 50-kHz USVs can be produced in general states associated with behavioral activation/arousal. For example, in addition to unpublished observations made by our team, Taylor and colleagues (2017) reported that a strong yet transient bout of 50-kHz USVs are elicited following an electrical footshock (~0.5 mA) which is generally agreed upon to be an aversive stimulus. After repeated presentations, the pattern of USVs invariably shifts to production of “negative affective” 22-kHz USVs, and thus novelty associated with initial footshock experience is suspected as the underlying factor driving 50-kHz USV emission. Here, it should be noted that the authors further define the acoustic features of shock-elicited 50-kHz USVs as belonging to a “constant frequency” category in contrast to “frequency-modulated”/“trill-like” 50-kHz USVs more commonly observed following acute or repeated psychostimulant injections (Ahrens et al. 2009; Wright et al. 2010). As rats throughout our studies were

familiar with their experimental test chambers, we do not believe that observed USV emissions were influenced by stimulus novelty. We additionally found that systemic suvorexant produced sporadic bouts of 22-kHz USVs which are generally tightly linked to experimental conditions associated with affective distress, fear and/or alarm.

Moreover, we conducted a cursory analysis on the proportion of 50-kHz USV call types (divided based on number of frequency modulations [i.e., ± 3 -kHz contiguous “sweep”, “jump” or “step”]) during MDPV self-administration and observed, consistent with earlier work in cocaine-injected rats, many calls possessed 1 or more frequency modulation—these call type profiles were largely unaffected by suvorexant (**Figure 12B**).

Functional connectivity: hypocretin/orexin afferents to rostrocaudal extent of ventral midbrain

To help further elucidate hcrt/ox-mediated circuits that participate in positive and negative affective states, we interrogated anatomically distinct ventral midbrain structures that are suspected to act in functional opposition. We sought to define for the first time the topographical organization of hcrt/ox afferents to VTA and RMTg to evaluate potential loci of hcrt/ox-mediated affective control. We observed comparable innervation densities across the two adjacent structures and explored functional differences of hcrt/ox transmission to each of VTA and RMTg. Specifically, we tested the hypothesis that hcrt/ox transmission in VTA positively mediates motivated cocaine-taking and participates in the encoding of rewarding experiences whereas hcrt/ox in RMTg brakes this transmission sequence to bias stimulus processing as aversively-valenced.

Geisler and Zahm (2005) describe the VTA as “a neuronal continuum with overlapping dendritic fields” as is evident from inputs arising from many anatomically diffuse sources (see also description of “isodendritic core” as articulated by Ramón-Moliner and Nauta 1966). Since 2009, several teams have recognized the caudal division of VTA as a unique structure based on cellular architecture and neurochemical composition and thus termed it based on anatomical location—the RMTg residing caudally/dorsolaterally to DA-producing midline VTA cells and caudally/dorsomedially to DA-producing SN cells. In fact, Lahti and colleagues (2016) ascribed the nuclear transcription factor FoxP1 as a relatively selective marker for RMTg which was later found to overlap selectively to psychostimulant-evoked Δ FosB. Inputs to VTA and RMTg of the ventral midbrain are of significance for their roles in encoding stimuli as rewarding or aversive. A comprehensive tracing study from hcr/ox afferents has not been conducted beforehand and thus was the aim of current investigation.

In 2009, Kaufling and colleagues (2009) published a report detailing inputs to RMTg using two retrograde tracer types (FluroGold [FG] and cholera toxin) deposited iontophoretically. Semiquantitative analyses revealed relatively strong input to RMTg from medial prefrontal cortex (mPFC), zona incerta, Lhb, LPO, hypothalamus, inferior division of superior colliculus, dorsal raphe nucleus (DRN), PAG, parabrachial nucleus, and laterodorsal tegmental nucleus. As described in [Section 4.2](#), cocaine injection was used to elicit Δ FosB within RMTg, and immunohistochemical confirmation of Δ FosB/GAD co-labeling was made whereas no cells were Δ FosB⁺ were co-labeled TH. Anterograde tracing using biotinylated dextran amines supported that fibers extending from mPFC, Lhb, LPO and hypothalamus appose cocaine-elicited Δ FosB⁺ nuclei within

RMTg whereas no such appositions were found along the amygdala→RMTg pathway. Figures 15 and 16 of this report clarify that virtually none of the FG⁺ cells traced from DRN contained the immunolabel against tryptophan hydroxylase—the rate-limiting enzyme used in serotonin biosynthesis—which otherwise predominates within DRN. Likewise, a small population (1.5%) of hcr/ox-immunoreactive cells within hypothalamus co-labeled with FG. This afferent proportion is lower than estimates derived from our retrograde tracing (6.7%) which may be due to differences in tracer type versus extent and rigor of tissue studied. The “retrobeads” used in the present study are latex microspheres conjugated to a fluorophore which are passively endocytosed by nearby terminals (e.g., Katz et al. 1984) similarly to FG whereas other work finds that the beta-subunit of cholera toxin may be actively transported and capable of trans-synaptic movement following lysosome-like vacuole packaging (e.g., van der Want et al. 1997). Additionally, our study was directed around comparing innervation between hcr/ox afferents to VTA and to RMTg whereas no such comparison work was performed by Kaufling and colleagues (2009). It should also be mentioned that continuous refinement on ventral midbrain structural heterogeneity continues to take place. For example, Petzel and colleagues (2017) provide elegant neuroanatomical tracing work revealing unique input to anterior and posterior divisions of VTA as well as to RMTg (furthest posterior) from LHb. Specifically, only the lateral division of LHb tends to innervate RMTg whereas mixed inputs across LHb target the posterior division of VTA. Our study assumed a relatively homogenous stretch of nuclei residing in the caudal division of ventral midbrain—future work utilizing anterograde tracing from the hcr/ox population across ventral midbrain will further elucidate relative innervation densities.

While no raw numbers are reported, Richardson and Aston-Jones (2012) show that VTA-projecting hcr/ox-immunoreactive cells are activated (interpreted by Fos immunoreactivity) upon exposure to a morphine-paired context during a place conditioning test session. Moreover, hcr/ox cells arising from the lateral hypothalamic division were most strongly activated following place conditioning testing, and a % Fos⁺ metric significantly correlated with the behavioral measure of preference score for the morphine-paired context. However, no report to-date has utilized a similar projection-specific Fos labeling approach in the context of psychostimulant addiction. Nonetheless, we suspected that putative hcr/ox afferents to ventral midbrain (including to VTA) are functionally recruited for reward processing psychostimulant drugs of abuse. From our tract-tracing data, we additionally suspected that innervation to the caudal division of ventral midbrain (RMTg) may promote a subjective state of opposing affective valence.

Functionally, we tested how hcr/ox transmission perturbation within VTA and RMTg influences the rate of 22- and 50-kHz USV emission as an analog measure of positive and negative affect. We additionally tested the extent to which ventral midbrain hcr/ox transmission perturbation impacts motivated cocaine-taking in rats trained to self-administer a low-dose (0.375 mg/kg/inf) or a relatively higher-dose (0.750 mg/kg/inf) of cocaine as prior work showed that OX1R blockade selectively impacts self-administration behavior at relatively higher doses only (España et al. 2010). USV data from intra-VTA suvorexant and intra-RMTg hcr/ox produced mixed, largely inconclusive results from data collected thus far. It should be noted that, to our knowledge, this study is the first to measure USVs during high-effort drug self-administration (i.e. progressive-ratio) test. Results from our earlier work (Simmons et al.

2017, 2018) show a robust presence of anticipatory 50-kHz USVs which diminishes to near-zero levels over the course of ~30 mins. These studies additionally captured a strong bout of 50-kHz USVs upon and following lever extension during which intravenous drug injections became available. The majority of rats evaluated in the current study, except intra-VTA cannulated rats self-administering low-dose (0.375 mg/kg/inf) cocaine, failed to emit appreciable bouts of 50-kHz USVs after lever extension. Interestingly, too, a comparable “low-dose” of self-administered cocaine (0.355 mg/kg/inf) was shown to lead to predominantly 22-kHz USVs under low-effort, FR-1 access conditions (Barker et al. 2010). At present, we attribute the lack of a “positive affective” response observed in present work to the employment of a high-effort, less predictable reward contingency task.

Technical considerations: retrograde tracing and recording of ultrasonic vocalizations

Newer technologies allowing monosynaptic retrograde tracing from neurochemically-defined cell populations (e.g., those that produce distinct neurotransmitters) enable improved understanding of how the VTA receives and conveys information. To date, we have relied in large part on conventional tracing methods that possess relatively promiscuous retrograde transport activity. Direct inputs to cells that produce DA, GABA and glutamate across ventral midbrain will address whether unique or overlapping cell populations (e.g., from hcrt/ox afferents) target the ventral midbrain. Ongoing efforts from our laboratory are attempting to deposit cholera toxin retrograde tracers conjugated to unique fluorophores to each of VTA and RMTg from single subjects.

It should be noted, here, that a majority of detected 22-kHz USVs from the experiment described in Chapter 8 were spectrographically “quiet” and suffered from poor signal:noise quality (1.05-1.10), and thus their presence should be interpreted cautiously. One possibility is that “quiet” 22-kHz USVs are organic signals from nearby animals and not the ones within the chamber being recorded from—all of our recordings were contained within wooden, sound-attenuating chambers but microphone sensitivities remain an untested contributing factor. Indeed, all detections carried a mean, monotonic frequency \sim 22-kHz and did not appear influenced from clearly-discernible artifact noises (e.g., spring leash tether contacting Plexiglas) which promoted their inclusion. Coupling the facts that 50-kHz USVs recorded from the same microphones possessed excellent signal:noise (1.20-1.30) and that the same microphones were able to capture shock-elicited USVs in a separate study without detection difficulty, we do not suspect the microphones suffer from an inability to detect 22-kHz USVs. Instead, it remains possible that 22-kHz USVs elicited under experimental conditions described in Chapter 8 are, in fact, quiet, monotonic and of varied lengths unlike acoustic characteristics of 22-kHz USVs described in earlier studies (e.g., Barker et al. 2010). Microphones with improved amplifier units, such as the CM16/CMPA model manufactured by Avisoft Bioacoustics, can likely detect these possible 22-kHz USVs with greater accuracy but are prohibitive for purchase by young laboratories carrying a cost \sim 13.7x greater than USB plug-and-play microphones (Dodotronic) used by our group. Recordings using improved microphones should be conducted to confirm the existence of 22-kHz USVs described in Chapter 8 as the functional implications from their emission are of great value.

Distribution of OX₁Rs and OX₂Rs across neurochemically-defined cell populations of ventral midbrain

An additional finding from intra-VTA suvorexant deposit was its tendency to enhance rates of anticipatory 50-kHz USVs—an effect that destabilizes with high-dose (3.0 µg/hemisphere) suvorexant pre-treatment—in low-dose cocaine self-administering rats suggesting hcrt/ox receptor blockade may augment positive anticipation for self-administered cocaine. Intra-VTA hcrt/ox receptor blockade studies thus far have utilized selective OX₁R antagonists and have invariably demonstrated that OX₁Rs positively mediate drug-taking and -seeking. Systemic blockade of OX₁Rs and OX₂Rs suppresses motivated responding in cocaine self-administering rats, and it appears too that dual hcrt/ox receptor blockade decreases positive affective to systemically-injected cocaine (Gentile et al. 2018). It remains possible that, while OX₁Rs may predominantly localize on VTA DA cells, OX₂Rs may more densely populate on VTA GABA interneurons that function to inhibit the VTA DA population. Pharmacological agents, including suvorexant, that antagonize both hcrt/ox receptor subtypes may elicit opposing effects on VTA DA cellular physiology and transmission to targets including NAcc. Under the simplistic view that net DA transmission in this manner effectively encodes reward signals and its suppression participates in encoding aversive stimuli, a mixed affective response from intra-VTA suvorexant could be generated. Studies utilizing selective blockade of hcrt/ox receptor subtypes on neurochemically-defined VTA populations could test the hypothesis blockade of VTA OX₂Rs disinhibits VTA DA to facilitate reward encoding.

Curiously, we observed that low-dose hcrt/ox (0.3 nmol/hemi) enhanced responding for low-dose self-administered cocaine whereas higher doses of intra-RMTg injected hcrt/ox (1.0 and 3.0 nmol/hemi) significantly decreased motivated cocaine-taking in high-dose cocaine self-administering rats. Reward-attenuating effects of intra-RMTg hcrt/ox (i.e. reducing motivated cocaine-taking) align with the idea that hcrt/ox functionally brakes cocaine-associated reward and reinforcement likely through augmenting activity of GABA-producing RMTg neurons that project and inhibit DA-producing neurons of VTA. In preliminary assessment, we failed to see any clear effect of intra-RMTg hcrt/ox on USVs during cocaine self-administration, so it is unclear as to whether RMTg hcrt/ox participates in the affective/emotional node underlying pathological motivation. It would be of great value to delineate the ventral midbrain cellular populations through which hcrt/ox transmits to—this knowledge is lacking at the moment.

In situ hybridization studies confirm that both OX₁Rs and OX₂Rs can be found within ventral midbrain, but to-date we do not know which neurochemically-defined cell populations hcrt/ox receptors are produced within. To help address this, we piloted a combined *in situ* hybridization (RNAscope) with immunohistochemistry study to visualize TH⁺, VGaT⁺, and VGluT2⁺ cells within ventral midbrain labeled along with probes targeting either hcrt-r1 or hcrt-r2 mRNA. However, poor signal:noise and non-specific labeling on negative-control tissue sections ended these experiments prematurely. It remains possible that OX₂Rs are densely populated on GABA-producing interneurons within VTA whereas OX₁Rs cluster on DA-producing cellular populations. If this is the case, blockade of both hcrt/ox receptor subtypes would produce a mixed

physiological effect within the structure which may explain a lack of intra-VTA suvorexant. While unsubstantiated at the moment, mixed effects of intra-RMTg hcr/ox pre-treatment on cocaine-taking between low- and high-doses of self-administered cocaine suggest that cocaine may influence the RMTg cellular population in a dose-dependent manner—specifically, our results support that low doses of self-administered cocaine bias the RMTg population to favor reward-seeking upon transmission of hcr/ox, whereas a bias towards promoting aversion would be produced following high doses of self-administered cocaine.

Beyond ventral midbrain: alternative targets of hypocretin/orexin in the mediation of psychostimulant-associated reward and reinforcement

While we have placed our attention solely on hcr/ox innervation within midbrain, effects of transmission to forebrain structures including to NAcc should not be discounted. Recent study finds that hcr/ox signals to D₂-containing NAcc cells to functionally contribute to innate risk avoidance (Blomeley et al. 2018) despite anatomical work showing relatively scant hcr/ox fiber density within striatum (e.g., Peyron et al. 1998). In this report, chemogenetic silencing of D₂-containing NAcc cells as well as systemic OX₁R blockade increased time spent in the center of an open field arena and decreased reward-seeking latency across a chamber paired with predatory odor. Notably, co-dependency of the hcr/ox→D₂(NAcc) circuit was interpreted from hcr/ox-driven risk avoidance requiring an intact D₂-containing NAcc cell population. Monosynaptic tracing using truncated G-segment Rabies virus confirmed ~25% of hcr/ox cells directly target D₂-containing NAcc cells whereas a relatively scarce proportion (~5%) of hypothalamic outputs contained MCH. Physiologically, photostimulation of hcr/ox terminals within

NAcc activated D₂-containing NAcc cells *ex vivo* which was abolished with bath application of hcrt/ox receptor blocking agents. This study reveals a pivotal role of hcrt/ox in risk avoidance by signaling directly to ventral striatal targets. Furthermore, neuropeptide proteomic analysis indicates that a functional role of hcrt/ox transmission to LHb is likely in large part via OX-B which has relatively greater affinity for OX₂Rs (Yang et al. 2018). While we speculate that the function of hcrt/ox in reward processing, including for abused drugs, relays in large part through midbrain structures, possible effects of direct hcrt/ox transmission to ventral striatum on reward-related behaviors should be considered. However, a technical limitation of the above-mentioned research is the use of a “selective” hcrt/ox cre mouse line—hcrt/ox mRNA is transiently expressed across many cells during development which leads in turn to translation of cre in cells that ultimately fail to produce hcrt/ox. Genetic tools for selective targeting of hcrt/ox cells at present carry considerable limitations—namely, poor and/or non-selective transfection.

Many nuclei within limbic and forebrain structures receive functionally-relevant transmission from hcrt/ox. In this thesis, we explored the idea that hcrt/ox may innervate VTA and RMTg to functionally participate in the production of opposing affective states upon stimulus presentation/receipt in the context of psychostimulant addiction. We provided evidence that, indeed, comparable innervation densities of hcrt/ox to each of VTA and RMTg can be observed, although functions remain uncertain. It appears that blockade of both OX₁Rs and OX₂Rs in the VTA produces no discernible effect on cocaine-taking, although some interesting dose-related effects on USVs are emerging. Intra-RMTg hcrt/ox transmission appears to be recruited in a relatively more significant manner to influence motivated cocaine-taking, although alterations in affective state at

the moment do not appear as underlying contributors. Explicit examination of hcr/ox receptor subtype expression within neurochemically-defined cellular populations of ventral midbrain will add important clarity to the above-mentioned results. Further, the effects of drug-taking history on hcr/ox receptor distribution on midbrain cellular populations should be assessed. Armed with this knowledge, more effective and individualized targeting strategies can be developed which could collectively promote pharmacotherapeutic intervention to better manage psychostimulant abuse.

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APPENDIX A

METHODOLOGICAL DETAILS ADAPTED FROM GENTILE ET AL. 2018.

SI.1) Locomotor activity

Rats were placed in ambulation chambers for a 30 min acclimation period prior to pre-treatment with suvorexant (30 mg/kg, IP) or vehicle at which point ambulation was recorded for an additional 30 mins. Thereafter, systemic cocaine (10 mg/kg, IP) or vehicle was injected, and ambulation was measured for 120 min. Thus, ambulation was measured across 180 min. Photodetectors, spanning width of behavioral chambers, and corresponding breaks were used to measure ambulation via Digiscan DMicro automated software program (Accuscan, Inc.; Columbus, OH, USA) from a nearby PC.

SI.2) Place conditioning

An apparatus consisting of two distinct contexts (via visual and tactile cues) with partition ability was used. Rats used for place conditioning were initially subjected to a 30-min pre-test whereby times spent on each of two contexts, freely accessible by removal of the partitioning door, were measured. The context that rats spent greater time on during the pre-test was designated as the “preferred” context and will hereafter be referred to as Context B. Following the pre-test, a series of counterbalanced 30-min daily conditioning trials ensued across 8 days (4 days/context) at which point rats were confined to one of two Contexts. For all rats and for all conditioning trials, a 30-min pretreatment preceded an acute injection after which rats were immediately placed in the assigned context. For all rats, Context B trials consisted of a vehicle (DMSO) pretreatment (-30 min) followed by an acute saline injection and placement into Context B. A forced-choice, biased design was used in which Context A was always the less

preferred context relative to times measured during the pre-test. Context A treatment assignments proceeded as follows: Veh-Sal, Veh-Coc, Suvo-Sal, Suvo-Coc. Following 8 conditioning trials, a 30-min post-test was conducted where rats could freely shuttle between Contexts A and B. Time spent on each side was measured once again.

SI.3) Intravenous drug self-administration: jugular vein catheterization, apparatus and behavioral procedures

Rats were maintained under isoflurane anesthesia, and ketoprofen (5 mg/kg, SC) was administered for pre-operative analgesia. The mid-scapular region of dorsal surface as well as neck region of ventral surface of rats were shaved and cleaned with betadine and alcohol. A midline incision was performed on the dorsal surface, and connective tissues were cleared to allow for placement of mesh back-mount containing 22-gauge stainless steel tubing with 90° elbow joint. A small incision was performed on ventral surface of rat, and the right jugular vein was thereafter isolated and secured. A tapered polyurethane catheter line which connected to back-mount tubing extending parallel to rat, secured with super glue, was fed using a hemostat to ventral incision site after pathway clearance with spatula. A sterile 22-gauge needle was used to make entry point within the lifted jugular vein. The catheter extended ~1-in into jugular vein, and biocompatible suture thread secured the catheter permanently within jugular vein. 0.2 mL of heparin- and antibiotic-containing saline was pushed through, and a gentle pull-back was performed to verify blood uptake. Dorsal and ventral incision sites were secured with 9-mm stainless steel wound clips, and wounds were first wiped with alcohol and then with topical antibiotic containing bacitracin zinc, neomycin sulfate and polymixin b sulfate. Each rat was supplemented with 2 mL of saline and was returned to vivarium

upon normal ambulation. After surgery, catheter lines were flushed with 100 μ L of filtered 0.9% saline containing heparin (100 USP/mL) and enrofloxacin (0.23 mg/mL) 5-7 d/wk prior to acquisition of drug self-administration.

During training and test sessions, rats were flushed prior to and following 2-h self-administration sessions. For the first 10 d, rats were trained to respond for intravenous cocaine (\sim 0.36 mg/kg/inf) under fixed-ratio 1 (FR-1) access conditions. After an infusion, a 20-s timeout period commenced at which point responses were not reinforced. Rats could respond for a maximum of 60 infusions during 2-h sessions. Following at least 3 consecutive sessions with \geq 20 infusions, rats were shifted to 4-h progressive-ratio (PR) sessions at which point response requirements for infusions progressively increased (e.g., 1, 1, 2, 4, 6, 9, 12 and 15 operant responses required) to assess motivational drive exerted by rats to maintain a cocaine “high” within each session. After rats exhibited consistent response behavior (\pm 1 infusion across \geq 3 consecutive sessions), a 3-d repeating suvorexant pre-treatment regimen (in order: FR-1, PR without pre-treatment, PR with pre-treatment) was utilized to evaluate effects of suvorexant (0, 3, 10, 30 mg/kg, IP) on motivated cocaine-taking using a within-subjects design (**Figure 6a**).

SI.4) Ultrasonic vocalization recording and analysis

To record USVs, condenser microphones (UltraMic200K [Dodotronic; Italy]) were affixed on top of Plexiglas behavioral chambers. In all recording instances, acoustic transmission was tested to ensure detection of USVs with minimal background interference. Microphones were connected to nearby recording PCs via mini USB cable. Audio was sampled at 192-kHz (to enable detection up to 96-kHz in frequency) and was

saved in 10-min “.wav” files for offline scoring and analysis. Spectrographic analysis proceeded offline by trained experimenters using RavenPro software (Cornell Laboratory of Ornithology; Ithaca, NY, USA) who manually inspected for putative USV detection. USVs were confirmed on the basis of appearance, and when necessary, playback proceeded at 1/20th recording speed for characteristic “whistle like” sound. USVs were categorized grossly by frequency of emission—USVs between 18- and 30-kHz in mean frequency (average of lower and upper frequency bounds of each detection) and greater than 15 ms in duration were classified as “22-kHz USVs” whereas USVs greater than 38-kHz in mean frequency and greater than 15 ms in duration were classified as “50-kHz USVs”. For some experimental subjects, USVs matching 50-kHz USV classification criteria were collected using a MATLAB-based automated detection program as described elsewhere (Barker et al. 2014b). Minimum correlation to template library was established at 0.30. Briefly, all calls detected using automated software were visually inspected by a trained experimenter, and artifact/non-organic detections were discarded prior to analysis.

SI.5) Fast-scan cyclic voltammetry

Mice were fully anesthetized with isoflurane gas (5% induction, 2-3% maintenance) mixed with oxygen and implanted with a carbon fiber microelectrode within ventral striatum (A/P +1.3; M/L +1.3; D/V -4.5). A Ag/AgCl reference electrode was lowered into cortical tissue of the contralateral hemisphere relative to the carbon fiber recording electrode. Finally, a bipolar stimulating electrode was gradually lowered within the DA-producing population of VTA (A/P -3.0; M/L +1.1; D/V -4.0) through tissue of the hemisphere ipsilateral to the carbon fiber recording electrode. The

stimulating electrode was lowered until a 60-Hz monophasic pulse (4 ms, 250 uA) elicited an appreciable DA response from the recording electrode embedded within the ventral striatum. A stable DA response (within 10% across 3 consecutive recordings, spaced 5-min apart) was required before proceeding to experimental treatments. Thereafter, mice were injected with either suvorexant (30 mg/kg, IP) or vehicle as pretreatment (-30 min) followed a second injection of cocaine (10 mg/kg, IP) after which ventral striatal DA efflux was recorded for an additional 60 min. Recording electrode potential was compared against reference via 0.8 V sweeps (-0.4 to 1.2 V) and cyclic voltammograms were generated every 100 ms via a 400 V/s scanning voltammeter/amperometer (Chem-Clamp, Dagan Corporation; Minneapolis, MN, USA). Magnitude and overflow of DA release were measured in part using Labview-based recording software (Demon Voltammetry and Analysis, National Instruments; Austin, TX, USA; see Yorgason et al. 2011).

SI.6) Metrics and statistics

For self-administration, the number of cocaine infusions earned and correct responses performed are expressed as a percentage relative to prior-day baseline data. One-way repeated measures ANOVA tested the effect of suvorexant dose (0, 3, 10, 30 mg/kg, IP) on aforementioned metrics. Bonferroni-corrected contrast analyses proceeded by comparing each dose of suvorexant pre-treatment (3, 10, 30 mg/kg, IP) against the effect of vehicle pre-treatment on cocaine infusions earned and correct responses performed. CPP data are expressed as “% Baseline” relative to pre-test time measured in Context B. One-way between-subjects ANOVA proceeded to test the effect of treatment group (Veh-Sal, Suvo-Sal, Veh-Coc, Suvo-Coc) on place preference, and Bonferroni-

corrected contrasts compared each of three groups to the Veh-Sal control group. USV data are expressed as normalized change-scores (Δ USV Score) which compares the number of 50-kHz USVs detected following the first injection of cocaine against USVs detected following the first injection of saline (i.e. $[B - A] / [A + B]$). To examine changes across the 30-min recording session, a two-way mixed ANOVA examining treatment group by time proceeded. For between-groups analysis, independent samples t-tests were performed testing each mean against “0”—the point of no-change when comparing cocaine- to saline-elicited USVs. Activity counts for the locomotor activity test were analyzed using one-way ANOVAs for pre-cocaine (-30 to 0 min) and post-cocaine (0 to 120 min) phases. Tukey’s HSD post-hoc tests were used to perform pairwise comparisons within each 5-min time interval during the post-cocaine phase, and Bonferroni-corrected t-tests were performed for pairwise comparisons during the pre-cocaine phase. Finally, ventral striatal DA efflux was expressed as a percentage of baseline comparing to extracellular DA content recorded following VTA stimulation. Two-way ANOVAs proceeded to compare DA efflux by treatment group and across time. For all ANOVAs and pairwise comparisons, significance was established if Type I error rate (α) was at or lower than 0.05.

APPENDIX B

METHODOLOGICAL DETAILS ADAPTED FROM SIMMONS ET AL. 2018.

S2.1) Experimental procedures

We initially assessed USVs following systemic injection of either cocaine (5, 10, 30 mg/kg, IP), MDPV (0.5, 1.0, 3.0 mg/kg, IP) or following a saline control injection. Rats were injected with one drug type/dose across 7 daily sessions. Following injection, rats were placed into Plexiglas chambers for 30 min. USVs were recorded following the last injection (day 7) for 30 min. For rats self-administering either cocaine or MDPV, USV recordings were triggered immediately as rats were placed into behavioral chambers, but initiation of the self-administration procedure commenced 5 min after placement into behavioral chambers. Thus, a 5-min recording session captured context-elicited “anticipatory” USVs (-5 to 0 min) as well as following lever extension and access to self-administered drugs (0 to 120 min).

S2.2) Ultrasonic vocalization recording and analysis

USV recording as described in supplemental [Section S1.4](#). For USV recording, each rat was recorded once on day 7 of systemic injections. For drug self-administering rats, USVs were recorded from individual subjects once during the second week of acquisition (i.e. between days 8 and 14).

S2.3) Metrics and statistics

To examine USVs elicited following repeated systemic injections of cocaine or MDPV, total 50-kHz USVs detected in 30-min recording sessions were analyzed using one-tail Mann-Whitney U-Tests against the saline-injected control group. To examine behaviors during drug self-administration, mixed-ANOVAs with infusion number as

within-subjects factor and drug type as between-subjects factor were used to examine changes in infusion latency and 50-kHz USVs across “load-up” (i.e. initial 10 infusions of self-administered drug). An independent samples t-test was applied to examine onset to first infusion of drug between cocaine and MDPV self-administration groups. Additional t-tests were used to examine differences in 50-kHz USVs between drug types within “anticipation” and “post-lever” time epochs. Statistical significance was determined based on Type I error rate (α) less than 0.05.

APPENDIX C

METHODOLOGICAL DETAILS ADAPTED FROM SIMMONS ET AL. 2017.

S3.1) Experimental procedures

Following 14 days of MDPV self-administration acquisition, rats were pre-treated with each of three doses of suvorexant (3, 10, 30 mg/kg, IP) or vehicle spaced 1-2 d apart according to a balanced Latin square design. Rats were pre-treated with suvorexant 15 min prior to being placed in the behavioral chamber (equating to 45 min prior to lever extension/onset of MDPV self-administration).

S3.2) Ultrasonic vocalization recording and analysis

USV recording proceeded as described in supplemental [Section S1.4](#). Briefly, each rat was recorded for 30 min prior to lever extension and onset of MDPV self-administration following pre-treatment with each of four drug doses. USVs were additionally recorded during the 120 min of MDPV self-administration. Unique to this study, 5-min samples during “anticipation” and “post-lever” time epochs (described below) were reviewed to classify 50-kHz USVs as belonging to one of three categories based on acoustic characteristics: fixed-frequency (FF) if no ≥ 3 -kHz contiguous fluctuation in frequency was observed or frequency-modulated (FM) if up to 2 (FM₁₋₂) or 3+ (FM₃₊) fluctuations were observed within a detected call. Typically, USVs with 3 or more fluctuations in frequency fit within the description of “trill-like” calls marked by rapid and contiguous frequency jumps from 40- to 80-kHz.

S3.3) Metrics and statistics

Infusions earned were analyzed via a one-way ANOVA with suvorexant pre-treatment dose as between-subjects factor. Non-parametric Wilcoxon signed-rank tests

were applied to examine 50-kHz USVs during “anticipation” and “post-lever” time epochs across suvorexant pre-treatment doses. The ratio of 50-kHz USVs elicited following suvorexant pre-treatment relative to within-subject elicitation of USVs following vehicle pre-treatment was computed and compared via Bonferroni-corrected one-sample t-tests to “0”—the point of no-change relative to vehicle pre-treatment. Correlations between the % change in infusions and % change in “anticipatory” or “post-lever” 50-kHz USVs were tested using the method of Spearman after Bonferroni adjustment for multiple comparisons. Pie charts were created for qualitative analysis of call type (FF, FM₁₋₂, FM₃₊) across suvorexant pre-treatment doses as well as by “anticipation” and “post-lever” time epochs.

APPENDIX D

ABBREVIATIONS

IIIv – third ventricle;

2-arachidonoyl glycerol – 2-AG;

3,4-methylenedioxymethamphetamine – MDMA (ecstasy);

3,4-methylenedioxypropylamphetamine – MDPV;

4-methylmethcathinone – 4-MMC (mephedrone);

5-choice serial reaction time task – 5CSRRT;

6-hydroxydopamine – 6-OHDA;

Acetylcholine – ACh;

Adenosine triphosphate – ATP;

Amy – amygdala;

Analysis of variance – ANOVA;

Bed nucleus of stria terminalis – BNST;

Central nervous system – CNS;

Cerebellum – Cb;

Cholecystokinin – CCK;

Cortex – Ctx;

Cyclic adenosine monophosphate – cAMP;

Δ^9 -tetrahydrocannabinol – THC;

Diacylglycerol – DAG;

Dopamine – DA;

Dorsal raphe nucleus – DRN;

Dorsal root ganglion – DRG;

Fixed frequency – FF;
Fixed-ratio – FR;
Food and Drug Administration – FDA;
Fornix – fx;
Frequency modulated – FM;
G-protein coupled receptor – GPCR;
 γ -aminobutyric acid – GABA;
Glutamic acid decarboxylase – GAD;
High-performance liquid chromatography – HPLC;
Hypocretin/orexin – hcrt/ox;
Hypocretin/orexin peptide, type 1 – hcrt-1;OX-A;
Hypocretin/orexin peptide, type 2 – hcrt-2;OX-B;
Hypocretin/orexin receptor, type 1 – hcrt-r1;OX₁R;
Hypocretin/orexin receptor, type 2 – hcrt-r2;OX₂R;
Inositol-1,4,5-triphosphate – IP₃;
Interpeduncular nucleus – IPN;
Intracranial self-stimulation – ICSS;
IP – intraperitoneal;
Kappa opioid receptor – KOR;
Lateral habenula – LHb;
Lateral preoptic area – LPO;
Laterodorsal tegmental nucleus – LDTg;
Lithium chloride – LiCl;

Locus coeruleus – LC;
Mammillothalamic tract – mtt;
Medial forebrain bundle – MFB;
Melanin-concentrating hormone – MCH;
Mu (μ) opioid receptor – MOR;
Neuropeptide Y – NPY;
Non-selective cation channel – NSCC;
Nor-binaltorphimine – norBNI;
Noradrenaline – NA;
Olfactory bulb – OB;
Paraventricular nucleus of the thalamus – PVT;
Pedunculopontine tegmental nucleus – PPTg;
Periaqueductal grey – PAG;
Phaseolus vulgaris leucoagglutinin – PHA-L;
Phencyclidine – PCP;
Phenylisopropylamine – amphetamine;
Phospholipase C – PLC;
Polymerase chain reaction – PCR;
Preoptic area – POA;
Progressive-ratio – PR;
Protein kinase C – PKC;
Rapid eye movement – REM;
Rostromedial tegmentum/rostromedial tegmental nucleus – RMTg;

Serotonin (5-hydroxytryptamine) – 5-HT;
Sodium-calcium exchanger – NCX;
Substantia nigra – SN;
Tetrodotoxin – TTX;
Transient receptor potential (channel) – TRP;
Trimethylthiazoline – TMT;
Ultrasonic vocalization – USV;
Variable-ratio – VR;
Ventral pallidum – VP;
Ventral tegmental area – VTA;
Vesicular GABA transporter – VGaT;
Vesicular glutamate transporter type-2 – VGluT2;