Year : 2010

Role of the hypocretin system in cocaine- and alcohol-related behaviors : evidence from hypocretin-deficient mice

STEINER, Nadia

STEINER Nadia, 2010, Role of the hypocretin system in cocaine- and alcohol-related behaviors : evidence from hypocretin-deficient mice

Originally published at : Thesis, University of Lausanne

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ROLE OF THE HYPOCRETIN SYSTEM IN COCAINE- AND ALCOHOL-RELATED BEHAVIORS: EVIDENCE FROM HYPOCRETIN-DEFICIENT MICE

Thèse de doctorat en Neurosciences

présentée à la

Faculté de Biologie et de Médecine
de l'Université de Lausanne

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Lausanne 2010

Programme doctoral interuniversitaire en Neurosciences des Universités de Lausanne et Genève

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Programme doctoral interuniversitaire en Neurosciences des Universités de Lausanne et Genève

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Président	Monsieur Prof.	Mehdi **Tafti**
Directeur de thèse	Monsieur Prof.	Benjamin **Boutrel**
Co-directeur de thèse		
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le Conseil de Faculté autorise l'impression de la thèse de

Madame Nadia Steiner

Master en biologie médicale de l'Université de Lausanne

intitulée

ROLE OF THE HYPOCRETIN SYSTEM IN COCAINE-AND ALCOHOL-RELATED BEHAVIORS: EVIDENCE BASED WITH HYPOCRETIN-DEFICIENT MICE

Lausanne, le 14 janvier 2011

pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof. Mehdi Tafti

Acknowledgements

First of all, I would like to thank Dr. Benjamin Boutrel, my thesis supervisor, for giving me the opportunity to do my thesis work in his lab.

Sincere acknowledgements to Prof. Mehdi Tafti, Prof. Michel Mühlethaler and Dr. Fulvio Magara for accepting to be members of the jury and evaluating my thesis work.

Thanks are due to Myriam Steinmann without her technical help this work could not have been completed. She taught me most of things about genotyping and breeding of mice. I also acknowledge Senda Mafuala and Ewelina Roulin for their technical help in the animal facility.

I would like to thank Prof. Pierre Magistretti and Olivier Halfon – along with Dr. Boutrel – who financially supported my research work.

I also wish to thank all my former and current colleagues at the Center for Psychiatric Neuroscience (CNP) who facilitated the accomplishment of this thesis work. Most of them were great fellows and have become friends.

I want to acknowledge the ADAS committee. It has been a nice experience as an active member and as a co-president. I really learn a lot.

Further, I gratefully thank my mother and my brother, as well as all my family for giving me the fullest support during all these years of my studies and my PhD and during good or bad moments that might happened in life. Noteworthy, without their help this thesis work would have never come off.

Last but not least, I would like to thank cordially all my friends. They were always very patient and understanding. Without their help and support I wouldn’t have succeeded.

Finally, I also want to thank all the persons that I forgot to mention in the present acknowledgments.

Résumé

L'addiction aux drogues est une maladie multifactorielle affectant toutes les strates de notre société. Cependant, la vulnérabilité à développer une addiction dépend de facteurs environnementaux, génétiques et psychosociaux. L'addiction aux drogues est décrite comme étant une maladie chronique avec un taux élevé de rechutes. Elle se caractérise par un besoin irrépressible de consommer une drogue et une augmentation progressive de la consommation en dépit des conséquences néfastes. Les mécanismes cérébraux responsables des dépendances aux drogues ne sont que partiellement élucidés, malgré une accumulation croissante d'évidences démontrant des adaptations au niveau moléculaire et cellulaire au sein des systèmes dopaminergique et glutamatergique. L'identification de nouveaux facteurs neurobiologiques responsables de la vulnérabilité aux substances d'abus est cruciale pour le développement de nouveaux traitements thérapeutiques capables d'atténuer et de soulager les symptômes liés à la dépendance aux drogues.

Au cours des dernières années, de nombreuses études ont démontré qu'un nouveau circuit cérébral, le système hypocrétinergique, était impliqué dans plusieurs fonctions physiologiques, tel que l'éveil, le métabolisme énergétique, la motivation, le stress et les comportements liés aux phénomènes de récompense. Le système hypocrétinergique est composé d'environ 3000-4000 neurones issus de l'hypothalamus latéral projetant dans tout le cerveau. Des souris transgéniques pour le gène des hypocrétines ont été générées et leur phénotype correspond à celui des animaux sauvages, excepté le fait qu'elles soient atteintes d'attaques de sommeil similaires à celles observées chez les patients narcoleptiques. Il semblerait que les hypocrétines soient requises pour l'acquisition et l'expression de la dépendance aux drogues. Cependant, le mécanisme précis reste encore à être élucidé. Dans ce rapport, nous rendons compte des comportements liés aux phénomènes de récompense liés à l'alcool et à la cocaïne chez les souris knock-out (KO), hétérozygotes (HET) et sauvages (WT).

Nous avons, dans un premier temps, évalué l'impact d'injections répétées de cocaïne (15 mg/kg, ip) sur la sensibilisation locomotrice et sur le conditionnement place préférence. Nous avons pu observer que les souris WT, HET et KO exprimaient une sensibilisation locomotrice induite par une administration chronique de cocaïne, cependant les souris déficientes en hypocrétines démontraient une sensibilisation retardée et atténuée. Il est intéressant de mentionner que les mâles HET exprimaient une sensibilisation comportementale intermédiaire. Après normalisation des données, toutes les souris exprimaient une amplitude de sensibilisation similaire, excepté les souris mâles KO qui affichaient, le premier jour de traitement, une sensibilisation locomotrice réduite et retardée, reflétant un phénotype hypoactif plutôt qu'une altération de la réponse aux traitements chroniques de cocaïne. Contre toute attente, toutes les souris femelles exprimaient un pattern similaire de sensibilisation locomotrice à la cocaïne. Nous avons ensuite évalué l'effet d'un conditionnement comportemental à un environnement associé à des injections répétées de cocaïne (15 mg / kg ip). Toutes les souris, quelque soit leur sexe ou leur génotype, ont manifesté une préférence marquée pour l'environnement apparié à la cocaïne. Après deux semaines d'abstinence à la cocaïne, les mâles et les femelles déficientes en hypocrétines n'exprimaient plus aucune préférence pour le compartiment précédemment associé à la cocaïne. Alors que les souris WT et HET maintenaient leur préférence pour le compartiment associé à la cocaïne. Pour finir, à l'aide d'un nouveau paradigme appelé IntelliCage®, nous avons pu évaluer la consommation de liquide chez les femelles WT, HET et KO. Lorsqu'il n'y avait que de l'eau disponible, nous avons observé que les femelles KO avaient tendance à moins explorer les quatre coins de la cage. Lorsque les souris étaient exposées à quatre types de solutions différentes (eau, 1mM quinine ou 0.2% saccharine, alcool 8% et alcool 16%), les souris KO avaient tendance à moins consommer l'eau sucrée et les solutions alcoolisées. Cependant, après normalisation des données, aucune différence significative n'a pu être observée entre les différents génotypes, suggérant que la consommation réduite d'eau sucrée ou d'alcool peut être incombée à l'hypoactivité des souris KO.

Ces résultats confirment que le comportement observé chez les souris KO serait dû à des compensations développementales, puisque la sensibilisation locomotrice et le conditionnement comportemental à la cocaïne étaient similaires aux souris HET et WT. En ce qui concerne la consommation de liquide, les souris KO avaient tendance à consommer moins d'eau sucrée et de solutions alcoolisées. Le phénotype hypoactif des souris déficientes en hypocrétine est probablement responsable de leur tendance à moins explorer leur environnement. Il reste encore à déterminer si l'expression de ce phénotype est la conséquence d'un état de vigilance amoindri ou d'une motivation diminuée à la recherche de récompense. Nos résultats suggèrent que les souris déficientes en hypocrétine affichent une motivation certaine à la recherche de récompense lorsqu'elles sont exposées à des environnements où peu d'efforts sont à fournir afin d'obtenir une récompense.

Abstract

Drug addiction is a multifactorial disorder affecting human beings regardless their education level, their economic status, their origin or even their gender, but the vulnerability to develop addiction depends on environmental, genetic and psychosocial dispositions. Drug addiction is defined as a chronic relapsing disorder characterized by compulsive drug seeking, with loss of control over drug intake and persistent maladaptive decision making in spite of adverse consequences. The brain mechanisms responsible for drug abuse remain partially unknown despite accumulating evidence delineating molecular and cellular adaptations within the glutamatergic and the dopaminergic systems. However, these adaptations do not fully explain the complex brain disease of drug addiction. The identification of other neurobiological factors responsible for the vulnerability to substance abuse is crucial for the development of promising therapeutic treatments able to alleviate signs of drug dependence.

For the past few years, growing evidence demonstrated that a recently discovered brain circuit, the hypocretinergic system, is implicated in many physiological functions, including arousal, energy metabolism, motivation, stress and reward-related behaviors. The hypocretin system is composed of a few thousands neurons arising from the lateral hypothalamus and projecting to the entire brain. Hypocretin-deficient mice have been generated, and unexpectedly, their phenotype resembles that of wild type mice excepting sleep attacks strikingly similar to those of human narcolepsy patients. Evidence suggesting that hypocretins are required for the acquisition and the expression of drug addiction has also been reported; however the precise mechanism by which hypocretins modulate drug seeking behaviors remains a matter of debate. Here, we report alcohol and cocaine reward-related behaviors in hypocretin-deficient mice (KO), as well as heterozygous (HET) and wild type (WT) littermates.

We first evaluated the impact of repeated cocaine injections (15 mg/kg, ip) on locomotor sensitization and conditioned place preference. We observed that WT, HET and KO mice exhibited behavioral sensitization following repeated cocaine administrations, but hypocretin deficient males displayed a delayed and attenuated response to chronic cocaine administrations. Interestingly, HET males exhibited an intermediate pattern of behavioral sensitization. However, after standardization of the post-injection data versus the period of habituation prior to cocaine injections, all mice displayed similar amplitudes of behavioral sensitization, except a reduced response in KO males on the first day, suggesting that the delayed and reduced cocaine-induced locomotor sensitization may reflect a hypoactive phenotype and probably not an altered response to repeated cocaine administrations. Unexpectedly, all female mice exhibited similar patterns of cocaine-induced behavioral sensitization. We then assessed the behavioral conditioning for an environment repeatedly paired with cocaine injections (15 mg/kg ip). All mice, whatever their gender or genotype, exhibited a robust preference for the environment previously paired with cocaine administrations. Noteworthy, following two weeks of cocaine abstinence, hypocretin-deficient males and females no longer exhibited any preference for the compartment previously paired with cocaine rewards whereas both WT and HET mice continued manifesting a robust preference. We finally assessed drinking behaviors in WT, HET and KO female mice using a novel paradigm, the IntelliCages®. We report here that KO females tended to less explore the four cage corners where water was easily available. When exposed to four different kinds of liquid solutions (water, 1mM quinine or saccharine 0.2%, alcohol 8% and alcohol 16%), KO mice tended to less consume the sweet and the alcoholic beverages. However, after data standardization, no significant differences were noticed between genotypes suggesting that the hypoactive phenotype is most likely accountable for the trend regarding the reduced sweet or alcohol intake in KO.

Taken together, the present findings confirm that the behavior seen in Hcrt KO mice likely reflects developmental compensations since only a slightly altered cocaine-induced behavioral sensitization and a normal behavioral conditioning with cocaine were observed in these mice compared to HET and WT littermates. With regards to drinking behaviors, KO mice barely displayed any behavioral changes but a trend for reducing sweet and alcoholic beverages. Overall, the most striking observation is the constant hypoactive phenotype seen in the hypocretin-deficient mice that most likely is accountable for their reduced tendency to explore the environment. Whether this hypoactive phenotype is due to a reduced alertness or reduced motivation for reward seeking remains debatable, but our findings suggest that the hypocretin-deficient mice barely display any altered motivation for reward seeking in environments where low efforts are required to access to a reward.

List of Abbreviations

ACTH	adrenocorticotropic hormone
AGRP	agouti gene related peptide
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
amyg.	amygdala
BNST	bed nucleus of the stria terminalis
CART	cocaine- and amphetamine-regulated transcript
CCK	cholecystokinin
CP	caudate putamen
CPP	conditioned place preference
CRF	corticotrophin releasing factor
DA	dopamine
DR	dorsal raphe
FR	fixed ratio
GPCR	G-protein coupled receptor
Hcrt	hypocretins
Hcrt-1	hypocretin-1
Hcrt-2	hypocretin-2
HcrtR1	hypocretin receptor 1
HcrtR2	hypocretin receptor 2
HET	heterozygous
HPA	hypothalamic-pituitary-adrenal
IC	IntelliCage®
icv	intracerebroventricular
ip	intraperitoneal
KO	knockout
LC	locus coeruleus
LDT/PPT	laterodorsal tegmental area/pedunculopontine tegmental nucleus
LH	lateral hypothalamus
LTD	long term depression
LTP	long term potentiation
MCH	melanin-concentrating hormone
MCHR1	melanin-concentrating hormone receptor 1
MCHR2	melanin-concentrating hormone receptor 2
mPFC	medial prefrontal cortex
NAcc	nucleus accumbens
NMDAR	N-methyl-D-aspartate receptor
NPY	neuropeptide Y
NREM	non-rapid eye movement
NTS	nucleus of the solitary tract
OXY	oxytocin
PFC	prefrontal cortex
POMC	proopiomelanocortin
ppHcrt	preprohypocretin
PVN	paraventricular hypothalamic nucleus
PVT	paraventricular thalamic nucleus
REM	rapid eye movement
rPP	rat pancreatic polypeptide
SCN	suprachiasmatic nucleus
TMN	tuberomammillary nucleus
Trib-2	Tribbles-2
TRH	thyrotropin releasing hormone
VLPO	ventrolateral preoptic nucleus
VTA	ventral tegmental area
WT	wild type

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1 Introduction

1.1 Addiction

1.1.1 General introduction

From the early stage of mankind, human beings learned to use plants for their nutritive, therapeutic, and psychotropic properties. Evidence found 3000 years BC shows that Mesopotamian could precisely describe numbers of disease (conjunctivitis, urinary problems, gynecologic disorders, venereal diseases or pulmonary troubles) and treat them with plants. For example, cypress, oleander, thyme and willow leaves were known for their anti-inflammatory and analgesic action. Further evidence shows that the psychoactive effects of hemp or poppy were also experienced. These plants that are still used nowadays were already found illustrated on the Mesopotamian palace. At that time, Mesopotamian physicians used their sedative and analgesic properties to perform minor surgeries. Evidence also depicted hemp and poppy cultivations in China around 2700 years BC. Chewing coca leaves has also been reported for alleviating signs of fatigue and altitude sickness in Equator and in Peru around 2500 years BC.

For centuries, geographic migrations have allowed a large widespread of psychotropic plants and their cultivations have been extended to regions poor in psychoactive plants. Progresses in chemistry and medicine allowed to describe, isolate and even synthesize number of psychotropic compounds during the 19^{th} century. These major advances did drastically change the pattern of cultivation, distribution and consumption of these plants leading to social problems and public health concerns. During the 19^{th} century, coca leaves extract (cocaine) reached Europe and was largely exploited for its medical use before becoming a must-have product that was widely commercialized. Cocaine was found on domestic markets in various forms such as cigarette, powder, beverage and soluble preparation for injection. During the 20^{th} century, cocaine use became a public health concern, but was not prosecuted until the seventies when it became a controlled substance. Nowadays, drugs of abuse are still a major public health concern with important economic issues, including preventions, repressions and treatments. Even if drugs of abuse are considered harmful, they remain easy to access and difficult to combat, as they widely circulate at moderate prices. In addition, newer synthetic drugs of abuse with high potential for addiction have been developed (for example, ecstasy, LSD and methamphetamine).

In summary, chemistry has made major progresses allowing the extraction and synthesis of the main psychoactive compounds found in psychotropic plants. Drug design also allowed the emergence of synthetic drugs with high potential for abuse. Hence, these new therapeutical approaches opened a promising window of opportunity but also raised a serious threat on public health.

1.1.2 The neurobiology of drug addiction

Drug abuse is a chronic relapsing disorder defined as a progressive neglect of alternative sources of reinforcement concomitant with the development of compulsive substance taking that persists despite evidence of harmful consequences. The inability to control substance taking is thought to be a complex disease process of the brain that results from recurring drug intoxication. It is also considered as a multifactorial psychiatric disease depending on environmental, genetic and psychosocial dispositions.

Addictive drugs act directly in the brain, specifically in the limbic system, where they overstimulate brain structures and induce molecular and cellular adaptations. These modifications are complex and involve drug-induced changes in gene transcription, membrane excitability and neuronal morphology leading to important behavioral consequences. Addiction is not triggered instantaneously upon exposure to drugs of abuse. It involves multiple, complex neural adaptations that develop over time ranging from hours to days and months. A key feature of the neurobiological research on drug addiction is a better comprehension of the molecular, pharmacological and circuitry changes underpinning biased decision making processes, and the overwhelming desire for the drug (craving) associated with the high risk of relapse to drug seeking after a period of abstinence.

Drugs of abuse often induce brain adaptations leading to sensitization, tolerance and affective/physical dependence. For the last 30 years, a growing body of evidence has converged to the identification of the mesocorticolimbic system as a critical neuronal network responsible for the addictive disorders.

1.1.3 The anatomy of the mesolimbic dopamine system

The ventral tegmental area (VTA) contains cell bodies of dopaminergic neurons projecting to the nucleus accumbens (NAcc), amygdala, hippocampus and prefrontal cortex (PFC). Defined as the mesocorticolimbic dopamine system [1], these neurons are critically implicated in brain mechanisms of reward, reinforcement and emotional arousal [2]. Their activity has been closely correlated to the availability of primary rewards such as food, water and sexual behavior [3]. Activation of the mesocorticolimbic dopamine pathway has been argued to represent a neuronal substrate for the rewarding or reinforcing properties of drugs of abuse [4–9]. As such, drugs of abuse can produce powerful interoceptive effects that cause them to act as primary reinforcers. Moreover, repeated exposure to drugs of abuse can produce behavioral and neurochemical sensitization, with subsequent drug exposures producing augmented behavioral responses [10–13]. The ability of the drug, and ultimately of related stimuli, to elicit behavior may be augmented with repeated administrations or intake of the drug. This phenomenon may depend on sensitization of dopamine release and resulting neuroadaptations within the NAcc produced by chronic drug intake [14–16]. However, recent data suggest that this "mesolimbic dopamine hypothesis" of drug abuse is only one factor of the neural circuit mediating the effects of illicit drugs on behavior [17].

1.1.4 The neurobiological hypotheses related to the transition from controlled to compulsive drug-seeking

Everitt and Wolf [18] argued that drugs of abuse significantly strengthen the learning involved in classical and operant conditioning. Therefore, the learned habit of performing a certain behavior in order to receive drug reinforcement is abnormally strong, and can be understood in terms of the aberrant engagement of Pavlovian and instrumental learning processes [18, 19]. This view is consistent with the role of midbrain DA neurons in functions more complicated than simple "reward". Midbrain DA neurons respond to unpredicted rewards and with training, this response transfers to stimuli predictive of rewards [20–22]. By signaling reward prediction errors, DA may act as a teaching signal for striatal learning [20]. Midbrain DA neurons innervate the striatal, cortical, and limbic regions implicated in addiction, so drug effects on DA neuronal activity may be important for synaptic "learning" throughout limbic-cortical circuitry [23].

In contrast, Markou and Koob [24] suggested that activity in brain reward circuitry desensitize as a result of chronic drug use. Koob and Le Moal [15, 16] proposed that the transition from controlled to compulsive drug use involves spiraling down of an "addiction cycle" that includes three components: preoccupation/anticipation, binge/intoxication, and withdrawal negative affect. Initial sensitization to the drug's effects may facilitate the preoccupation/anticipation stage. However, with increased access to drug, self-administration turns to binge use, and the withdrawal/negative affect stage appears as opponent processes work to return the internal milieu to normal. The individual takes even more drug, and withdrawal symptoms become stronger, which in turn cause the preoccupation for the drug to become even stronger. This spiral through the cycle of drug administration and addiction continues until homeostatic mechanisms are not sufficient to oppose the continuous effects of drug administration. At this point, allostatic mechanisms are activated such that basal reward function or sensitivity is shifted downward, which means that more drugs is needed to produce euphoria than when the drug was used for the first time. The result of this cycle is that the need for the drug, and the loss of control over drug consumption, is ever growing.

In contrast to hedonic/withdrawal views of addiction, Robinson and Berridge [10, 25, 26] suggested that the transition to addiction is due to the incentive-motivational consequences of drug-induced alterations in NAcc-related circuitry that mediate incentive salience. The drug and the cues associated with it acquire heightened/sensitized incentive value and become ever more salient to the drug user. Through this process, drugs of abuse gain disproportionate control over behavior. They hypothesize that it is specifically sensitization of incentive salience of representations of drug cues and drug-taking that causes the compulsive pursuit of drugs and persisting vulnerability to relapse in addiction.

Finally, Jentsch and Taylor [27, 28] have proposed that impulsivity, resulting from frontostriatal dysfunction, plays an important role in addiction, and acts synergistically with sensitization to produce compulsive drug-seeking behavior. Because it is believed that striato-cortical circuits are important in the inhibition of common responses in contexts in which they are not relevant,

the dysfunction of the striato-thalamo-orbitofrontal circuit secondary to chronic use could participate in the inappropriately intense motivation to procure and self-administer the drug in addicted subjects. According to this hypothesis, recent evidence indicates that repeated drug exposure alters cortical cognitive function and leads to loss of inhibitory control mechanisms. Monkeys treated repeatedly with cocaine exhibit a specific perseverative pattern of responding, selecting irrelevant information in the face of new reinforcement contingencies [29]. More recently, Vanderschuren and Everitt confirmed that drug-seeking becomes compulsive after prolonged cocaine self-administration in rats [30].

Akin to this latter hypothesis, over the last decade, Volkow and colleagues have collected data based on imaging studies of substance abusers and concluded that addiction might be a disease of compulsion that involves the orbitofrontal cortex (OFC) [31–33].

Perhaps the most striking evidence that confirms a key role played by the OFC in addiction is the observation that cocaine self-administration in mice lacking the DA transporter (i.e. the main molecular target of cocaine) results in c-fos activation of the anterior olfactory nuclei and the piriform cortex, and to a lesser extent the orbital cortex, without inducing any c-fos activation of the NAcc (considered a target of the reinforcing effects of cocaine) [34].

1.1.5 The glutamate and dopamine systems

Drugs of abuse induce long term molecular and morphological modifications following acute and chronic administration which occur in both the dopaminergic and the glutamatergic system. The dopamine neurotransmitter is critical for the neural adaptations which underlie the generation of behavioral responses to relevant events [35]. On the other hand, glutamate homeostasis also affects synaptic activity and plasticity by controlling extracellular glutamate levels in the synaptic and perisynaptic extracellular environment [36]. Drugs of abuse cause long term changes on behavior by altering neuronal function and plasticity in brain circuits [36]. Long term potentiation (LTP) and long term depression (LTD) are important plasticity mechanisms associated to learning and memory. LTP and LTD are important candidate mechanisms for the drug induced responses during the development of addiction [37, 38]. Both LTP and LTD are mediated by AMPARs and N-methyl-D-aspartate receptors (NMDARs) [37, 38]. Interactions between addictive drugs and synaptic plasticity in different brain regions contribute to specific aspects of the addiction cycle, such as drug seeking and taking, craving, withdrawal and relapse. Three classical procedures, the behavioral sensitization, the conditioned place preference and the self-administration, have been widely used to assess the impact of drugs of abuse on synaptic plasticity. Behavioral sensitization is commonly assayed as multiple addictive drugs increased locomotor activity which is progressively augmented with repeated administrations which is characteristic of neuronal adaptations. Conditioned place preference assesses the rewarding effects of drugs of abuse intoxication by evaluating the potency of addictive drugs to trigger drug seeking behavior. And self-administration is an operant procedure which promotes drug seeking/drug taking, withdrawal and relapse behaviors and evaluates the sustained neuronal adaptations over months.

Following the first cocaine exposure, dopamine neurons undergo synaptic plasticity. A single exposure to cocaine induces potentiation of the glutamatergic system through AMPARs and NMDARs which lasts for 5 days in dopamine neurons [39]. Chronic substances of abuse treatments maintain similar potentiation relative to the first drug exposure which consolidate neuronal modifications. The glutamatergic system is implicated in many behavioral outcomes since NMDARs transgenic mice model or blockade of the NMDARs specifically in the VTA prevents behavioral sensitization, conditioned place preference and self-administration [40–42]. In addition, the dopaminergic system showed neuronal adaptation following addictive drugs exposures. Dopamine receptor redistribution has been observed in mesolimbic structures, especially in the NAcc, the PFC and in the VTA. All these regions express DA receptor 1 (D1) and receptor 2 (D2). It seems that D1 is induced by phasic firing which privileges long term potentiation (LTP) and D2 is induced by tonic, basal, firing which facilitates long term depression (LTD) like state. Chronic cocaine also causes long term changes in both the NAcc and the PFC. For example, in the striatum, dopamine receptors D2 are downregulated following chronic exposure to cocaine [43]. Imaging experiments in primates and human brains demonstrate that psychostimulant abusers showed low levels of D2 receptor [44–46]. DA phasic firing modulates many behavioral responses [47]. By inducing phasic firing with the help of optogenetic tools in dopaminergic neurons, Tsai et al. [48] were able to induce conditioned place preference in mice. In addition, blockade of dopamine receptors or the use of knock out transgenic mice support the hypothesis that dopamine is implicated in behavioral sensitization, conditioned place preference and self administration [49, 50].

Since cocaine and alcohol treatments affect brain circuits activation and behavior reflecting modifications at the cellular level, large range of studies focused on molecular changes considering gene transcription, protein expression or posttranslational modifications. For example, following chronic exposure, two transcription factors, ΔFosB and CREB, accumulates in the NAcc cells [51]. Both proteins are transcriptional activators which enhance transcription signaling promoting the expression of numbers of gene such as growth factor, hormones, etc which modulate synaptic plasticity, growth and survival. Since both have important role in many processes such as neuronal growth, it is not surprising that both induce long lasting morphological modifications in addictive brain, including reduction in the size of cell bodies and in neurofilament amounts, as well as spine density [51]. These evidence suggest that both transcription factors might be responsible for morphological adaptation which could be reflected through the abnormal brain activities observed by imaging approaches in addicted individuals [52].

Understanding the basic neurobiological processes sustained by natural and pharmacological reward will promote a better knowledge for drug of abuse-induced machinery and thus for treatments. For more than twenty years, reward processing was mainly focused on two specific neurocircuits, the glutamatergic and dopaminergic systems, which partially explain how substances of abuse hijack natural reward circuitry ranging from molecular mechanisms to behavioral outcomes. Recently, a new system was discovered, the hypocretin system, which demonstrated

implication in many behavioral states and interestingly in reward processing.

1.2 The hypocretin system

More than 10 years ago, two different groups have discovered independently a new family of neuropeptides, the hypocretins, also known as orexins. Since then, a growing number of evidence has implicated the hypocretins in a variety of physiological processes, such as arousal, metabolism, reward, sleep and stress.

Early observations came from Gautvik et al. [53]. The group focused on establishing a database composed of all mRNAs that are only expressed in the hypothalamus; this structure has an important role for maintaining basal body homeostasis and especially feeding behaviors. This open system search allowed highlighting 22 novel mRNAs highly enriched in the hypothalamus. *In situ* hybridization on coronal sections was performed using all clones including the clone of the hypocretin peptides and revealed that hypocretins were restricted to a few neurons distributed bilaterally in the lateral hypothalamic area [54]. In the meantime, the group of M. Yanagisawa was searching for potential ligands of orphan G-protein coupled receptors (GPCRs); these receptors are main pharmacological targets by inhibiting upstream pathways. A systemic biochemical approach was performed to screen endogenous peptide ligands for multiple orphan GPCRs leading to the identification of two peptides which they named orexins. Autoradiography of *in situ* hybridization on coronal sections showed that the orexins neurons were located in the lateral part of the hypothalamus (LH) [55]. Interestingly, both groups observed a similar pattern of expression; the localization of both peptides was restricted to the dorsal and lateral part of the hypothalamus, in particular in the perifornical region (PeF) and the dorsomedial hypothalamic nucleus (DMH), indicating that both peptides were identical [54,55]. In the current report, the term hypocretin will be used.

The hypocretin family is composed of a precursor, the preprohypocretin (ppHcrt), which after post-translational processing is cleaved into two neuropeptides, hypocretin-1 (Hcrt 1) and hypocretin-2 (Hcrt 2), also known as orexin-A (Ox-A) and orexin-B (Ox-B) respectively. The human and rat gene sequences are composed of two exons with an intervening intron which encodes a 130-residue mRNA. In both species, the prepropeptide is composed of three parts, a secretory signal sequence of 33 amino acids followed by hypocretin-1 and hypocretin-2 sequences. Maturation of the prepropeptide starts with the removal of the signal sequence followed by proteolytic cleavages which give birth to two neuropeptides Hcrt-1 composed of 33 amino acids of 3,562 Da, and Hcrt-2, a 28 amino acids peptide of 2,937 Da (figure 1.1) [55, 56]. To our knowledge, no alternative splicing variants of the prepropeptide hypocretin were reported either in human or in rodents. Bioinformatic investigations based on amino acids sequences showed that hypocretins are homologous and highly conserved throughout species suggesting important physiological roles [57, 58].

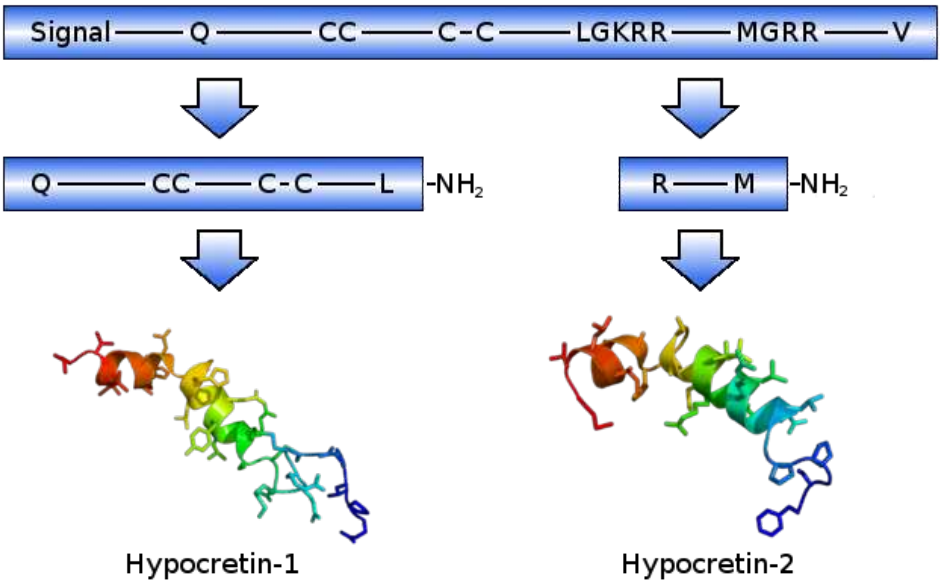


Figure 1.1: **Hypocretin peptides.** Maturation of preprohypocretin in hypocretin-1 and hypocretin-2.

Hypocretins expression takes place early in the development and is age-dependent. Rodent preprohypocretin mRNA is found at low concentration at embryonic day 18 (E 18) and is weakly detected at birth (PD 0) [54, 59]. From PD 0 to PD 15, mRNA levels increase gradually and are strongly enhanced between PD 15 and 20 until reaching a plateau at 3 weeks of age which are maintained during adulthood [54, 59, 60]. Hypocretins expressions are stable from 3 weeks to 12-month of age and begin to decline after 12 months and keep decreasing with aging [61–63]; between PD 400 and PD 800, hypocretin neurons decrease of 15% and of 29% between PD 800 and PD 1000 [62]. In the present report, all animals used were tested during adulthood.

Hypocretin neurons are found in many brain regions (see figure 1.2) and application of hypocretin peptides promotes neuronal activity through two G-protein coupled receptors (GPCRs), hypocretin receptor 1 (HcrtR1) and hypocretin receptor 2 (HcrtR2), also known as orexin 1 receptor (OX1R) and orexin 2 receptor (OX2R) respectively [55]. The hypocretin-1 peptide binds to both HcrtR1 and HcrtR2 with a high affinity, whereas hypocretin-2 binds selectively to HcrtR2 with a similar high affinity [55]. Similar to Hcrt-1 and Hcrt-2 peptides, HcrtR1 and HcrtR2 are highly conserved throughout species; hypocretin receptors were detected in human, rodent, pig, bovine and fish. The genomic sequence of the mouse HcrtR1 consists of nine exons spanning on 9kb and the mouse HcrtR2 consists of eight exons of more than 40kb [64]. G protein coupled receptors have characterized conformation structure; they are composed of a N terminal extracellular domain followed by 7 transmembrane domains linked by intracellular and extracellular loops and ended by a C terminus intracellular domain which mediates downstream molecular pathway through G-proteins, including G_s, G_q and $G_{i/o}$ subtypes [65–67].

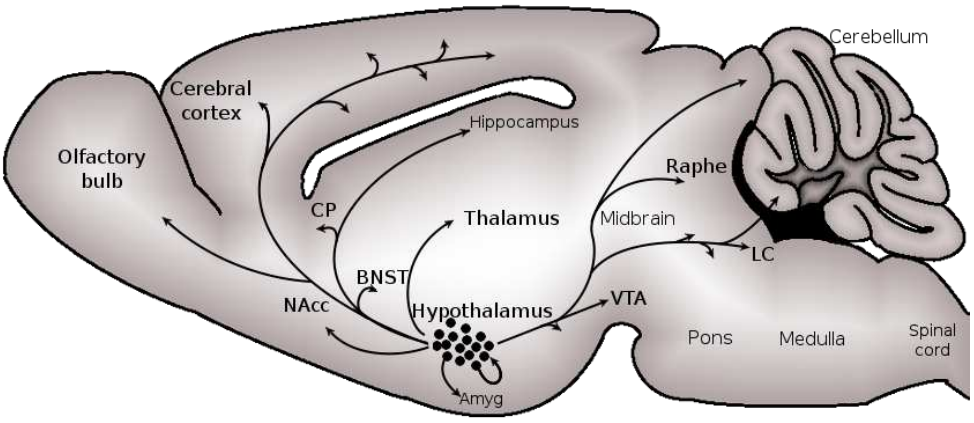


Figure 1.2: **Hypocretin neurons projection.** Schematic drawing of the hypocretin system on a sagittal section through the rat brain. Illustration depicts the principal structures receiving dense innervation of hypocretin neurons where dots represents hypocretin cell bodies. Amyg.: amygdala; BNST: bed nucleus of stria treminalis; CP: caudate putamen; LC: locus coeruleus; NAcc: nucleus accumbens; VTA: ventral tegmental area.

Establishment of the anatomical expression pattern of the neuropeptides (see figure 1.2) and receptors (figure 1.3) gives indications on the physiological roles of hypocretins. Interestingly, the hypocretin system which is composed of a few thousand neurons projects throughout the brain. Hypocretin fibers and hypocretin receptor expression patterns are consistent. However, differential HcrtR1 and HcrtR2 distributions were found; HcrtR1 and HcrtR2 expression pattern are complementary in many cases, but overlapping distributions can also be observed in certain brain regions [68–72] (figure 1.3).

Hypocretin neurons and receptors were found within the tuberomammillary nucleus (TMN), the locus coeruleus (LC), the dorsal raphe (DR) and the laterodorsal tegmental area / pedunculopontine tegmental nucleus (LDT/PPT), as well as the ventrolateral preoptic nucleus (VLPO); these structures are involved in arousal and sleep regulation. They were also found in the PFC, the amygdala, the BNST, the hippocampus, the paraventricular hypothalamic nucleus (PVN) and the VTA. Hypocretin fibers and receptors were also observed in motor related subcircuit, the caudate putamen (CP) and the substantia nigra. They could be found in autonomic centers, the periaqueductal gray matter, the nucleus of the solitary tract (NTS) and the parabriachal nucleus and the septum. [68–78]. Evidence showed hypocretin neurons and receptors in the olfactory bulb [79] and the cerebral cortex. In turn, hypocretin neurons receive afferences from many of these brain regions [78, 80].

Hypocretin peptides and receptors were also observed in the peripheral tissues of both human and rat, including the lung, the myenteric plexus of the enteric nervous system [81] the endocrine cells of the gut [81, 82], the pancreas [82, 83], the adrenal gland [84–86], adipose tissues [87], epidermis and testis [84, 88, 89].

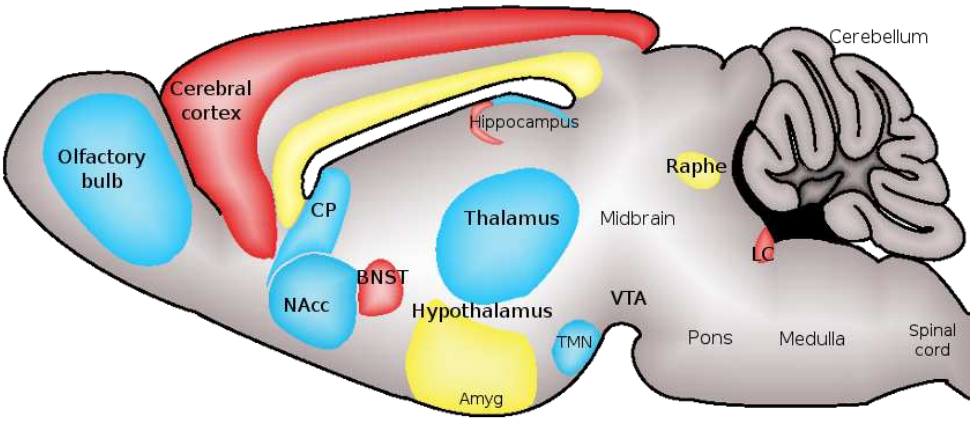


Figure 1.3: **Hypocretin receptors distribution.** Schematic drawing of the hypocretin receptors distribution on a sagittal section through the rat brain. It illustrates the principal structures where hypocretin receptors were observed. HcrtR1 is shown in red, HcrtR2 in blue and HcrtR1 and HcrtR2 overlapping expression is illustrated in yellow. Amyg.: amygdala; BNST: bed nucleus of stria treminalis; CP: caudate putamen; LC: locus coeruleus; NAcc: nucleus accumbens; TMN: tuberomammillary nucleus; VTA: ventral tegmental area.

The anatomical profile of the hypocretin system suggests that it mainly targets monoaminergic circuits, including cholinergic, dopaminergic, noradrenergic and serotoninergic systems [73–75]. These systems are responsible for homeostatic regulations and behavioral outputs. Hypocretin neuronal population could be divided in two fields regarding afferent projections, suggesting a specialization of those neurons in mediating specific responses in respect of the stimuli involved [78]. The medial part seems to be linked to waking and stress and the lateral part seems to be connected with structures that are implicated in reward related behaviors [90–92]. However, this hypothesis is not supported by many authors. Nevertheless, it seems that the hypocretin system is able to sense and integrate environmental stimuli and, because of its key position in the brain, induces the activation of the different centers mediating arousal, emotion, energy metabolism, reward, sleep and stress. In order to better understand the functional roles of hypocretins, transgenic animals (constitutive ppHcrt KO mice or the hypocretin/ataxin3 mice), agonists (synthetic Hcrt-1 or Hcrt-2) or antagonists have been developed. The first pharmacological hypocretin antagonist, SB-334867 which is still widely used, significantly inhibits HcrtR1 mediated response in a concentration-dependent manner [93]. SB-334867 can penetrate the blood brain barrier, in spite of its low bioavailability [94], and following intraperitoneal administration of 30 mg/kg maintains good exposure over 4h [95]. However, SB-334867 did also show some affinity for 5-HT_{2B} and 5-HT_{2C} receptors and other GPCRs [94].

1.3 The physiological roles of the hypocretin system

1.3.1 Arousal and sleep

Arousal and sleep are regulated through the activity of suprachiasmatic nucleus (SCN) and of monoaminergic neurons in the TMN, the LC, the DR, the VTA, and the LDT/PPT, as well as the VLPO [96]. The SCN is thought to orchestrate the circadian clock and its main target is the dorsomedial part of the hypothalamus, which hosts in its vicinity the hypocretin-producing

neurons [97–99]. Moreover anatomical evidence showed that the SCN innervated the medial part of hypocretin-containing cells field [78]. Interestingly, hypocretins levels vary according to the circadian cycle with high levels of peptides during the wake-active period and low levels during the sleep period [100, 101]. Lesioning the SCN disrupts Hcrt circadian rhythm [102]. The DR, the LC, and the TMN , defined as the wake-active centers, are activated by the SCN and interact with the hypocretin system. *In vitro* electrophysiological observations showed that all these regions changed their firing rate after hypocretin application [103–108], except the VLPO [109] and intracerebroventricular (icv) administration of hypocretins in the structures mentioned above during the light period has been reported to increase arousal and decreased REM and non-rapid eye movement (NREM) sleep time [103, 110–112]. In contrast, pharmacological blockade of both hypocretin receptors, HcrtR1 and HcrtR2, promotes sleep [113]. Thus, during the wake-active period, activation of hypocretin neurons by the SCN results in the depolarization of the waking promoting centers. Hypocretins may mediate activation of the LC/noradrenaline system through HcrtR1 [114, 115], the TMN/histaminergic system through HcrtR2 [108, 116] and the DR/serotoninergic system through both HcrtR1 and HcrtR2 [107]. These monoaminergic systems subsequently inhibit the VLPO and the hypocretin neurons. In addition, hypocretins neurons promote arousal through the activation of HcrtR2 in the LDT/PPT [106, 117–119]. These evidence strongly suggest that hypocretins are mainly driving wakefulness. On the other hand, during the sleep-active phase, the activity of SCN and hypocretin neurons decreases which indirectly abolishes the inhibition of the VLPO [120]. The VLPO appears to play a critical role in the initiation of NREM sleep and maintenance of both NREM and REM sleep. The VLPO sends inhibitory projections to the arousal centers and the hypocretin neurons, therefore promoting sleep. These evidence indicate that the hypocretin system mainly drives arousal state and when it is impaired, altered sleep/wake pattern is observed.

Hypocretins are implicated in narcolepsy, a neurological disorder defined as excessive daytime sleepiness associated with unstable boundaries between sleep and wakefulness. In other word, narcolepsy is characterized by a sudden recurrent uncontrollable compulsion to sleep (sleep attacks). These sleep attacks can last from a few seconds to more than an hour. Narcolepsy is often associated with cataplexy, which is characterized by a sudden loss of muscle tone and sleep paralysis. Narcolepsy is tightly associated with genetic dispositions and especially related with the HLA system. Further, progressive loss of hypocretin neurons has been shown to trigger the disease [121–124]. Narcolepsy patients with cataplexy carry the HLA haplotype *DRB5*0101-DRB1*1501-DQA1*0102-DQB1*0602* allele [125] and have reduced or undetectable hypocretin levels in their cerebrospinal fluid. It is speculated that an autoimmune process kills the Hcrt-producing neurons. Recently, the identification of circulating Tribbles 2 (Trib2)-specific antibodies, known to be involved in multiple autoimmune disorders, gives new insight of the mechanisms involved in the development of narcolepsy. Trib2-specific antibody titers were higher in the narcoleptic patients than that of controls and Trib2-specific antibodies showed specific immunoreactivity with hypocretin neurons in the mouse hypothalamus, providing the first evidence that narcolepsy is an autoimmune disorder [126]. Interestingly, narcoleptic patients almost never carried a *DRB1*1301-DQB1*0603* haplotype, suggesting a

protective allele against other HLA associated disorders [127]. In addition, mutation of the HcrtR2 also caused narcolepsy in humans and in agreement with this observation, narcoleptic dogs have disrupted HcrtR2 gene [128]. The generation of transgenic mice confirmed the implication of the hypocretin system in narcolepsy. Constitutive preprohypocretin, HcrtR2 or dual HcrtR1/HcrtR2 knockout mice and hypocretin neuron-ablated mice, also exhibited narcoleptic-like phenotype [129–133]. These transgenic lines exhibit more transitions from wakefulness to rapid eye movement (REM) sleep during the dark phase. The sleep and the active cycles are fragmented and reduced relative to WT recordings [129–133] and rescuing the hypocretin system with hypocretin gene transfer or administration of synthetic hypocretin restore the normal sleep/wake pattern [132]. Using optogenetic tool, Adamantidis et al. [134] promoted wakefulness by photostimulating the LH. LH photostimulation promoted rapid awakening in WT animals than that of ppHcrt KO mice. Interestingly, heterozygous animals issued form the backcross of constitutive ppHcrt KO mice, did not exhibit any narcoleptic-like phenotype, even though hypocretin-1 and hypocretin-2 expression levels are reduced mimicking the progressive decrease of hypocretins levels observed in narcoleptic patients [122–124]; hypocretin levels in HET animals reach 75% and 83% respectively relative to hypocretin levels in WT animals [55]. On the other hand, overexpression of the human preprohypocretin gene in mice also altered the sleep/wake cycles. Transgenic mice overexpressing Hcrt exhibited reduced REM sleep during both light and dark periods compared to WT [135, 136]. These evidence suggest that the hypocretins are intimately linked to wake/sleep regulation. This confirms that either the absence or the high expression of hypocretins induced impairment in arousal/sleep cycles.

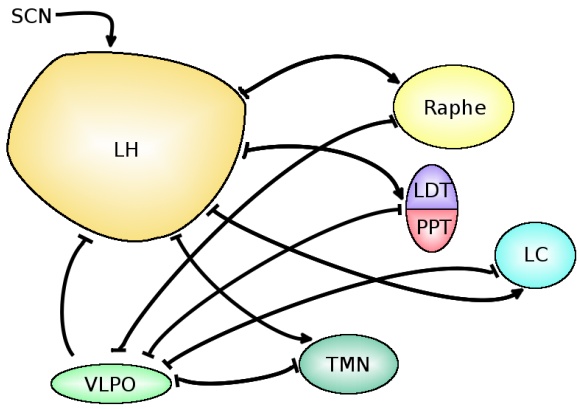


Figure 1.4: **The hypocretin system: Arousal/Sleep.** Schematic drawing of the structure involved in arousal/sleep. During the wake-active period, activation of hypocretin neurons by the SCN depolarizes the LC, the LDT/PPT, the raphe, and the TMN. The LC, LDT/PPT, the raphe and the TMN inhibit the VLPO, thus promoting wakefulness. During the sleep-active phase, the activity of SCN and hypocretin neurons decreases abolishing the inhibition of the VLPO. The VLPO promotes sleep by inhibiting the LC, LDT/PPT, the raphe, the TMN and the LH.

1.3.2 Attention

Levels of awareness and sleep involve interactions between the thalamus and the cortex. Patients with impaired sleep-waking cycles exhibit attentional deficits [137]. The executive attention network of the medial prefrontal cortex permits the sharing of attention among different potential sources of information [138]. Damage to part of this region is thought to result in concentration

difficulties [139]. The majority of patients with such thalamic lesions exhibit deficits on tests assessing fronto-executive functions [140]. Interestingly, narcoleptic patients exhibit attention difficulties [141]. The Hcrt system is anatomically connected to thalamic nuclei and prefrontal cortex suggesting a role for Hcrt in attention [78]. Hcrt peptides and receptors are found in the paraventricular thalamic nucleus (PVT) which in turn projects to the ventral part of medial prefrontal cortex (mPFC), particularly the infralimbic and prelimbic cortices, nucleus accumbens, and amygdala [142]. Hypocretins have recently been shown to depolarize thalamic neurons through the HcrtR2 [142, 143]. Hcrt-1 application stimulates cortical neurons [144]. In addition, Hcrt was shown to excite thalamocortical terminals in the prefrontal cortex [145]. Further evidence established hypocretins may be involved in attention [146]. Nasal delivery of the synthetic Hcrt-1 peptide reverses the attentional performance deficits produced by sleep deprivation, whereas pharmacological blockade of HcrtR1 decreased performance [147]. Since Hcrt fibers have been shown to innervate both the NAcc [148] and the insula [149], it is tempting to speculate that Hcrt may contribute to define behavioral strategies by optimizing the processing of environmental signals in attention-demanding tasks with regard to past experience. Hence, the Hcrt system may enhance cognitive arousal and attentional performance for improving prediction making, and drive sustained attention for achieving the goal-oriented behavior whatever the context is: reward seeking or punishment avoidance [150, 151].

1.3.3 Feeding behavior

It has been suggested that perturbation of the sleep/wake cycles leads to impaired feeding behavior [152–154]. In addition, the SCN, which is known to induce circadian rhythm, also regulates the endocrine system [83, 97]. Large range of hormonal orexinergic peptides, such as ghrelin and anorexinergic peptides, such as cholecystokinin, insulin and leptin are involved in the maintenance of the appropriate energy balance regarding the environment and the needs [155]. Most of these circulating endocrine peptides involved in energy metabolism are regulated in function of the circadian cycle and activates the LH. The hypothalamus, especially the medial and the lateral part, controls appetite; lesions of these specific nuclei lead respectively to obese and anorexia phenotypes [156]. Further, the LH peptides, hypocretins, are related to the incretin family, especially with secretin [57, 58], but an alternative assumption postulates that hypocretins may be related to the bombesin family [157]. Nevertheless, both peptide families are implicated in the regulation of feeding behaviors. Electrophysiological data support the idea that hypocretin-containing neurons are regulated by anorexinergic and orexinergic mediators. The application of glucose and leptin have been shown to induce hyperpolarization associated with decrease in firing rates in a dose dependent manner, while choleocystokinin and ghrelin depolarize dose-dependently hypocretin neurons and increase the spiking frequency [158, 159]. Icv injection of synthetic Hcrt peptides enhanced food intake [55, 160, 161], whereas treatment with the pharmacological antagonist SB-334867 reduced food consumption [95]. In contrast, no difference in food intake was observed, when hypocretins were administered systemically [162]. In transgenic lines, hypocretins seem to promote both energy expenditure and food intake; constitutive ppHcrt KO or hypocretin/ataxin3 KO mice are hypophagic, but do have normal body weights compared to WT animals. In addition, hypocretin/ataxin3 KO mice lose less

weight under fasting conditions and develop obesity over time, while ppHcrt KO tend to be overweighed [163]. These evidence suggest that hypocretins may contribute to the maintenance of appropriate energetic stores during the wake active phase.

Orexinergic and anorexinergic peptides activates the LH, but also the arcuate nucleus. The arcuate nucleus plays an integrative role in feeding behaviors by receiving signals from peripheral tissues. Two populations of neurons are found in the arcuate nucleus, the neuropeptide Y (NPY) / agouti gene related peptide (AGRP) producing neurons and the cocaine- and amphetamine-regulated transcript (CART) / proopiomelanocortin (POMC) containing neurons [155,164]. The hypocretin neurons are anatomically linked to the arcuate nucleus and contact both NYP/AGRP and CART/POMC neurons [73, 78, 165, 166]. Stimulation of the NPY/AGRP neurons activates hypocretin neurons which, in turn, send positive feedback to the latter. Then both NPY/AGRP- and hypocretin-producing neurons inhibit CART/POMC neurons [167, 168]. Taken together, these observations suggest that the arcuate and the lateral hypothalamus act synergistically to stimulate the anabolic pathway and promote feeding behavior during the wake active periods.

Further, NPY/AGRP, CART/POMC and hypocretin neuronal populations also innervate the paraventricular nucleus of the hypothalamus which, in turn, sends feedback projections to the arcuate nucleus and the LH. The PVN is a complex structure considered to be an integrator of the autonomic and neuroendocrine centers. It is composed of different populations of neurons, namely the magnocellular neurons, the parvocellular neurons, the corticotrophin releasing factor (CRF) producing neurons, the oxytocin (OXY) containing cells, the thyrotropin releasing hormone (TRH) synthesizing neurons [169, 170]. Interestingly, CRF activation mediates metabolic stress resulting from negative energy balance and fasting. Direct application of Hcrt-1, but not Hcrt-2, into the PVN induced food intake behaviors through the activation of the magnocellular, the parvocellular, and the CRF-containing neurons [171–173]. The SCN also projects to the PVN and the lateral hypothalamus and the activation of both the hypocretin and the CRF systems synchronize food seeking and food intake with the wake active period [97, 174]. CRF and hypocretins also mediate many ranges of behavioral stress responses, such as increase in the locomotor activity. Thus, concomitant activation of the hypocretin and the CRF system might induce subsequently hyperlocomotion and hyperarousal in order to privilege food seeking behaviors.

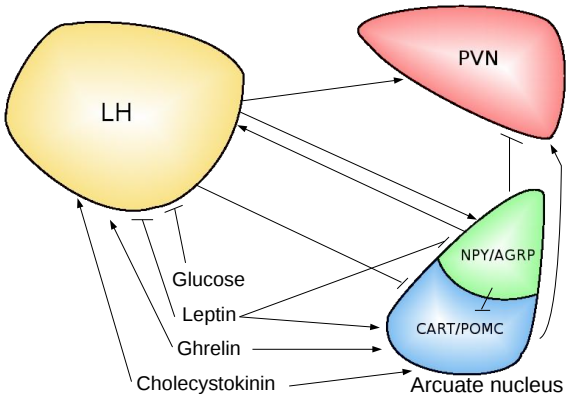


Figure 1.5: **The hypocretin system: feeding behavior.** Schematic drawing of the structure involved in feeding behaviors. The arcuate nucleus, composed of the NPY/AGRP and the CART/POMC neuronal populations, and the Hcrt neurons of lateral hypothalamus regulate the energy homeostasis. Both nuclei are anatomically and functionally linked. Hcrt neurons are inhibited by glucose and leptin and activated by cholecystokinin and ghrelin. In the arcuate nucleus, leptin inhibits the NPY/AGRP neurons and activates the CART/POMC neurons, while ghrelin inhibits the CART/POMC neurons and activates the NPY/AGRP neurons. Both the arcuate nucleus and the LH integrated signals to provide coherent feeding behaviors.

1.3.4 Stress

Negative energy balance induces an acute stress response promoting subsequent hyperlocomotion and hyperarousal in order to stimulate food seeking behaviors. Stress is triggered by emotional, physical or environmental negative affects and is mediated through the activation of the hypothalamic-pituitary-adrenal (HPA) axis. The activation of the HPA axis stimulates the release of adrenocorticotropic hormone (ACTH) in the pituitary gland and subsequently increases the secretion of adrenal cortisol in humans and adrenal corticosterone in rodents. Cortisol and corticosterone are the major downstream mediator of the stress response triggered by the activation of CRF-producing neurons which are located in the PVN, BNST and the amygdala. CRF activation promotes many ranges of sympathetic tone, including blood pressure, heart rate, respiratory regulation and behavioral stress responses, such as chewing, gnawing, grooming, locomotor activity and fighting.

The lateral and the paraventricular nuclei of the hypothalamus are closely connected [78, 80] suggesting that the hypocretin system might be involved in the activation of the brain stress response pathways. Indeed, application of Hcrt-1 in the HPA axis strongly stimulates CRF-expressing neurons and *in vivo* administration of Hcrt-1 increase the circulating corticosterone levels [172, 175–177]. Further, Hcrt administration in rodent enhances grooming and hyperlocomotion which are considered to be behavioral landmarks of stress related responses [103, 104, 178–181]. Indeed, high levels of hypocretins is correlated to stress prone state in humans and in rodents [182–184]. By reducing the expression of hypocretins with siRNA or treatment with a dual antagonist of the hypocretin receptors reduced dose dependently hypocretin levels and the associated sympathetic responses to stress [184]. Further, in agreement with this observation, hypocretin deficient mice exhibit reduced stress related response compared to WT [185–187]. However, not all forms of stress are triggered by the hypocretins, exploration or conditioned fear, but not cold exposure or immobilization, increase the circulating hypocretin levels and

subsequently the heart rate and the blood pressure [188, 189].

Stressful events seem to activate the paraventricular nucleus of the hypothalamus, the lateral hypothalamus and the amygdala resulting in the release of CRF and Hcrt peptides. Both CRF and Hcrt may act synergistically to enhance arousal and locomotor activity; CRF and Hcrt peptides stimulate the NTS, [190–193], which regulates the cardiovascular, gastrointestinal tract and respiratory systems [194], and activation of the hypocretin pathway leads to the release of monoamines, notably that of serotonin and noradrenalin promoting the arousal [103, 110–112]. The concomitant action of CRF, Hcrt, noradrenaline and serotonin trigger increased blood pressure, heart rate, respiratory frequency, muscle tone and hyperarousal. The augmentation of blood flow gathers all resources to promote energy supply to muscles to initiate motor actions and hyperarousal trigger the maximum of attention to define the best strategies.

Stressful stimuli could result from diverse sources. For example, drug seeking behavior can trigger the brain stress pathways. Stress has been reported to contribute to the early neural adaptations which facilitates the development of drug addiction [195]. A role for the brain stress pathways has to be taken into account and notably the CRF-producing neurons mediating the reinstatement of previous reward seeking behaviors.

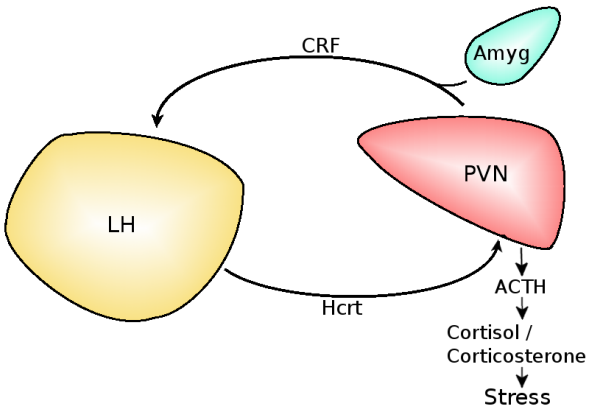


Figure 1.6: **The hypocretin system: Stress.** Schematic drawing of the structure involved in stress. Emotional, physical or environmental negative affects activate the PVN and the amygdala. The activation of CRF-producing neurons induces the activation of the Hcrt neurons. Release of Hcrt in the PVN promotes the stress response through the activation of the hypothalamic-pituitary-adrenal (HPA) axis. The activation of the HPA axis stimulates the release of adrenocorticotropin hormone (ACTH) in the pituitary gland and subsequently increases the secretion of adrenal cortisol in humans and adrenal corticosterone in rodents.

1.3.5 Reward related behaviors

Addictive drugs can drastically affect brain circuits especially the mesolimbic brain circuitry, known for evaluating the incentive salience which determines the intensity of behavioral responding [196]. Interestingly, all mesolimbic structures project to the hypocretin system [78] and the latter sends reciprocal feedbacks. The VTA is the main source of dopamine which mediates incentive learning. Interestingly, one of the largest inputs to the VTA dopamine neurons originates in the lateral hypothalamus and more precisely from hypocretin-containing neurons [78, 197]. However the VTA is moderately innervated by Hcrt fibers compared to the LC and the raphe [73–75]. The innervation of the VTA by hypocretin neurons is predominantly

ispsilateral from the lateral nucleus of the hypothalamus. The VTA mainly sends projections to the NAcc which mediates the reinforcing effects of drugs of abuse. Hypocretin neurons receive strong input from the NAcc shell [78]. Moreover, hypocretin neurons do also receive large input from the amygdala, the BNST, the cerebral cortex and the hippocampus [68, 73–75, 78].

The hypocretinergic system has not only an anatomical interaction with the dopaminergic and glutamatergic systems but also functional interactions. Application of hypocretin induces changes in spiking frequency pattern in many mesolimbic structures [198, 199]. Application of Hcrt-1 or -2 into the VTA potentiates NMDAR EPSCs in dopamine neurons via both HcrtR2 and HcrtR1 [200, 201]. Hcrt-1 induces late-phase increase in postsynaptic AMPAR-mediated synaptic transmission [200]. Hcrt-2 receptor activation could initiate through NMDAR activation or other mechanisms that facilitate AMPAR trafficking [201]. Direct administration of Hcrt-1 or Hcrt-2 in the VTA also induces a dose dependent release of DA, with an increased release of DA after Hcrt-1 application [202]. Not all neurons in the VTA respond to hypocretin peptides; indeed, application of hypocretin-1 activated preferentially the caudomedial part of the VTA which in turn projects to the PFC and NAcc shell [203]. Both the PFC and NAcc shell are stimulated by DA release following direct administration of Hcrt in the VTA [180, 204]. In addition, the PFC projects to the NAcc shell. The PFC and the NAcc shell are also potently activated by hypocretins. In the PFC, hypocretins depolarize cortical neurons [205–207], whereas icv administration of hypocretins in the NAcc induces hyperpolarization [208, 209]. However, hypocretin-2 was more efficient than Hcrt-1 in stimulating both PFC and NAcc through HcrtR2 [142, 208, 209]. Imaging approach in patients suffering from narcolepsy-cataplexy shows that both the PFC and NAcc exhibit abnormal brain activation in response to rewarding stimuli compared to healthy patients [210]. The switch controlled to compulsive drug seeking is mediated by a transition from goal directed behavior driven by the NAcc to habits formation regulated by the dorsal striatum [211]. It has been reported that the dorsal striatum can also be activated by hypocretins, since application of hypocretin-1 in striatal neurons upregulates AMPARs, but not NMDARs, which may mediate a delayed synaptic LTP [212]. The amygdala and the BNST are critical for establishing learned associations of motivationally relevant events and interact with the NAcc and the PFC. Amygdala and the BNST are innervated by the hypocretin system and both Hcrt-1 and Hcrt-2 potently induce depolarization of these structures through HcrtR2 [193, 213]. Hypocretin neurons receive in turn large afferents from the amygdala and the BNST [78]. All dopaminergic and glutamatergic structures involved in reward processing respond electrophysiologically to hypocretins application, especially to the Hcrt-2 peptide, through HcrtR2 which is consistent with hypocretin neurons and receptors distributions, meaning that hypocretin peptides may be implicated in reward related synaptic plasticity by inducing long term changes in these structures. Drugs of abuse drastically modify the mesolimbic neurocircuitries by triggering synaptic plasticity processes and the hypocretin system may contribute to the development of drug addiction. Interestingly, most of narcoleptic patients treated with amphetamine-like drug do not abuse their medication [214, 215], suggesting that addictive drugs may interact with hypocretins in order to promote reward related behaviors.

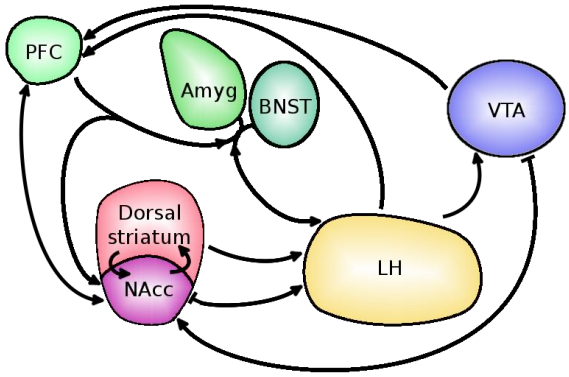


Figure 1.7: **The hypocretin system: Reward related behaviors.** Schematic drawing of the structure involved in reward related behaviors. Positive or negative sensory stimuli induced by drugs of abuse treatment in a specific context affect the amygdala, the hippocampus and the PFC. All these regions activates the VTA. The VTA mainly sends projections to the NAcc mediating the reinforcing effects of drugs of abuse and over time engage habits through the activation of the dorsal striatum. Hypocretin neurons send projections to the VTA, but also to the amygdala and the PFC. In turn, it receives strong input from the amygdala and the NAcc. The cross communication between the LH and the mesolimbic structures reinforce the dopamine signaling and promote drug seeking and drug-taking behaviors which are key features for the development of drug addiction.

Wide range of behavioral procedures are used to dissect drug addiction development and expression. Here, we present three classical paradigms commonly used: the behavioral sensitization, the conditioned place preference and the self-administration.

Behavioral sensitization

Multiple different addictive drugs induce hyperlocomotion and behavioral sensitization, defined as a gradually escalating behavioral and motivational response to a fixed drug dose. The locomotor response induced by daily acute drug administration is progressively enhanced with repeated administration which generally persists for weeks or months even after cessation of drug administration, suggesting the involvement of neuronal plasticity processes. However, when hypocretins are lacking, behavioral sensitization seems to be affected. Indeed, constitutive ppHcrt KO mice failed to exhibit any robust behavioral sensitization to morphine [204, 216] or to psychostimulant drugs, including methamphetamine [217], methylphenidate [217] and 3,4-methylenedioxymethamphetamine (MDMA) [217]. Rodents treated with the HcrtR1 antagonist SB-334867 failed to develop normal amphetamine- [218] and cocaine-induced hyperlocomotion [200]. However, acute blockade of HcrtR1 with SB-334867 does not impair ongoing behavioral sensitization [200]. These suggest that hypocretins seems to facilitate behavioral sensitization and therefore drug-induced synaptic plasticity.

Conditioned place preference

The conditioned place preference (CPP) procedure is a form of Pavlovian conditioning in which distinctive environmental cues become associated with a temporal and motivational event. Place conditioning is most often used to study the positive motivational effects of drug of abuse acute intoxication. As a result of this association, the environmental cues are assumed to acquire the ability to evoke a conditioned motivational response similar to that elicited by addictive drug administration. Rodents are more likely to approach and spend more time in the context

associated with positive conditioned stimulus. In such a paradigm, hypocretin neurons seem to be activated by the evocation of the rewarding effect of the stimulus, as the immediate early gene c-Fos is express following conditioning for food, cocaine or morphine, but not in control conditions or novelty [91]. Interestingly, Fos-positive hypocretin neurons were found in the lateral part of the hypocretin-containing cells field [91]. Pharmacological treatment with the antagonist of HcrtR1 prior the test session attenuated expression of conditioned place preference for morphine [219]. Following active extinction, direct administration of hypocretins in the VTA promotes the reinstatement of the previous extinguished morphine preference [91]. Regarding constitutive ppHcrt KO mice, conditioned place preference for morphine was attenuated even following conditioning with high dose of morphine [204]. In addition, WT pretreated with the HcrtR1 antagonist, SB-334867, displayed a reduced preference for the compartment paired with repeated morphine injections [204], whereas ppHcrt KO female mice demonstrate similar preference than WT for the context paired with repeated exposure to 15mg/kg cocaine [216]. It has been observed that Hcrt may act in the development of associative learning in the conditioned place preference paradigm, especially in the early phase of conditioning. Excitotoxic lesions in the LH and/or application of SB-334867 into the VTA impair the expression of CPP for morphine [219]. Rats trained for CPP show a decrease in hypocretin mRNA expression, especially in the lateral part of the hypocretin-producing neurons, following repeated pairings between the context and cocaine [92]. Finally, application of hypocretin-1 and hypocretin-2 in the VTA potently induces a dose-dependent preference for the drug paired compartment [202]. These observations suggest that hypocretins are involved in mediating learned association during the first step of the conditioning phase and potentiate the VTA to the rewarding effect induced by drugs of abuse or food reward. Interestingly, direct application in the LH of rat pancreatic polypeptide (rPP), a neuropeptide involved in feeding behavior, activates hypocretin neurons through Y4 receptors [220] and reinstates extinguished morphine preference [91].

Self-administration

The self-administration paradigm is an operant task, that selectively highlights particular aspects of the drug experience and the processes contributing to the development of addiction including acquisition, withdrawal and relapse. Self-administration studies are powerful measures considered to mimic conditions of addiction whereby the subject is controlling drug delivery. Several reports explored the role of Hcrt in drug intake with the help of the antagonist of the HcrtR1, SB-334867. In contrast, only few reports were performed with constitutive or conditional Hcrt knockout animals.

Hcrt seems to play a role in alcohol consumption and alcohol seeking [221–228]. Administration of synthetic Hcrt in the LH stimulates alcohol intake [223]. Under fixed ratio (FR) schedule of reinforcement, blockade of HcrtR1 dose-dependently attenuates responding to alcohol [221, 225]. Moreover, SB-334867 potently reduces olfactory cue- and stress-like-induced reinstatement of alcohol seeking behavior [221, 224]. These observations suggest that hypocretin might be involved in alcohol related behaviors.

Hcrt is also critical for cocaine seeking and taking behaviors. Hcrt-1 dose-dependently reinstates cocaine seeking behavior [229]. Systemic administration of HcrtR1 antagonist, but

not HcrtR2 antagonist, attenuates cue-, context- and stress-induced reinstatement for cocaine [226,229–233]. Similar to behavioral sensitization, blockade of HcrtR1 does not alter the acquired drug seeking behaviors in rats trained to self-administer cocaine. These evidence suggest that drug seeking and drug intake are most likely mediated through the activation of the Hcrt system.

All these evidence suggest that Hcrt may facilitate the expression of many aspects of the drug addiction cycle. However, it seems that Hcrt have a drastic impact on DA transmission through the VTA. Peripheral and intra-VTA blockade of HcrtR1 disrupts baseline DA signaling and reduces baseline DA activity and DA responses to cocaine [227], suggesting that intact Hcrt neurotransmission is necessary to support normal DA responses to cocaine. Application of Hcrt in the VTA has been shown to induce DA release in the PFC and NAcc which are a key structure in the development of drug addiction [180, 204]. Intra-VTA infusions of Hcrt-1 facilitate DA release to cocaine in the NAcc and induce c-fos activation in VTA DA neurons that project to the NAcc shell [234]. Thus, Hcrt increases DA neuron activity in the VTA and, in agreement with a few reports, Hcrt increases both burst and firing of DA neurons [199]. Further, Hcrt KO mice show reduced levels of basal dopamine in forebrain structure [217] and in NAcc [227] and reduced evoked dopamine release and uptake in the NAcc following cocaine and morphine injections [204,234]. The DA and Hcrt systems may act synergistically to regulate reward seeking behaviors.

1.3.6 Brief summary

The hypocretin system is composed of a few thousand neurons restricted in the lateral hypothalamus and the perifornical area. Hypocretin-containing neurons project throughout the brain with a prominent input to basal forebrain structures involved in arousal, stress, motivation, and reward [73, 148]. Their interaction with autonomic, neuroendocrine and neuroregulatory systems [75, 148, 235–242] strongly suggests they act as neuromodulators in a wide variety of neural circuits. The hypocretins have been implicated in the modulation of noradrenergic [103, 110, 114], cholinergic [106], serotoninergic [105, 243], histaminergic [116, 244] and dopaminergic systems [104, 199] and in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis [75, 241, 245–248]. Evidence from multiple experiments indicates that Hcrt neurons in the lateral hypothalamus receive inputs from diverse sensory and limbic systems to provide a coherent output that results in the stability of the states of vigilance [249].

Again, chronic drug intoxication may induce changes in basic needs priorities, and the Hcrt system may be part of a common mechanism for adapting and/or ranking priorities and eliciting appropriate levels of alertness to drive attention processes and trigger goal-directed behaviors according to these new priorities [151].

1.3.7 Further considerations: The melanin-concentrating hormone (MCH) system

Noteworthy, the lateral hypothalamus also hosts in its vicinity the melanin-concentrating hormone (MCH) system. These two systems have distinct distributions and do not overlap [250–252], but share several similarities. Like the hypocretin system, the MCH neurons sends projections throughout the brain [253] and MCH peptide binds two different GPCRs, MCH receptor 1 (MCHR1) and MCH receptor 2 (MCHR2), but only MCHR1 is expressed in rodents. Activation of both receptors mediates responses through $G_{i/o}$, G_q and G_s proteins [254, 255]. Similar to the Hcrt system, it has been implicated in numbers of physiological states, such as arousal/sleep [256–260], energy metabolism [261–264], and finally in reward-related behaviors [265–273].

Both Hcrt and MCH systems are expressed in the hypothalamus which is a key structure in mediating autonomic and neuroendocrine signaling. Hypocretins and MCH are involved in the regulation of many physiological states and seem to be complementary in many cases. In addition, the MCH system interacts with the hypocretin neurons; MCH peptides have an inhibitory influence on hypocretin neurons [274, 275]. These observations suggest that these two neuronal populations could directly control sleep-wake regulation, energy expenditure and motivation for reward.

2 Rationale

Drug addiction is a complex behavior driven by multiple parameters, including genetic and environmental dispositions, affecting a large range of the population. For the past few years, drug addiction has been dissected with a special emphasis on the dopaminergic and glutamatergic systems. Substances of abuse drastically modify catecholaminergic transmission by inducing neuronal adaptations following acute or chronic use. Even though major advances have been leading to a better comprehension of the processes triggered by drug addiction, not all aspects of the development and expression could be explained regarding dopamine and glutamate. Recent evidence has shown that the hypocretin system might represent a promising candidate target for both fundamental research and clinical practice. Drugs of abuse withdrawal symptoms could be reduced with Modafinil treatment, a medication for narcolepsy. Indeed, Modafinil activates the hypocretin system, since an increase in c-Fos is observed. In addition, most of narcoleptic patients treated with amphetamine-like drug do not abuse their medication [214,215], suggesting that addictive drugs may interact with hypocretins in order to promote reward related behaviors.

Electrophysiological and behavioral data suggest that the hypocretin system, especially Hcrt-1 and HcrtR1, contributes to the development and the expression of drug addiction. So far, an increasing number of evidence supports the hypothesis that hypocretins mediate a wide range of adaptations triggered by drug abuse, considering that either their blockade or absence induce impaired responses to reward related behaviors. Most of the studies on drug addiction considering the hypocretin system were carried out on rodents treated with antagonists, mainly with the HcrtR1 antagonist SB-334867 although it shows low affinity for 5-HT_{2B} and 5-HT_{2C} receptors and others GPCRs [94]. Only a few reports were performed on constitutive ppHcrt KO mice and only one report used the hypocretin ablated neurons mice. To our knowledge, no study was reported with the constitutive preprohypocretin knock out mice regarding cocaine and alcohol. And transgenic animals are powerful tools to dissect the role of complex neural processes. In addition, the hypocretin system is well conserved throughout the species, allowing translational approach to humans. In order to further understand the role of Hcrt in both cocaine- and alcohol-related behaviors, constitutive ppHcrt KO mice, HET and their WT littermates were tested for either cocaine, including behavioral sensitization and conditioned place preference, or social alcohol consumption. The first part of this project was to assess in both male and female whether constitutive ppHcrt KO mice could develop normal cocaine related behaviors and whether reduced levels of hypocretins might affect the development of drug-induced responses compare to their WT littermates. The second part of the present report was to assess alcohol consumption in female KO, HET and WT mice. In order to measure alcohol consumption, we used a novel automated apparatus allowing us to monitor a large number of mice for several consecutive days where social interactions were allowed during the ongoing procedure.

3 Materials and Methods

3.1 Animals

Founder hypocretin knockout mice, provided by Dr. Yanagisawa (Houston) [129], were on a C57Bl/6J-129/SvEV background and their offspring were backcrossed with C57Bl/6J mice for at least eight generations. In our experiments, we used ppHcrt KO, HET and WT mice that were produced from heterozygous crosses. All animals were housed in a controlled environment (22 ± 1°C, humidity 50 ± 5%)and were kept under a reverse light/dark cycle (lights off at 10 am and lights on at 10 pm) with free access to food and water. All experiments were conducted under the dark phase and were performed in accordance with the Swiss Federal Act on Animal Protection and Swiss Animal Ordinance and as approved by cantonal ethics committee.

3.2 Genotyping

Genomic DNA was obtained from tail biopsies. Tail biopsies were incubated in TENS buffer (100mM Tris-HCl pH 8.0, 5mM EDTA-Na_2 pH 8.0, 0.5% SDS, 100mM NaCl, 200 μg/ml proteinase K) overnight at 55°C. After centrifugation, the supernatant was recovered and DNA was precipitated with isopropanol. Then, DNA was harvested by centrifugation and the pellet was once washed with 70% ethanol before being resuspended in ddH_2O. Mice were genotyped using PCR amplification with a neo primer 5'-CCG CTA TCA GGA CAT AGC GTT GGC-3', or a genomic primer 5'-GAC GAC GGC CTC AGA CTT CTT GGG-3', and genomic primer 3'-TCA CCC CCT TGG GAT AGC CCT TCC-5' common to knock out and WT mice.

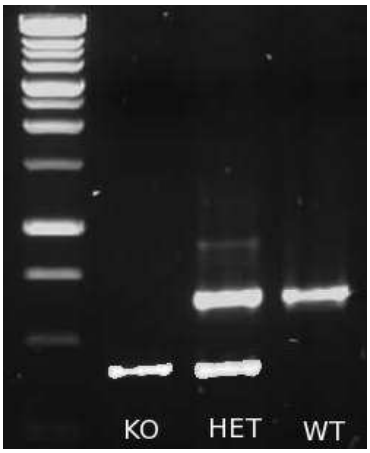


Figure 3.1: **Hypocretin-deficient mice genotyping.**

3.3 Locomotor Sensitization

All treatment and behavioral testings were performed during the dark cycle. Prior the start of the experiment, males and females, aged between 10 and 18-weeks, were habituated for 15min in a large circular openfield ($\oslash = 72.5$ cm). Mice were then tested for 7 consecutive days during

which all animals were further habituated to the openfield paradigm for 15 min prior to the 60 min testing session. All animals were challenged with intraperitoneal (ip) injections of saline on day 1 and either with 15 mg/kg cocaine or with saline as the control condition from day 2 to day 7. Hcrt KO mice (n = 30), HET (n = 30) and WT littermates (n = 31) were given either cocaine (15 mg/kg) or saline (see table 3.1 for details). Mice were first exposed to the arena for 15 min before injection and following injection, animals were put back in the arena where they were videotaped for 1h. Locomotor activity was then assessed with Ethovision 3.0 software (Noldus ®).

Treatments	Genotypes	Males	Females
Saline	WT	8	8
	HET	8	6
	ppHcrt KO	9	7
Cocaine 15 mg/kg	WT	8	7
	HET	9	7
	ppHcrt KO	8	7

Table 3.1: **Locomotor sensitization.** Summary of the number of animals used in each group

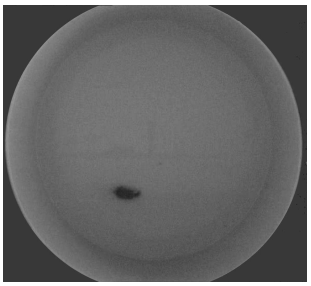


Figure 3.2: **Locomotor sensitization paradigm.** The locomotor sensitization paradigm consisted of a large circular arena. The distances traveled of each animal in the arena were assessed with Ethovision 3.0 software (Noldus ®).

3.4 Locomotor Sensitization with Running Wheel

All treatment and behavioral testings were performed during the dark cycle. Prior the start of the experiment, mice were first habituated to the running wheels for 5 consecutive days in a 120 min session in order to allow mice to learn how to coordinate their movements and speed with wheel running. The number of wheel turns was recorded every 5 min epoch in a 120 min session. Following two days of rest in their home cage, all animals were further habituated for 60 min prior the 60 min testing session for 7 consecutive days. The number of wheel turns was recorded every 5 min epoch for each 60 min session.

Genotypes	Females
WT	6
HET	7
ppHcrt KO	5

Table 3.2: **Locomotor sensitization with running wheel.** Summary of the number of animals used in each group

Figure 3.3: **Running Wheel apparatus.** The running wheel apparatus consisted of a home cage supplemented with a wheel. The running wheel apparatus provides a measurement of voluntary locomotor activity over a fixed period of time.

3.5 Evaluation of Motor Skills with Rotarod

KO mice, HET and WT females were tested in a rotarod to assess their locomotor skills. Animals were tested for 3 consecutive days. The first day, ten- to twelve-weeks aged mice were trained with 5rpm in a 5min session. The second day, mice were tested for 8 different speeds (5, 10, 15, 20, 25, 30, 35, 40rpm) in a 2 min session. The last day mice were tested using the acceleration mode (5 - 60rpm) for a 600 s (10 min) session. For each testing mode, each mouse was given 3 trials.

Genotypes	Females
WT	6
HET	7
ppHcrt KO	5

Table 3.3: **Rotarod.** Summary of the number of animals used in each group

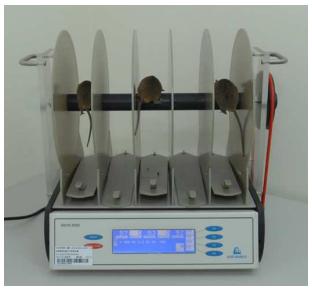


Figure 3.4: **Rotarod apparatus.** The rotarod apparatus consist of beam that can rotate at fixed or accelerating speed. Mice are placed on the beam and the latency to fall provides a measurement of their motor coordination.

3.6 Conditioned Place Preference

The paradigm consisted of a Y-shape box with two-equal sized compartments (15 x 15 x 15 cm) separated by a neutral triangular space. The two compartments had different visual (dots vs stripes) and tactile (metal grid floor vs spotted floor) cues. The place conditioning schedule was conducted in four phases: habituation, preconditioning test, conditioning and post conditioning test. The habituation session was performed to familiarize animals to handling and manipulation as well as to the arena; mice were allowed to freely explore the arena for 20 min. The preconditioning test consisted of a 20 min session during which mice were recorded using a digital video camera and the time spent in each compartment was assessed with Ethovision 3.0 software (Noldus ®). Any animal spending more than 70% of total time in one compartment were excluded to avoid confounding factor of endogenous preference or aversion. The conditioning phase was performed for 4 consecutive days. Each day, animals were treated with either cocaine 15 mg/kg or saline and confined to one compartment for 20 min in the morning session and treated with saline (intraperitoneally) and confined to the other compartment for 20 min in the evening session. Post conditioning test consisted of a 20 min session in which mice were recorded using a digital video camera and the time spent in each compartment was assessed by Ethovision 3.0 software (Noldus®). Mice were also exposed to the arena 2 weeks after posttest to assess whether they still exhibit a preference for the compartment previously associated with cocaine. Mice aged between 10 and 18-weeks were distributed as the following groups: ppHcrt KO (n = 38), HET (n = 34) and WT mice (n = 33) were given either cocaine or saline (for more details, see table 3.4).

Treatments	Genotypes	Males	Females
Saline	WT	11	8
	HET	9	7
	ppHcrt KO	8	7
Cocaine 15 mg/kg	WT	12	7
	HET	10	8
	ppHcrt KO	11	7

Table 3.4: **Conditioned place preference.** Summary of the number of animals used in each group

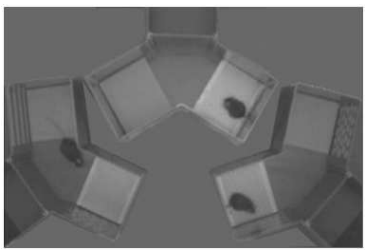
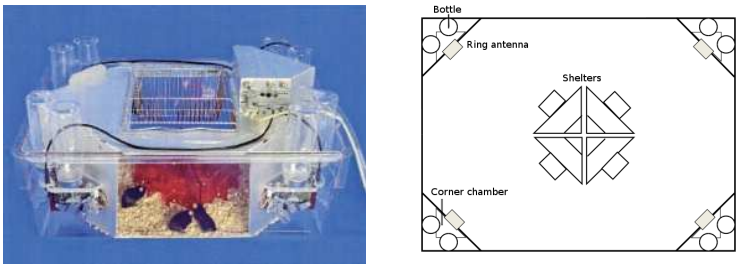


Figure 3.5: **Conditioned place preference apparatus.** The paradigm consisted of a Y-shape box with two-equal sized compartments (15 x 15 x 15 cm) separated by a neutral triangular space. The two compartments had different visual (dots vs stripes) and tactile (metal grid floor vs spotted floor) cues.

3.7 IntelliCage®

3.7.1 IntelliCages paradigm description

The IntelliCage (IC) apparatus is a novel automated system assessing spontaneous behavior in social groups of mice. The apparatus provides four right-angled triangular recording chambers that fit into the corners of the housing cage (55 x 37.5 cm at the base, 58 x 40 cm at the top, 20.5 cm for height). Each recording chambers contains two openings, each permitting access to bottles. Prior the start of the experiment, animals were implanted with subcutaneous transponders which enable recognition of each individual at the entrance of each corner via an antenna. Each recording chamber monitored animals' visits, nosepokes and licks which were implemented through the software provided with the IntelliCage. Four triangular mouse shelters were placed in the center of each cage allowing mice free access to food.



(a) (b)

Figure 3.6: **IntelliCage apparatus.** (a). Illustration of the IntelliCage apparatus. (b). Schematic drawing of the IntelliCage apparatus shows the four triangular corners with a ring antenna enabling recognition of each animal transponder. The circular antenna give access to the inner chambers, each holding two bottles where animal's nosepokes and licks were recorded. In the middle, plastic housing shelters provide access to the food hopper.

3.7.2 Experimental design

The IntelliCages allow hosting and recording large social groups of mice. To avoid or minimize any aggressive behavior or any bias resulting from hierarchy between animals, only female mice were used. In the present report, each IC contained 10 mice with balanced number of ppHcrt KO, HET and WT mice. The IC procedure was divided in different stages. In order to familiarize mice with the apparatus, animals were exposed in the IntelliCage for one week where they were allowed to freely explore each corner in which water was available. Animals exhibiting any preference for one corner in terms of proportion of visits, nosepokes and licks were discarded from the experiment. After one-week habituation and two days of resting in home cage, different liquid solutions were introduced in the IntelliCage apparatus. Each corner corresponds to one specific solution which was the following: 1) water, 2) quinine 1mM or 0.2% saccharine, 3) alcohol 8% w/v and 4) alcohol 16% w/v. Different alcohol solutions were used to test the propensity of mice to drink alcohol and quinine was used to test gustatory sensitivity. Animals were exposed in the IntelliCage for 3 sessions which consisted of 3 consecutive days exposure in IC followed by 4 days rest in their home cages. Then animals experienced two weeks of alcohol abstinence, before being exposed in the IC apparatus for 3 consecutive days where alcohol 8% w/v or 16% w/v were freely available in order to test whether mice exhibit a relapse-like behavior.

Genotypes	Females
WT	7
HET	7
ppHcrt KO	6

Table 3.5: **IntelliCages.** Summary of the number of animals used in each group

3.8 Data and Statistical Analysis

Data shown are presented as group (WT, HET and ppHcrt KO) mean ± SEM. Normalization of the cocaine sensitization data is obtained by dividing the difference between the distance traveled post injection and the distance traveled prior injection with the total locomotor activity during both periods ($\frac{prior-post}{prior+post}$).

Statistical analyses were performed with the R program (version 2.11.1). Significance threshold is considered at α-level<0.05. A repeated measures ANOVA was performed followed by the TukeyHSD post hoc test or by the Student's t test. If the data did not reach ANOVA assumption criteria, data were first transformed. If the transformed data did not follow a normal distribution, the equivalent non parametric statistical test (Kruskal-Wallis) was performed.

4 Results

4.1 Behavioral sensitization in ppHcrt KO mice

Previous studies have demonstrated that hypocretin disruption led to a reduction of the drug induced increased locomotor activity. [204, 216–218]. Further, rats treated with the HcrtR-1 antagonist SB-334867 displayed a reduced sensitization to the psychostimulant-induced hyperlocomotion [200, 218]. Finally, the Hcrt levels have been shown to be differentially regulated in male and female mice. Therefore, we performed an exhaustive study with male and female mice of the three genotypes, WT, HET and ppHcrt KO. All animals were injected with cocaine at the dose of 15 mg/kg ip or with saline, and their locomotor activity was assessed for seven daily sessions of 60 min. Session 1 consisted of saline treatment to assess the basal locomotor activity and from session 2 to session 7, animals were treated with either cocaine or saline (control condition).

4.1.1 Males

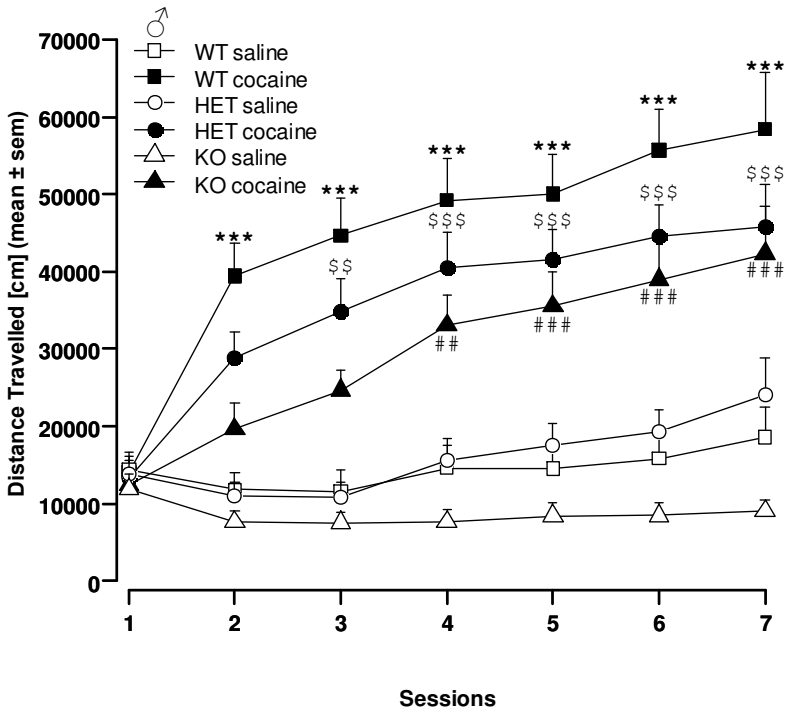


Figure 4.1: **Locomotor sensitization in males.** Locomotor activity was assessed after ip injection of 15 mg/kg cocaine (filled symbols) or saline (open symbols) in WT (square), HET (triangle) and ppHcrt KO mice (circle). Results are displayed as the mean ± SEM of the total distance traveled during 1h test session. Asterisks, dollars and sharps denote a p-value<0.05 relative to session 1. Tukey post hoc test: * p-value<0.05, ** p-value<0.01 and *** p-value<0.001 versus session 1 in the WT cocaine treated group. # p-value<0.05, ## p-value<0.01 and ### p-value<0.001 versus session 1 in the KO cocaine treated group. $ p-value<0.05, $$ p-value<0.01 and $$$ p-value<0.001 versus session 1 in the HET cocaine treated group.

Basal locomotor activities on session 1 in the group assigned in the cocaine treatment (wt: 14089 ± 2119 cm, n = 8; ppHcrt KO: 12444 ± 906 cm, n = 8; het: 13345 ± 1013 cm, n = 9; p-value>0.05) were statistically not different from the saline-treated groups (WT: 14339 ± 2251 cm, n = 8; ppHcrt KO: 11902 ± 1883 cm, n = 9; HET: 13799 ± 1752 cm, n = 8; post hoc test: p-value>0.05) (figure 4.1). All the control groups did not exhibit any significant increase in their locomotor activity after saline administration over sessions (post hoc test: p-values>0.05). WT and HET animals showed a slight increase in the distance traveled during session 7 which was not significant relative to baseline values (session 1; post hoc test: p-values>0.05). WT mice treated with cocaine exhibited a robust sensitization; the distances traveled in the arena were significantly different from the first cocaine administration (session 2; post hoc test: p-value<0.001) to the last cocaine treatment (session 7; post hoc test: p-value<0.001) relative to session 1 ($F_{(1,308)}$(treatments)=563.6, p-value<0.001). KO mice exhibited a delayed and attenuated locomotor sensitization to cocaine injections (($F_{(2,308)}$(genotypes)=36.7, p-value<0.001)); mice displayed a significant increased locomotor activity from session 4 to session 7 (compared to session 1). Noteworthy, HET animals exhibited an intermediate response to cocaine-induced hyperlocomotion (figure 4.1). On session 7, all mice exhibited similar locomotor activity (58389 ± 7359 cm for WT, 45834 ± 5483 cm for HET and 42303 ± 6106 cm for KO mice). No statistical differences were observed between genotypes (post hoc test: p-value>0.05).

4.1.2 Females

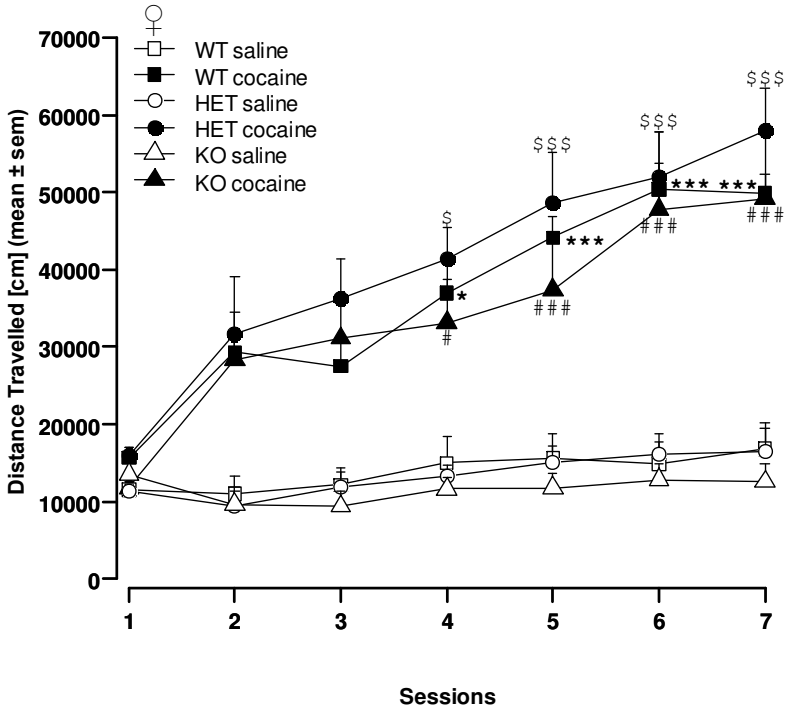


Figure 4.2: **Locomotor sensitization in females.** Locomotor activity was assessed after ip injection of 15 mg/kg cocaine (filled symbols) or saline (open symbols) in WT (square), HET (triangle) and ppHcrt KO mice (circle). Results are displayed as the mean ± SEM of the total distance traveled during 1h test session. Asterisks, dollars and sharps denote a p-value<0.05 relative to session 1. Tukey post hoc test: * p-value<0.05, ** p-value<0.01 and *** p-value<0.001 versus session 1 in the WT cocaine treated group. # p-value<0.05, ## p-value<0.01 and ### p-value<0.001 versus session1 in the KO cocaine treated group. $ p-value<0.05, $$ p-value<0.01 and $$$ p-value<0.001 versus session 1 in the HET cocaine treated group.

No differences were detected in basal locomotor activity between groups (session 1) (figure 4.2). In the saline-treated groups, the traveled distances remained stable over sessions. No differences were observed between genotypes (post hoc test: p-value>0.05). In the cocaine-treated groups, all animals displayed a significant cocaine-induced hyperlocomotion ($F_{(1,252)}$(treatments) =476.6, p-value<0.001). For all cocaine-treated groups, the traveled distances in the arena became significantly different from session 1 after the third injection of cocaine (session 4; post hoc test: p-value<0.05). Even though no robust differences are observed between genotypes over sessions, statistical analysis highlighted a significant genotype effect ($F_{(2,252)}$(genotypes)=5.35, p-value<0.01) indicating that HET exhibited a higher locomotor activity following saline and cocaine injections than that WT and KO.

4.1.3 Comparison between males and females

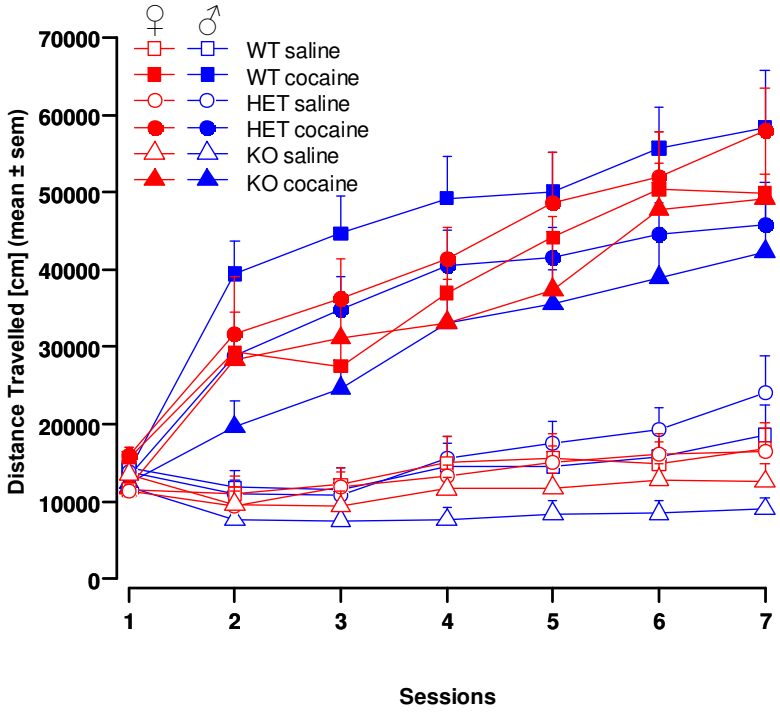


Figure 4.3: **Locomotor sensitization: Comparison males vs females.** Locomotor activity was assessed after ip injection of 15 mg/kg cocaine (filled symbols) or saline (open symbols) in WT (square), HET (triangle) and ppHcrt KO mice (circle) in males (blue) and females (red). Results are displayed as the mean ± SEM of the total distance traveled in 1h test session. For statistical analysis, see figures 4.1 for males and 4.2 for females.

Figure 4.3 summarizes all the data collected with male and female mice. On session 1, the distances traveled in the one hour testing session by males and females were similar irrespective of genotypes. In the saline-treated groups, the distances traveled were maintained stable in all groups over the 7 sessions. However, male ppHcrt KO tend to exhibit lower locomotor activity relative to all groups, even compared to female ppHcrt KO ($F_{(2,560)}$(gender x genotype)=9.0, p-value<0.001). Following the first cocaine administration (session 2) and after repeated exposures to cocaine (15 mg/kg), all animals did exhibit hyperlocomotion ($F_{(1,560)}$(treatments)=1097.6, p-value<0.001 and $F_{(6,560)}$(sessions)=45.9, p-value<0.001). However, WT males exhibited a

more robust cocaine behavioral sensitization and KO males did show a delayed and attenuated behavioral sensitization compared to the other cocaine-treated groups, which was highlighted by a significant genotype effect ($F_{(2,560)}$(genotypes)=35.3, p-value<0.001).

4.2 Amplitude of behavioral sensitization in ppHcrt KO mice

Before each test session, animals were exposed in the arena during 15 min and the locomotor activity was assessed. Immediately after ip injections of either cocaine or saline, animals were returned to the arena for the 60 min test session. In order to evaluate the amplitude of the behavioral sensitization, the locomotor activity of the first 15 min of each test session was related to the daily activity (for more detail, see section 3.8). Figures 4.4, 4.5 and 4.6 represent normalized data. Negative values mean a decrease in the distance traveled during the first 15 min post injection compared to the 15 min of habituation before the injection. In contrast, positive values indicate an increase in the distance traveled during the first 15 min post injection compared to the 15min of habituation before the injection.

4.2.1 Males

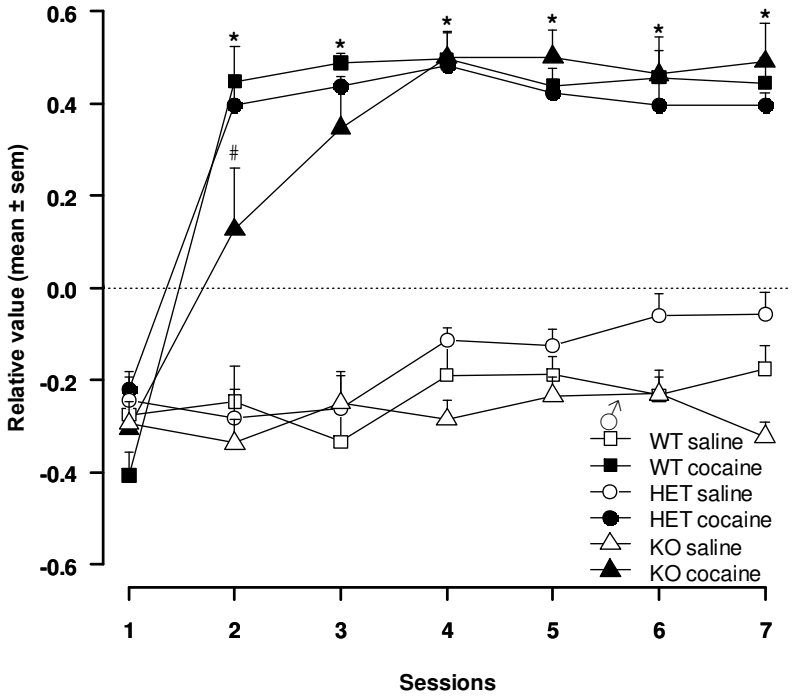


Figure 4.4: **Locomotor sensitization in males: Data normalization.** Representation of normalized data between the first 15 min of the test session and the 15 min prior the injection. Groups treated with 15mg/kg cocaine are represented by filled symbols and control groups by open symbols, each groups including WT (square), HET (triangle) and ppHcrt KO mice (circle). Values under zero represent reduced locomotor activity and above zero represent increased activity following cocaine injections. Results are displayed as the mean ± SEM of normalized data. Asterisks denote a p-value$<$0.05 relative to session 1. Sharp denotes a p-value$<$0.05 versus WT cocaine-treated group, HET cocaine treated group and the other sessions of KO cocaine treated group.

On session 1, all groups of mice were treated with saline injections to assess the basal activity (figure 4.4). The basal locomotor activity was similar in all male groups. The saline-treated groups displayed a reduced locomotor activity following the treatment over sessions. In contrast, the cocaine-treated groups demonstrated an increase in the distance traveled during the first 15 min post injection compared to the 15 min habituation prior to cocaine administration

($F_{(1,308)}$(treatments)=1143.1, p-value<0.001). Behavioral sensitization amplitudes were similar in WT and HET, an observation underestimated on figure 4.1, where HET male mice did show a reduced locomotor activity induced by cocaine administration compared to WT littermates. Interestingly, ppHcrt KO mice displayed a behavioral sensitization to cocaine with a similar amplitude to that observed in WT and HET mice. Only session 2 remained significantly different compared to session 1 and sessions 4 to 7 ($F_{(2,308)}$(genotypes)=7.0, p-value<0.01), confirming that KO males exhibit a reduced and delayed sensitization to the locomotor effects of cocaine, but to a lesser extent than initially suggested on figure 4.1.

4.2.2 Females

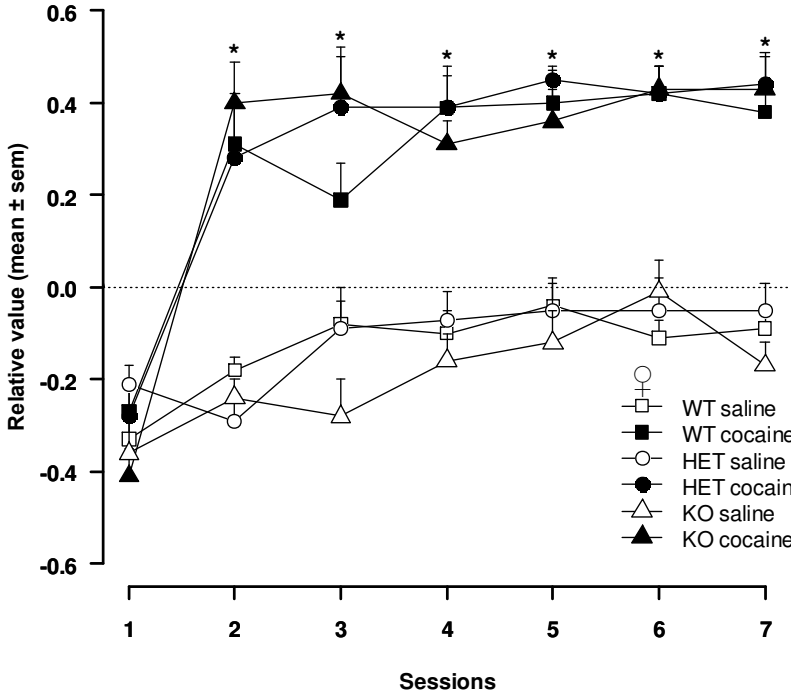


Figure 4.5: **Locomotor sensitization in females: Data normalization.** Representation of normalized data between the first 15 min of the test session and the 15 min prior the test session. Groups treated with 15mg/kg cocaine are represented by filled symbols and control groups by open symbols, each groups including WT (square), HET (triangle) and ppHcrt KO mice (circle). Values under zero represent reduced locomotor activity and above zero represent increased activity following injections of either cocaine or saline. Results are displayed as the mean ± SEM of normalized data. Asterisks denote a p-value<0.05 relative to session 1.

Consistent with our former observations, the basal locomotor activity following saline administration on session 1 was reduced in all females (post hoc test: p-value>0.05). In the saline-treated groups, the locomotor activity was similar over sessions (figure 4.5; post hoc test: p-value>0.05). In the cocaine-treated groups, all mice exhibited hyperlocomotion following acute and chronic intermittent exposure to cocaine ($F_{(1,252)}$(treatments)=14.2, p-value<0.001). No statistical differences were observed between ppHcrt KO, HET and WT females ($F_{(2,252)}$(genotypes) =0.1, p-value>0.05), which is consistent with our previous observations on figure 4.2.

4.2.3 Comparison between males and females

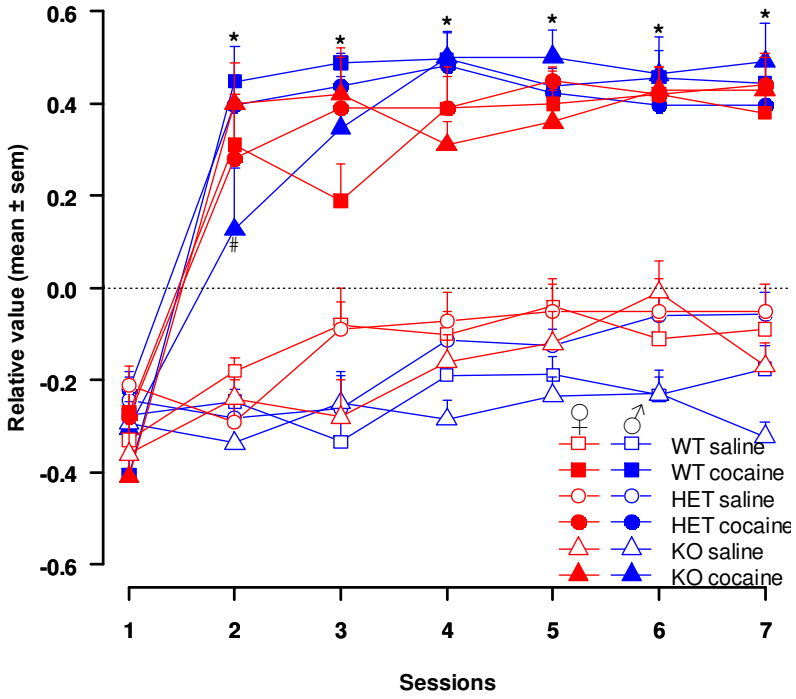
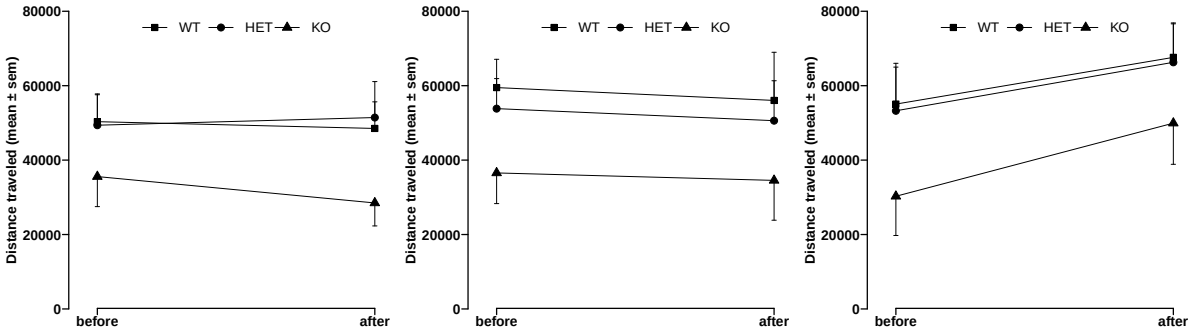


Figure 4.6: **Locomotor sensitization: Comparison males versus females.** Locomotor activity was assessed after ip injection of 15 mg/kg cocaine (filled symbols) or saline (open symbols) in WT (square), HET (triangle) and ppHcrt KO mice (circle) in males (blue) and females (red). Results are displayed as the mean ± SEM of the total distance traveled in 1h test session. Asterisks denote a p-value<0.05 relative to session 1. Sharp denotes a p-value<0.05 versus WT cocaine-treated group, HET cocaine treated group and the other sessions of KO cocaine treated group.

Behavioral sensitization amplitudes in males and females are represented on figure 4.6. No statistical differences were observed between gender ($F_{(1,560)}$(gender)=1.6, p-value>0.05). Behavioral sensitization amplitudes were significantly affected by genotypes ($F_{(2,560)}$(genotypes)=9.1, p-value<0.001), meaning that KO animals seems to have a lower locomotor activity following saline and cocaine injections, and ($F_{(1,560)}$(treatments)=1536.9, p-value<0.001), indicating that the amplitude of the cocaine-treated groups were statistically different relative to the saline-treated groups.

4.3 Behavioral sensitization with running wheel in female ppHcrt KO mice

It has been reported that wheel running augments drug-induced locomotor sensitization. In order to test whether the environment has an influence on behavioral sensitization in female, we used running wheels as an alternative to the openfield. To avoid any confounding effects due to novelty, animals were first trained for five consecutive days to learn how to coordinate their movements and speed with wheel running. Then, animals followed a similar procedure as the locomotor sensitization procedure, except for the duration of the habituation phase prior each test session which was extended to one hour.



(a) Session 1 (b) Session 2 (c) Session 7

Figure 4.7: **Locomotor sensitization with running wheels in females** Locomotor activity was assessed before and after ip administration of (a). saline injections, (b). the first cocaine injection (15 mg/kg) and (c). after chronic intermittent treatment with cocaine (15 mg/kg) in WT (filled square; n = 6), HET (filled circle; n = 7) and ppHcrt KO (filled triangle; n = 5) mice. Results are displayed as the mean ± SEM of the total distance traveled during habituation (before) and the test session (after).

The basal locomotor activity in running wheel was assessed in session 1 (figure 4.7a). The distances traveled during habituation (before) and following saline injection (after) were similar in HET and WT animals. No statistical differences were found between WT and HET ($F_{(1,30)}$ (tests)=0.1, p-value>0.05). The distances traveled by ppHcrt KO mice during habituation and the test session were reduced compared to both WT and HET ($F_{(2,30)}$(genotypes)=3.5, p-value=0.044). However, in our sample size, multiple comparison did not highlight any statistical differences between groups (post hoc tests: p-value>0.05).

Following the first ip administration of cocaine, WT, HET and ppHcrt KO female mice did not exhibit any hyperlocomotion (figure 4.7b) ($F_{(1,30)}$(tests)=0.2, p-value>0.05). Even though the distances traveled by ppHcrt KO mice were reduced relative to WT mice, these differences were not significant ($F_{(2,30)}$(genotypes)=2.9, p-value=0.07).

After repeated exposures to cocaine, WT, HET and ppHcrt mice exhibited behavioral sensitization in running wheel (figure 4.7c). WT and HET mice showed a similar locomotor activity. The distances traveled by ppHcrt KO mice seemed to be reduced compared to HET and WT mice. However, these differences were not significant $F_{(1,30)}$ (tests)=3.3, p-value=0.07 and

$F_{(2,30)}$(genotypes)=2.6, p-value=0.09 in our sample size.

In order to compare data obtained in the locomotor sensitization procedure in a openfield with the present data, normalization was performed (see section 3.8 for more details). As previously mentioned, negative values mean a decrease in the distance traveled during the first 15 min post injection compared to the 15 min of habituation before the injection. In contrast, positive values indicate an increase in the distance traveled during the first 15 min post injection compared to the 15min of habituation before the injection.

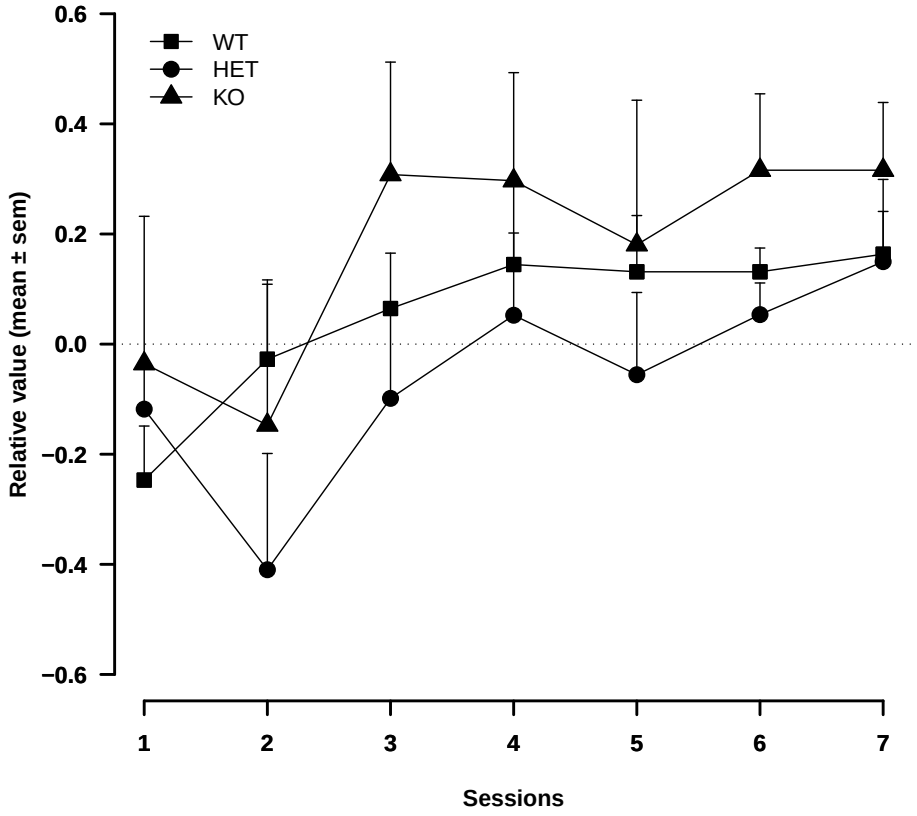
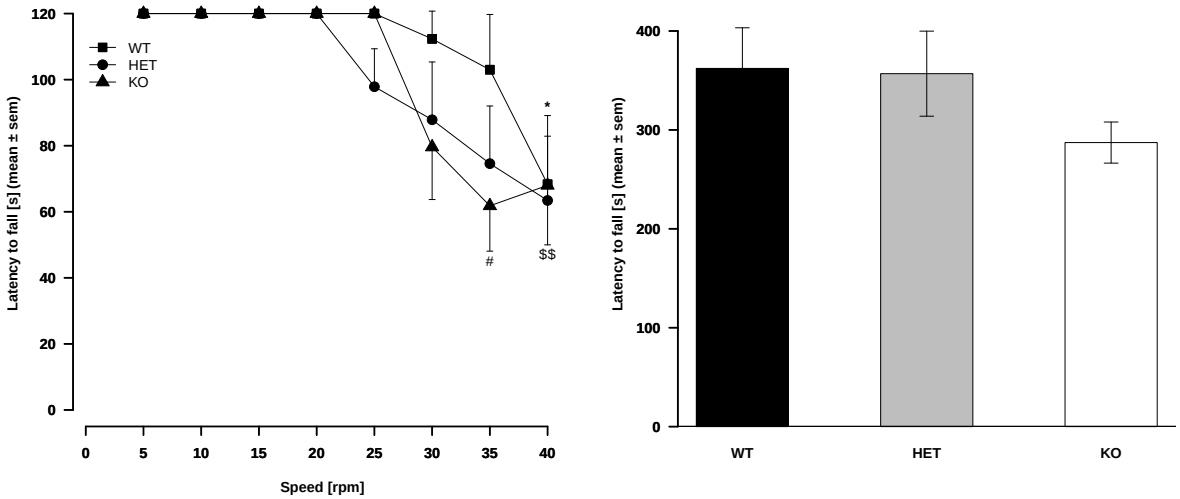


Figure 4.8: **Locomotor sensitization with running wheels in females: Data Normalization.** Representation of normalized data between the first 15 min of the test session and the 15 min prior the test session. All groups were treated with 15mg/kg cocaine and WT are represented with filled square(n = 6), HET with filled circle (n = 7) and ppHcrt KO mice with filled triangle (n = 5). Values under zero represent reduced locomotor activity and above zero represent increased activity. Results are displayed as the mean ± SEM of normalized data.

The basal locomotor activity was assessed on session 1. The distance traveled in running wheel was similar between all genotypes; no statistical differences were observed (figure 4.8; post hoc test: p-value>0.05). From session 2 to session 7, female mice were treated intraperitoneally with 15 mg/kg cocaine following the daily habituation phase. On session 2, cocaine injection did not induce any changes in the locomotor activity between genotypes; WT, HET and KO mice did not exhibit any hyperlocomotion. From session 3, ppHcrt KO mice and to a lower extent WT exhibited a slight increase in their locomotor activity following cocaine injection ($F_{(6,105)}$(sessions)=3.5, p-value<0.01). In contrast, HET animals did not show any cocaine-induced locomotor sensitization ($F_{(2,560)}$(genotypes)=45.9, p-value<0.01). These results contrasts with our previous observations (figure 4.5).

4.4 Deficit in coordination at high speed on the rotarod in female ppHcrt KO mice

The locomotor sensitization procedure with running wheel points out locomotor differences between KO and WT female mice. To test whether ppHcrt KO mice exhibit deficit in locomotor skills, we used the rotarod paradigm. Here, fixed and accelerating modes were performed on WT, HET and KO mice.



(a) fixed speed mode (b) accelerated speed mode

Figure 4.9: **Evaluation of motor skills in females on the rotarod.** Latency to fall off the rotarod in [s] is represented for (a) fixed speed mode. Results are displayed as the mean of the latency to fall in [s] ± SEM. Tukey post hoc test: * p-value=0.06 and # p-value<0.05 relative to 5, 10, 15, 20 and 25 rpm in WT and KO mice, respectively. $ p-value<0.05 and $$ p-value<0.01 relative to 5, 10, 15 and 20 rpm in HET. (b). accelerating mode. Results are displayed as the mean of the latency to fall in [s] ± SEM.

On the fixed speed mode (figure 4.9a), ppHcrt KO, HET and WT mice performed well on speeds up to 20 rpm. At 25 rpm, HET animals seemed to fall off earlier than both ppHcrt KO and WT mice. However, no significant differences were observed between WT, HET and ppHcrt KO mice ($F_{(2,120)}$(genotypes)=2.5, p-value=0.08). Although no statistical difference was detected, HET and ppHcrt KO mice tended to have more difficulty to stay on the rotarod compared to WT animals. At the highest speed, all mice fell off before the end of the trial ($F_{(7,120)}$(speeds)=13.5, p-value<0.001). At 40 rpm, the latency to fall for WT, HET and ppHcrt KO were 68.3 ± 20.8 s, 63.4 ± 19.4 s and 68.0 ± 18.0 s, respectively.

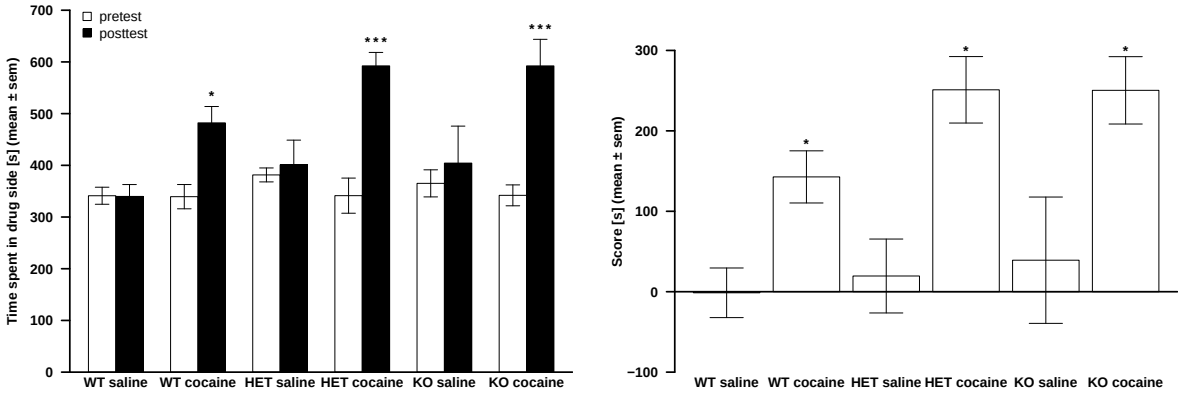
On the accelerating mode (figure 4.9b), ppHcrt KO mice seemed to fall off earlier than WT and HET mice. WT, HET and ppHcrt KO mice fell at 362.2 ± 41.1 s, 356.9 ± 43.0 s and 287.2 ± 20.8 s, respectively. However, the latencies to fall between genotypes were not statistically different ($F_{(2,15)}$(genotypes)=1.2, p-value>0.05). The average speed at which mice fell off was for WT, HET and ppHcrt KO mice at 38 ± 4 rpm, 37 ± 4 rpm and 32 ± 2 rpm respectively. No statistical differences were found in speed between genotypes ($F_{(2,15)}$(speed)=1.1, p-value>0.05). Comparison of data acquired from fixed speed and accelerating tasks showed similar results for ppHcrt KO and WT mice in both tests. In contrast, HET were able to run faster on the

accelerating mode than the fixed speed task (figure 4.9a).

4.5 Conditioned place preference in ppHcrt KO mice

It has been reported that ppHcrt KO mice did not exhibit any preference for the compartment previously paired with repeated morphine injection [204, 216]. To determine whether ppHcrt KO mice would exhibit a preference for cocaine, we tested mice in an unbiased CPP procedure. During the pretest procedure, animals were allowed to freely explore the entire arena in a drug free state. The time spent in the compartment in which conditioning would be proceeded was assessed. After four repeated conditionings either with saline or cocaine, animals were exposed again in the entire arena in a drug free state and the time spent in the compartment paired with repeated conditionings was monitored. The graphs below represent an histogram of the time spent in the compartment associated with repeated saline (control condition) or cocaine injections during pretest and posttest (figure 4.10a) and the score (figure 4.10b), which is defined as the difference between the time spent in the compartment paired with cocaine during posttest minus the time spent during pretest.

4.5.1 Males



(a) Pretest vs Posttest

(b) Score

Figure 4.10: **Conditioned Place Preference in males.** Cocaine induced a rewarding effect in WT, HET and KO mice. (a). The results are displayed as the time spent in [s] in the compartment associated with repeated cocaine or saline injections during pretest and posttest. Each bar represents the mean time ± SEM of 8-12 mice. Tukey post hoc test: * p-value<0.05 and *** p-value<0.001. (b). Data are represented by the scores, defined by the difference in [s] between the postconditioning phase and the preconditioning phase. Each bar represents the mean time ± SEM of 8-12 mice. Tukey post hoc test: * p-value<0.05.

During pretest, all mice spent similar amount of time in the compartment of reference; no statistical differences were observed (post hoc test: p-value>0.05). In the saline-treated groups, the time spent in the compartment associated with saline injections were similar to pretest (post hoc test: p-value>0.05). In the cocaine-treated groups, all animals exhibited a robust conditioned place preference ($F_{(1,110)}$(treatments)=15.2, p-value<0.001); however the rewarding effect of cocaine following tended to be more robust in ppHcrt KO and HET mice compared to WT, although these differences were not significant ($F_{(2,110)}$(genotypes)=1.2, p-value=0.052). The time spent in the compartment paired with cocaine injections during posttest in WT, HET and ppHcrt KO mice were 482 ± 32 s, 592 ± 26 and 592 ± 51 s respectively compared to pretest (339 ± 23 s for WT, 331 ± 34 for HET and 342 ± 20 s for KO).

The score was 250 ± 42 s for ppHcrt KO mice , 251 ± 41 s for HET and 143 ± 32 s for WT mice. The score was statistically different relative to saline control groups (p-value<0.05). The control group did not exhibit any statistical preference or aversion after repeated pairings with saline; the time spent in the compartment associated with the treatments during the pretest and posttest were similar (figure 4.10b; p-value>0.05).

We also assessed the locomotor activity during both pretest and posttest by measuring the distance traveled in the conditioning box.

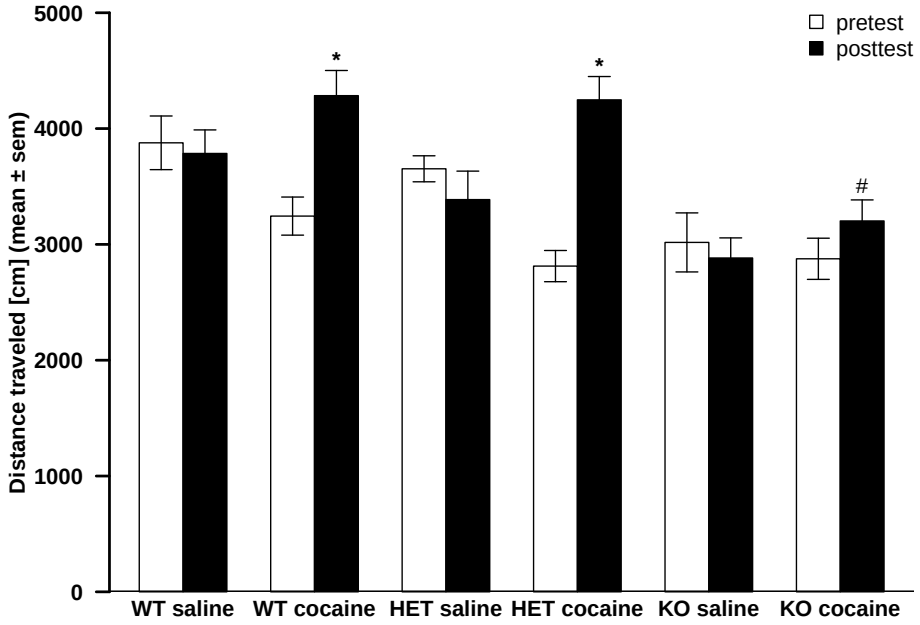
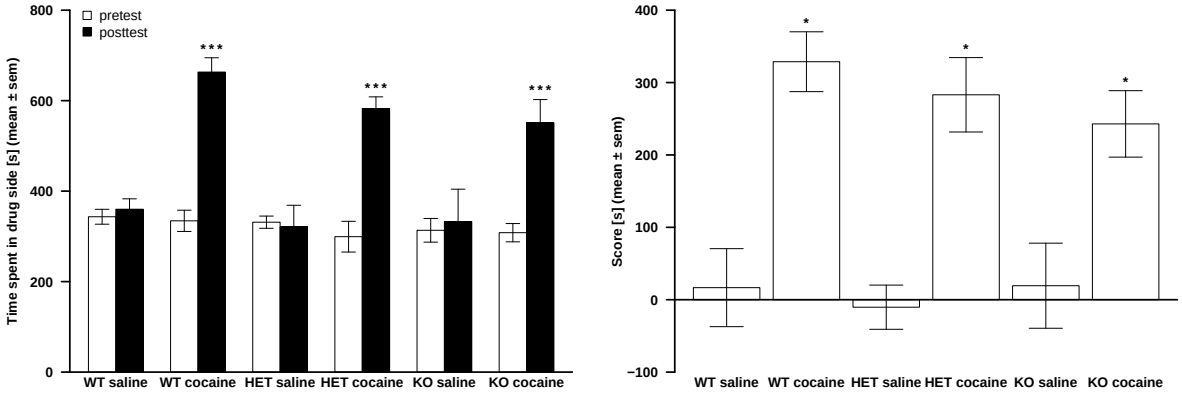


Figure 4.11: **Conditioned Place Preference in males: Distance traveled in the arena during pretest and posttest.** The distance traveled in the arena was measured during pretest and posttest to evaluate exploratory behavior. The distances traveled in the arena are represented as bars corresponding to the mean ± SEM of 8-12 mice. Tukey post hoc test: * p-value<0.05 versus pretest and # p-value<0.05 versus the cocaine-treated groups posttest values.

During pretest, all mice readily explored the arena (figure 4.11). All saline-treated animals exhibited similar locomotor activities during pretest and posttest sessions. On the other hand, the cocaine-treated groups exhibited a significant increase in locomotor activity during posttest ($F_{(1,110)}$(tests)=15.6, p-value<0.001 and $F_{(1,110)}$(treatment x tests)=25.0, p-value<0.001), except for the ppHcrt KO mice ($F_{(2,110)}$(genotypes)=18.8, p-value<0.001). The ppHcrt KO mice did not display any hyperlocomotion following repeated cocaine administrations; the distance traveled in the arena during posttest was similar to pretest. These results were consistent with data obtained with the locomotor sensitization procedure on figure 4.1.

4.5.2 Females

This procedure was also performed in females in order to determine whether gender could affect conditioned place preference in ppHcrt KO mice.



(a) Pretest vs Posttest

(b) Score

Figure 4.12: **Conditioned Place Preference in females.** Cocaine induced a rewarding effect in all genotype, as conditioned place preference was observed in all genotypes. In the control groups, no preference was observed following repeated saline injections. The results are displayed as the time spent in [s] in the compartment associated with repeated cocaine injections during pretest and posttest for each group tested. Each bar represents the mean time ± SEM of 7-8 mice. Tukey post hoc test: * p-value<0.05, and *** p-value<0.001

The time spent in the compartment associated with drug treatment during pretest was similar in all groups (figure 4.12a). Following conditioning, the saline-treated groups did not exhibit any significant preference or aversion. In the cocaine-treated groups, all animals exhibited a robust preference for the compartment associated with repeated cocaine injections ($F_{(1,76)}$(treatments)=25.0, p-value<0.001), whatever their genotype ($F_{(2,76)}$(genotypes)=1.9, p-value>0.05). The time spent during posttest in the compartment paired with repeated cocaine injections was 551.03 ± 39.14 s for ppHcrt KO mice , 582.43 ± 47.93 s for HET and 663.17 ± 54.92 s for WT mice.

The score was 242 ± 46 s for KO , 283 ± 51 s for HET and 328 ± 41 s for WT mice. The score was statistically different relative to saline-treated groups (p-value<0.05). The control group did not exhibit any statistical preference or aversion after conditioning with saline; the time spent in the compartment during the pretest and posttest were similar (figure 4.12b; p-value>0.05).

The distance traveled in the arena was also measured in pretest and posttest to evaluate the exploratory behavior of female mice.

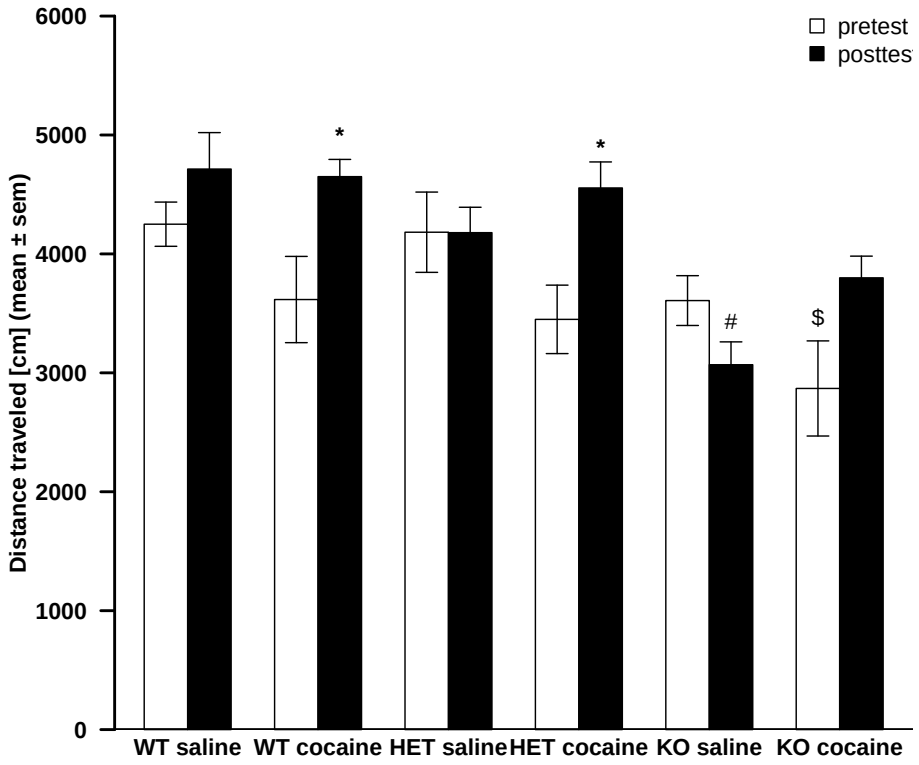
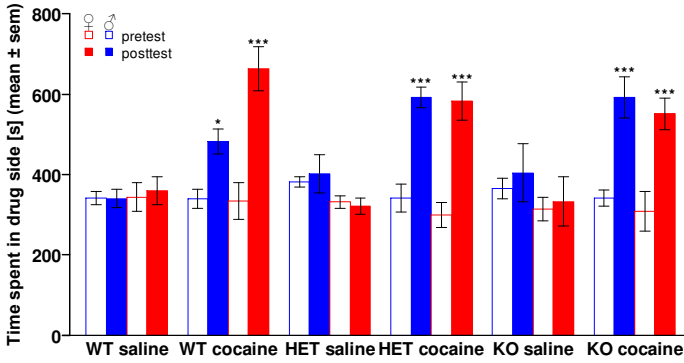


Figure 4.13: **Conditioned Place Preference in females: Distance traveled during pretest and posttest.** The distance traveled in the arena was measured during pretest and posttest to evaluate exploratory behavior. The distances ed in the arena are represented as bars corresponding to the mean ± SEM. Tukey post hoc test: * p-value<0.05 versus pretest. $ p-value<0.05 versus pretest in WT and HET saline-treated groups. # p-value<0.05 versus posttest, except the KO cocaine group posttest value.

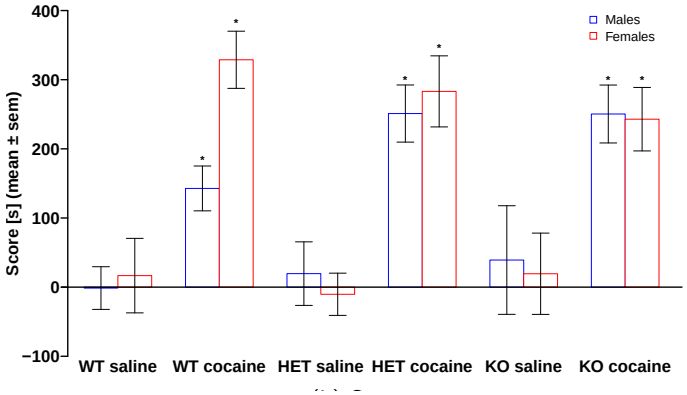
In the saline-treated groups, WT and HET females did exhibit similar locomotion activity during posttest compared to pretest. In contrast, the distance traveled by KO mice significantly differed from WT and HET posttest values. As expected, the cocaine treatment induced a significant increase in the locomotor activity ($F_{(1,76)}$(tests)=12.9, p-value<0.001 and $F_{(1,76)}$(treatment x tests)=13.1, p-value<0.001), but KO females exhibited a reduced cocaine effect ($F_{(2,76)}$(genotypes)=16.9, p-value<0.001) compared to HET and WT littermates (see figure 4.2).

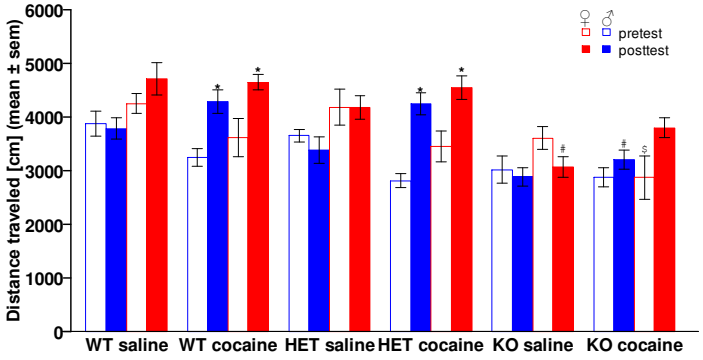
4.5.3 Comparison males between females

Comparisons between gender are illustrated on figure 4.14.



(a) Pretest vs Posttest





(c) Distance traveled

Figure 4.14: **Conditioned Place Preference: Comparisons males vs females.** (a). Cocaine induced a rewarding effect in all genotypes in males (blue) and females (red), as conditioned place preference was observed in every genetic background and in both gender. In the control groups, no preference was observed following repeated saline injections. (b). Preference for the drug associated side is represented by the scores, defined by the difference in [s] between the postconditioning phase and the preconditioning phase. (c). The distance traveled in the arena was measured during pretest and posttest to evaluate exploratory behavior. The results are displayed as the time spent in [s] in the compartment associated with repeated cocaine injections during pretest and posttest for each group tested. Each bar represents the mean time ± SEM of 7-8 mice. Tukey post hoc test: * p-value$<$0.05 and * p-value$<$0.001 versus pretest. $ p-value$<$0.05 versus pretest in WT and HET saline-treated groups. # p-value$<$0.05 versus posttest, except the KO cocaine group posttest value.

All the different groups exhibit similar exploratory behaviors during pretest. In the cocaine-treated groups, the time spent in the compartment paired with cocaine during posttest was increased in all groups. Significant treatments effect ($F_{(1,186)}$(treatments)=39.2, p-value<0.001), testing sessions ($F_{(1,186)}$(tests)=76.1, p-value<0.001) and treatment x testing sessions interaction ($F_{(1,186)}$(treatments x tests)=61.5, p-value<0.001) were detected. However, the rewarding effect of cocaine was independent of genetic background ($F_{(2,186)}$(genotypes)=0.3, p-value>0.05) and gender ($F_{(1,186)}$(gender)=1.4, p-value>0.05).

As expected, mice did not exhibit any preference for the environment paired with saline injection and in the other hand, all mice, whatever their genotype and gender, displayed a preference for the environment paired with cocaine administration (figure 4.14b).

Considering the distance traveled in the arena (figure 4.14c), WT and HET animals showed robust locomotor activity in the arena as compared to ppHcrt KO mice ($F_{(2,186)}$(genotypes)=34.6, p-value<0.001). Locomotor activity was influenced by the testing sessions ($F_{(1,186)}$(tests)=28.5, p-value<0.001) and the treatment ($F_{(1,186)}$(treatments x tests)=37.3, p-value<0.001). Overall, female mice manifested an increased exploratory behavior compared to males in the CPP apparatus ($F_{(1,186)}$(gender)=29.1, p-value<0.001).

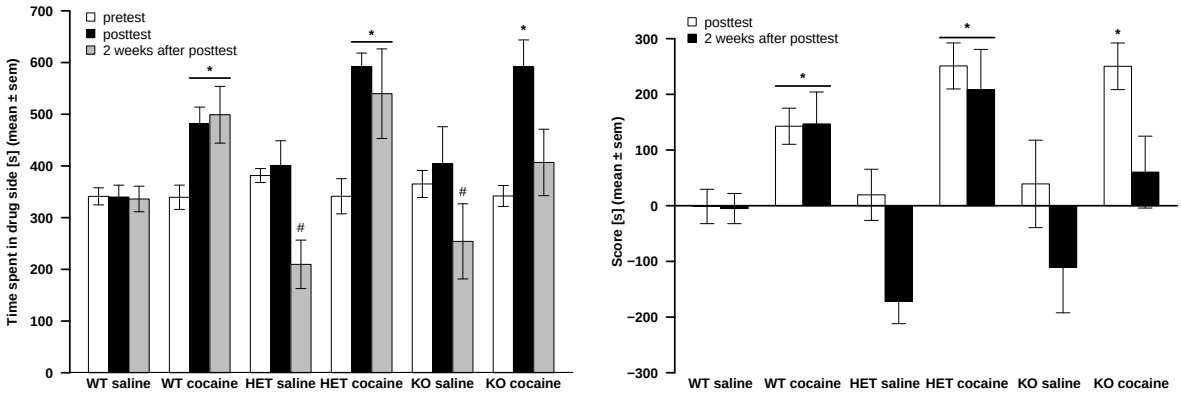
4.6 Conditioned place preference in ppHcrt KO mice : a longitudinal approach

A few studies reported that conditioned place preference could be maintained for up to 4 weeks in rodents [276, 277]. The place preference induced by the drug persists in the absence of active extinction procedure. In the current report, mice were retested two weeks after conditioning to check whether rewarding effect could be maintained in KO, HET and WT mice. After two weeks of passive extinction in their home cage, mice were once again exposed to the CPP arena and the time spent in the compartment previously paired with drug treatment was measured (summarized in table 4.15).

4.6.1 Males

Treatments	Genotypes	Pretest	Posttest	After 2-weeks of passive extinction
Saline	WT	341 ± 16	340 ± 23	336 ± 25
	HET	381 ± 13	401 ± 48	210 ± 47
	ppHcrt KO	365 ± 26	404 ± 72	254 ± 73
Cocaine 15 mg/kg	WT	339 ± 23	482 ± 32*	499 ± 55*
	HET	341 ± 34	592 ± 26*	540 ± 87*
	ppHcrt KO	342 ± 20	592 ± 51*	407 ± 64

Table 4.1: **Conditioned Place Preference in males.** Summary of the time spent in the environment paired with repeated exposures to either saline or 15 mg/kg of cocaine during pretest, posttest and the testing session 2 weeks after the last conditioning. Tukey post hoc test: * p-value<0.05 versus pretest.



(a) Pretest vs Posttests

(b) Score

Figure 4.15: **Conditioned Place Preference in males: Assessment of place preference two weeks after conditioning.** Cocaine induced a rewarding effect in all mice. (a). The results are displayed as the time spent in [s] in the compartment associated with repeated cocaine injection during pretest and posttest for each group tested. (b). Preference for the drug associated side is represented by the scores, defined by the difference in [s] between the post-conditioning phase and the preconditioning phase. For both graphs, each bar represents the mean time ± SEM of 8-12 mice. Tukey post hoc test: * p-value<0.05 versus pretest only. # p-value<0.05 versus pretest and posttest.

Two weeks after conditioning, the saline-treated groups spent similar or reduced amount of time in the compartment associated with repeated saline injections; HET and ppHcrt KO mice

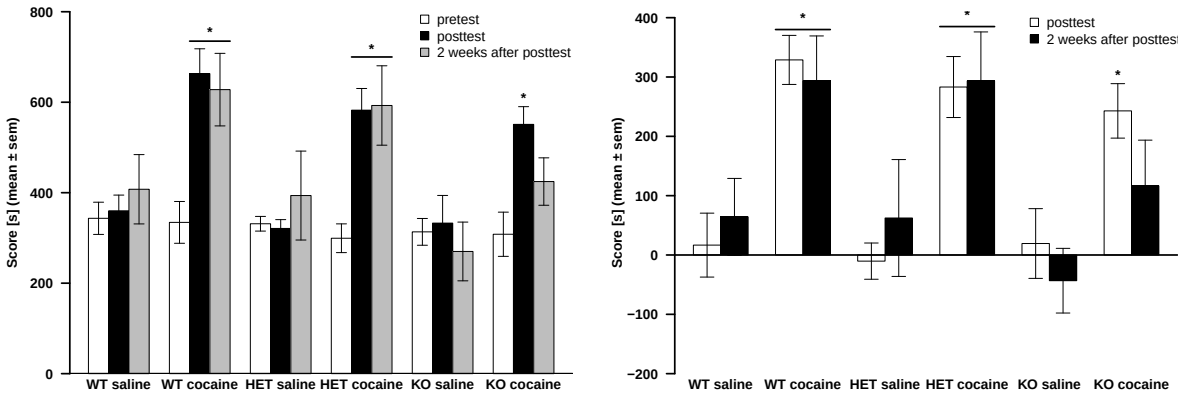
exhibited a significant reduction in the time spent in the compartment paired with saline injection compared to the pretest. Two weeks after conditioning, WT and HET mice previously treated with cocaine maintained their preference ($F_{(1,153)}$(treatments)=42.7, p-value<0.001); the time spent in the compartment previously paired with cocaine was similar to that obtained during posttest and was still statistically significant compared to pretest ($F_{(2,153)}$(tests)= 16.6, p-value<0.001 and $F_{(2,153)}$(treatments x tests=16.7, p-value<0.001). In contrast, ppHcrt KO mice stopped expressing a preference for the compartment previously paired with repeated cocaine treatments ($F_{(4,153)}$(genotypes x tests)=3.4, p-value<0.05).

4.6.2 Females

To determine whether passive extinction could be affected by gender, the procedure was repeated with female mice (summarized in table 4.2).

Treatments	Genotypes	Pretest	Posttest	After 2-weeks of passive extinction
Saline	WT	343 ± 36	360 ± 35	408 ± 77
	HET	331 ± 16	321 ± 19	394 ± 98
	ppHcrt KO	313 ± 30	333 ± 61	270 ± 65
Cocaine 15 mg/kg	WT	334 ± 46	663 ± 55*	628 ± 80*
	HET	299 ± 32	582 ± 48*	593 ± 88*
	ppHcrt KO	308 ± 49	551 ± 39*	425 ± 52

Table 4.2: **Conditioned Place Preference in females.** Summary of the time spent in the environment paired with repeated exposures to either saline or 15 mg/kg of cocaine during pretest, posttest and the testing session 2 weeks after the last conditioning. Tukey post hoc test: * p-value<0.05 versus pretest.



(a) Pretest vs Posttests

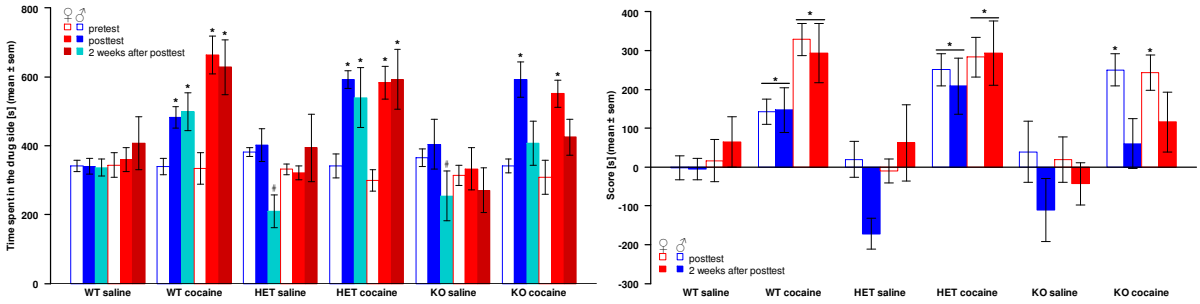
(b) Score

Figure 4.16: **Conditioned Place Preference in females: Assessment of place preference two weeks after conditioning.** Cocaine induced a rewarding effect in all animals. (a). The results are displayed as the time spent in [s] in the compartment associated with repeated cocaine injection during pretest and posttest for each group tested. (b). Preference for the drug associated side is represented by the scores, defined by the difference in [s] between the postconditioning phase and the preconditioning phase. For both graphs, each bar represents the mean time ± SEM of 8-12 mice. Tukey post hoc test: * p-value<0.05 versus pretest.

After experiencing two weeks of passive extinction, the saline-treated groups spent similar

amount of time compared to pretest in the compartment of reference. In the cocaine-treated groups, WT and HET mice maintained a significant place preference for up to two weeks following conditioning ($F_{(1,114)}$(treatments) =32.1, p-value<0.001, $F_{(2,114)}$(tests)=12.4, p-value<0.001 and $F_{(2,114)}$(treatments x tests)=11.3, p-value<0.001). In contrast, female ppHcrt KO mice did not exhibit any robust preference for the context previously paired with repeated cocaine administration ($F_{(2,114)}$(genotypes)=4.2, p-value<0.05).

4.6.3 Comparison between males and females



(a) Pretest vs Posttests

(b) Score

Figure 4.17: **Conditioned Place Preference in females: Comparison males versus females.** Cocaine induced rewarding effect in all genotype and in both gender, males (blue) and females (red), as conditioned place preference was observed in all genetic background and in both gender. (a). The results are displayed as the time spent in [s] in the compartment associated with repeated cocaine injection during pretest and posttest for each group tested. (b). Preference for the drug associated side is represented by the scores, defined by the difference in [s] between the postconditioning phase and the preconditioning phase. For both graphs, each bar represents the mean time ± SEM of 8-12 mice. Tukey post hoc test: * p-value<0.05, versus the respective pretest session.

Figure 4.17 summarizes the data collected in males and females. During posttest, cocaine treatment significantly influenced drug seeking behavior through the different testing sessions ($F_{(1,267)}$(treatments)=74.4, p-value<0.001, $F_{(2,267)}$(tests)=24.1, p-value<0.001 and $F_{(2,267)}$(treatments x tests)=26.9, p-value<0.001) whatever their gender and genotypes ($F_{(1,267)}$(gender)=1.1, p-value>0.05 and $F_{(2,267)}$(genotypes)=2.4, p-value=0.08). However, after experiencing two weeks of passive extinction, both KO females and males did not display any preference for the compartment previously paired with cocaine injections ($F_{(4,267)}$(genotypes x tests)=3.5, p-value<0.01), whereas WT and HET did exhibit a robust preference compared to pretest.

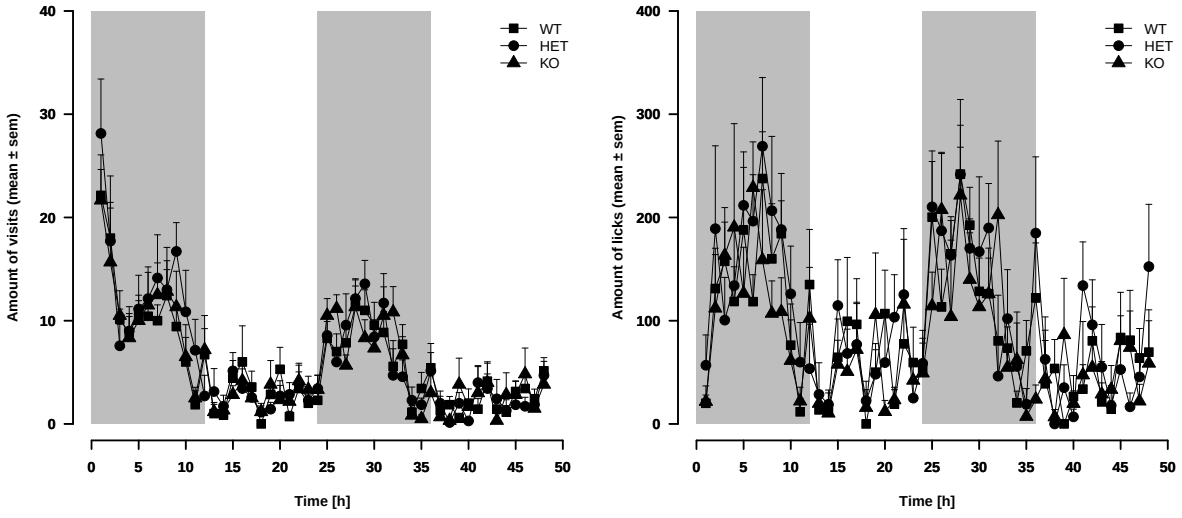
4.7 Drinking behaviors in ppHcrt KO female mice

Only few investigations reported the relationship between alcohol-seeking and taking and the hypocretin system. Previous approaches showed that Hcrt may play a role in alcohol-related behaviors. Hcrt-1 injected in the LH stimulated voluntary alcohol intake and pretreatment with the selective antagonist of HcrtR1 (SB-334867) reduced operant alcohol self-administration and suppressed a cue induced reinstatement of alcohol-seeking in rats [221, 223–225]. Further, repeated intake of alcohol leads to a reduction in Hcrt gene expression in the LH [278]. So far, no reports described alcohol-related behaviors in constitutive ppHcrt KO mice.

Female ppHcrt KO mice, HET and WT littermates were tested in a novel apparatus where animals behavior could be monitored in terms of the number and duration of visits, nosepokes and licks. This apparatus promotes social interactions, as up to twelve mice can be housed in a single IntelliCage (IC).

4.7.1 Habituation phase with water

During the habituation phase in the IC, only water was provided at each corner. Each mouse activity was recorded and the number of corner visits was hourly assessed to determine the exploratory behavior of each genotype in the IntelliCages during the first 48h (figure 4.18a). It has been reported that hypocretin deficient mice exhibit disrupted sleep/wake cycles which possibly affect circadian rhythm pattern. This procedure allowed determining how ppHcrt KO mice behave over time compared to WT mice.

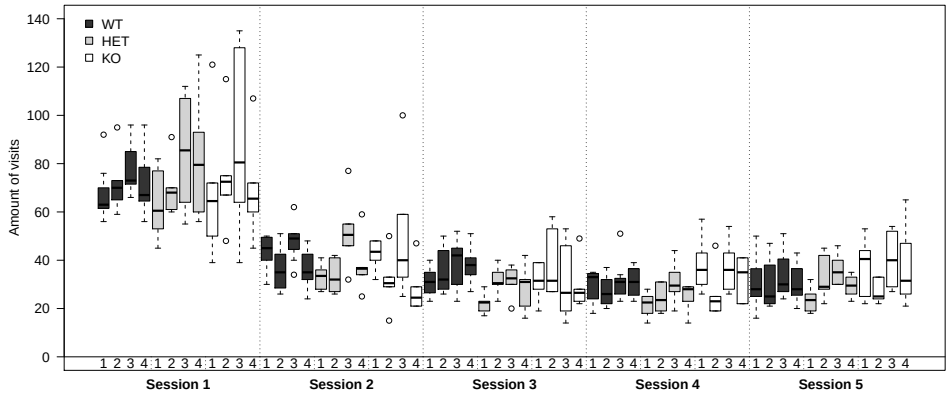


(a) Visits pattern (b) Licks pattern

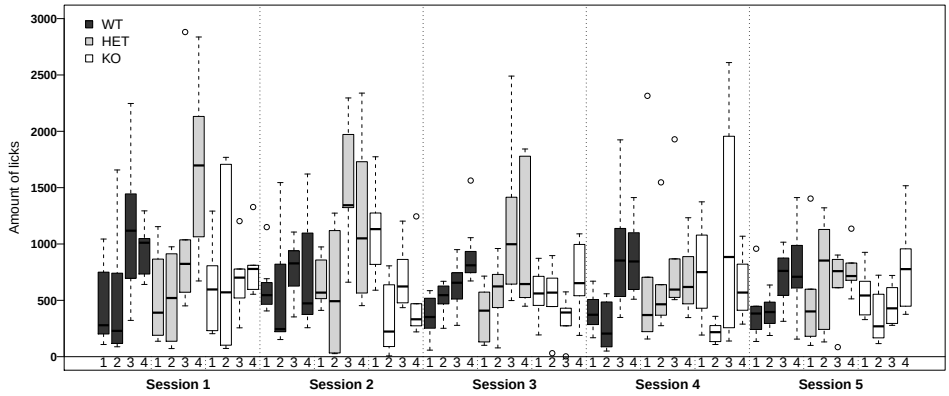
Figure 4.18: **Intellicages®: Basal activity in females.** The total number of visits (a) and licks (b) of ppHcrt KO mice (filled triangle; n=6), HET (filled circle; n=7) and their WT (filled square; n=8) littermates was measured each hour of the habituation phase. Here the first 48h of the first habituation session are represented as the mean visits or licks ± SEM of 6-7 mice. The grey zones represent the dark phase and the non-colored zones illustrate the light phase.

In the first three hours of exposition in the IC, the activity was enhanced due to novelty-induced exploration as compared to the following hours (figure 4.18a). The number of corner visits decreased over time and reached baseline values. The exploratory behavior of WT, HET and ppHcrt KO mice was similar during the first 48h session in the IC and a circadian rhythm was observed ($F_{(2,815)}$(genotypes)=0.8, p-value>0.05). High exploratory behavior was observed during the dark phase and low activities were found during the light phase ($F_{(47,815)}$(time)=19.3, p-value<0.001).

We then looked at the liquid consumption (figure 4.18b). Liquid consumption was variable between subjects of the same genotype and followed a circadian rhythm. It seemed that HET females consumed more water than WT and KO mice ($F_{(2,815)}$(genotypes)=4.1, p-value<0.05). The number of licks were significantly increased during the dark phase relative to the light phase ($F_{(47,815)}$(time)=7.3, p-value<0.001).



(a) Visits pattern



(b) Licks pattern

Figure 4.19: **Intellicages®: Habituation phase in females.** The daily activity (a) in the IntelliCage apparatus was measured, as well as the total number of licks (b), was assessed for each genotype (ppHcrt KO mice (white; n=6), HET (grey; n=7) and WT (black; n=7)) at each corner (corner 1, corner 2, corner 3 and corner 4) for five consecutive days.

During the habituation phase, WT, HET and ppHcrt KO mice were also tested to assess whether mice exhibited any preference for the different corners (figure 4.19a). Water was pro-

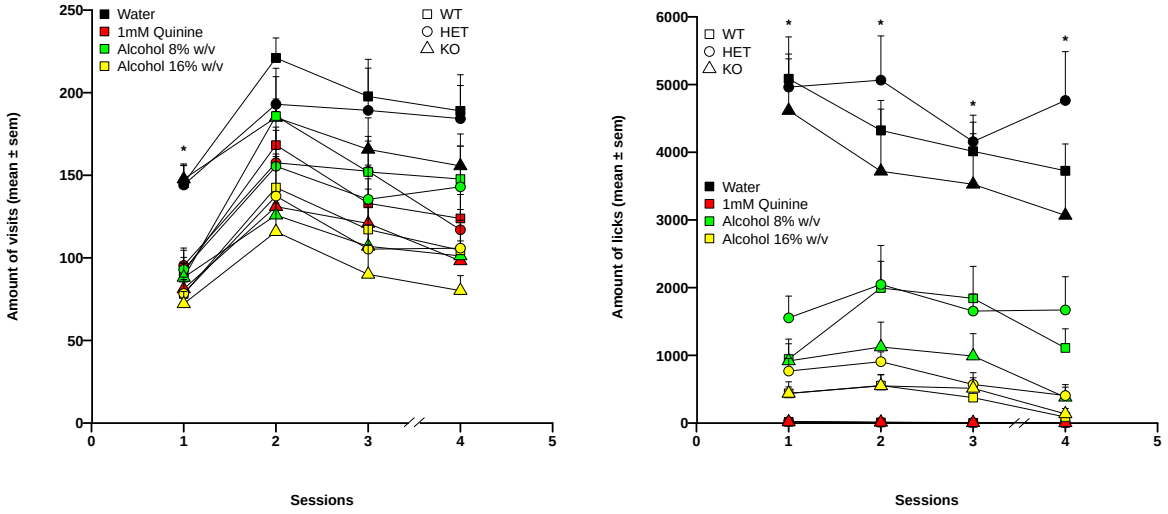
vided at each corner. The number of visit was influenced by the time spent in IntelliCages (figure 4.19a). The number of visits during session 1 was significantly higher compared to the following sessions ($F_{(4,320)}$(sessions)=138.6, p-value<0.001). However, it seemed that HET mice displayed lower amount of visits over sessions ($F_{(4,320)}$(genotypes)=138.6, p-value<0.001).

In contrast, the number of licks varied between corners and genotypes over the sessions (figure 4.19b). However, the variability between animals tended to decrease over time ($F_{(4,320)}$(sessions)=2.2, p-value>0.05). Drinking behavior was influenced by genetic background, it seemed that HET mice consume more water than WT and KO mice ($F_{(2,320)}$(genotypes)=5.4, p-value<0.01).

4.7.2 Testing phase with different liquid solutions

Amount of visits and licks

After two days resting in their home cages, animals returned in the IC where novel liquid solutions (water, 1mM quinine, alcohol 8% and alcohol 16%) were introduced (figure 4.21). Each corner was associated to one specific liquid solution and the number of visits and licks were averaged from three consecutive days repeated three times with a four days resting period in the home cage between each session (figure 4.21). In the previous procedure, we observed that two weeks of passive extinction was sufficient to suppress the preference for an environment previously paired with cocaine injections in ppHcrt KO animals, so we monitored again mice behavior two weeks after the last exposure to the IC (the challenge session).

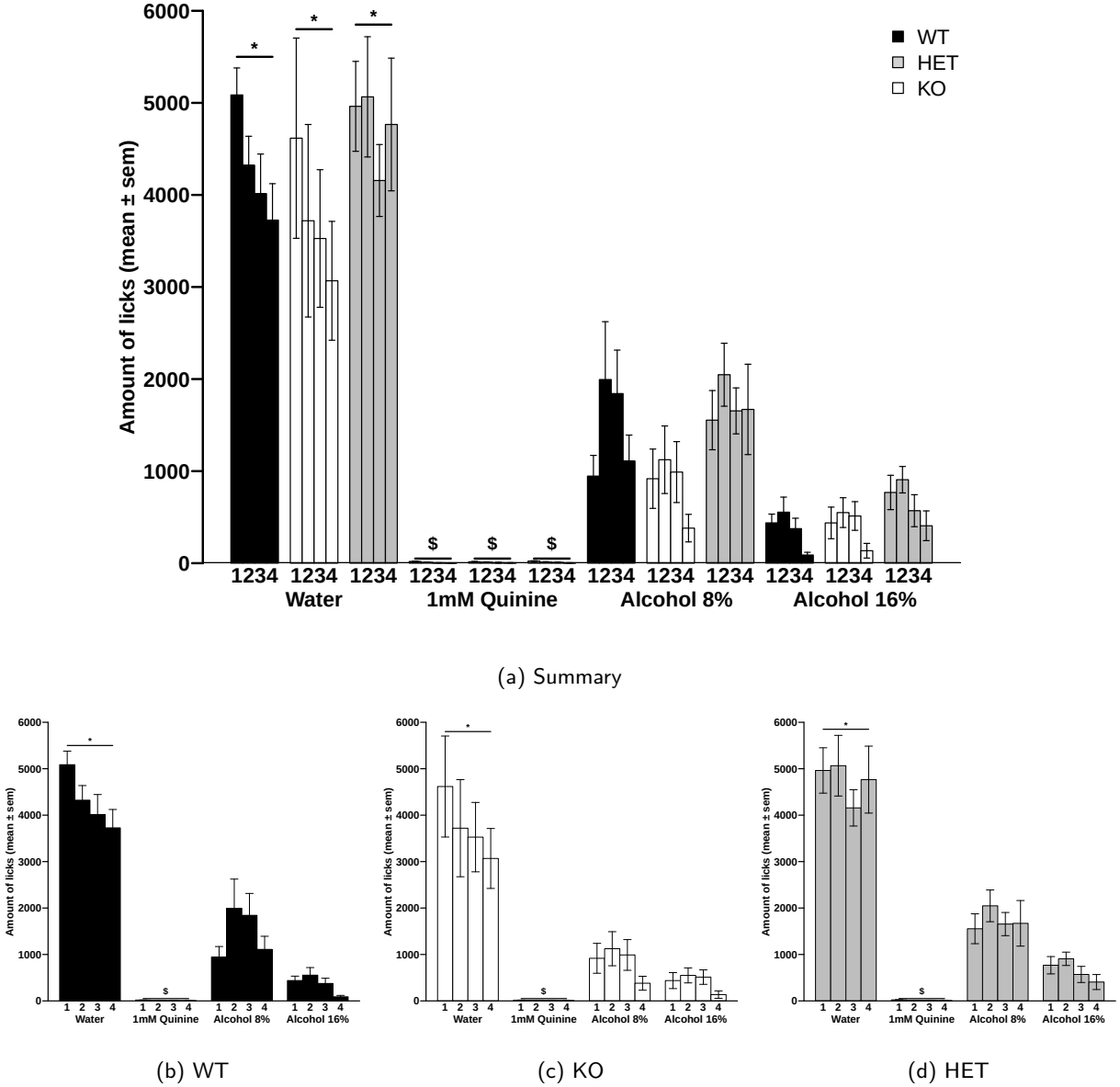


(a) Visits (b) Licks

Figure 4.20: **Intellicages®: Exploration and liquid consumption behaviors in females.** Female mice were exposed in the IntelliCages for 3 testing sessions (session 1 to 3) before experiencing alcohol abstinence for two weeks in their home cage (session 4). The numbers of visits (a) and licks (b) of WT (square; n=7), HET (circle; n=7) and ppHcrt KO mice (triangle; n=6) were assessed by counting the number of entries and licks made at the four different corners where water (black), 1mM quinine (red), alcohol 8% (green) and alcohol 16% (yellow) were provided. The data are represented as the mean ± SEM. Asterisks denote a p-value<0.05 versus the other solutions.

The liquid solutions provided at each corner significantly influence the exploratory pattern of mice ($F_{(3,272)}$(liquid solutions)=43.3, p-value<0.001). On session 1, the corner associated with water had the highest number of visits compared to the other liquid solutions corner (figure 4.20a). No statistical differences in the number of visits were found between genotypes within each corner (post hoc test: p-value>0.05). On sessions 2 and 3, the number of visits was higher in the water corner compared to the other liquid solutions corners. Interestingly, the number of visit in the corner paired with alcohol 8% increased slightly, whereas the number of entries in the corner paired with alcohol 16% decreased (figure 4.20a). On session 2 and 3, ppHcrt KO mice showed low level of exploratory behavior relative to WT ($F_{(2,272)}$(genotypes)=9.5, p-value<0.001). In contrast, HET animals exhibited similar activity to that of WT. During the challenge session (session 4), ppHcrt KO mice, HET and WT animals exhibited similar exploratory behaviors compared to session 3. However, ppHcrt KO mice tended to exhibit a lower exploratory behavior relative to WT and HET, but this tendency was not statistically significant in our sample size (figure 4.20a).

The amount of licks was influenced by the liquid solutions available ($F_{(3,272)}$(liquid solutions)=579.2, p-value<0.001) (figure 4.21a). Animals always exhibited a robust preference for water and a robust aversion for 1mM quinine solution (figure 4.20b). The number of licks for both water and 1mM quinine were significantly different from the other liquid solutions during the whole procedure (figure 4.21a). Animals showed a slight preference for alcohol 8% w/v compared to alcohol 16%. In WT, the number of licks for water decreased over time with a concomitant increase for alcohol 8% (figure 4.21b). KO mice did also show a reduction in water consumption, but the number of licks for alcohol 8% and 16% remained stable from session 1 to session 3 (figure 4.21c). HET animals maintained similar pattern of licking behaviors among each corner over time, except for alcohol 16% (figure 4.21d). During the challenge session, mice did not demonstrate any robust rebound in the intake for alcohol 8% and alcohol 16% (figures 4.22c and 4.22d). The number of licks was decreased for alcohol 8% and alcohol 16% in WT and ppHcrt KO ($F_{(2,272)}$(genotypes)=15.5, p-value<0.001).



(a) Summary

(b) WT (c) KO (d) HET

Figure 4.21: **Intellicages®: Liquid consumption behaviors in females.** Female mice were exposed in the IntelliCages for 3 testing sessions (session 1 to 3) before experiencing alcohol abstinence for two weeks in their home cage (session 4). The amounts of licks of WT (black; n=7), HET (grey; n=7) and ppHcrt KO mice (white; n=6) were assessed by counting the number of licks made at the four different corners (water, 1mM quinine, alcohol 8% and alcohol 16%). The data are represented as the mean ± SEM. * p-value<0.05 versus the other liquid solutions (preference) and $ p-value<0.05 versus the other liquid solutions (aversion).

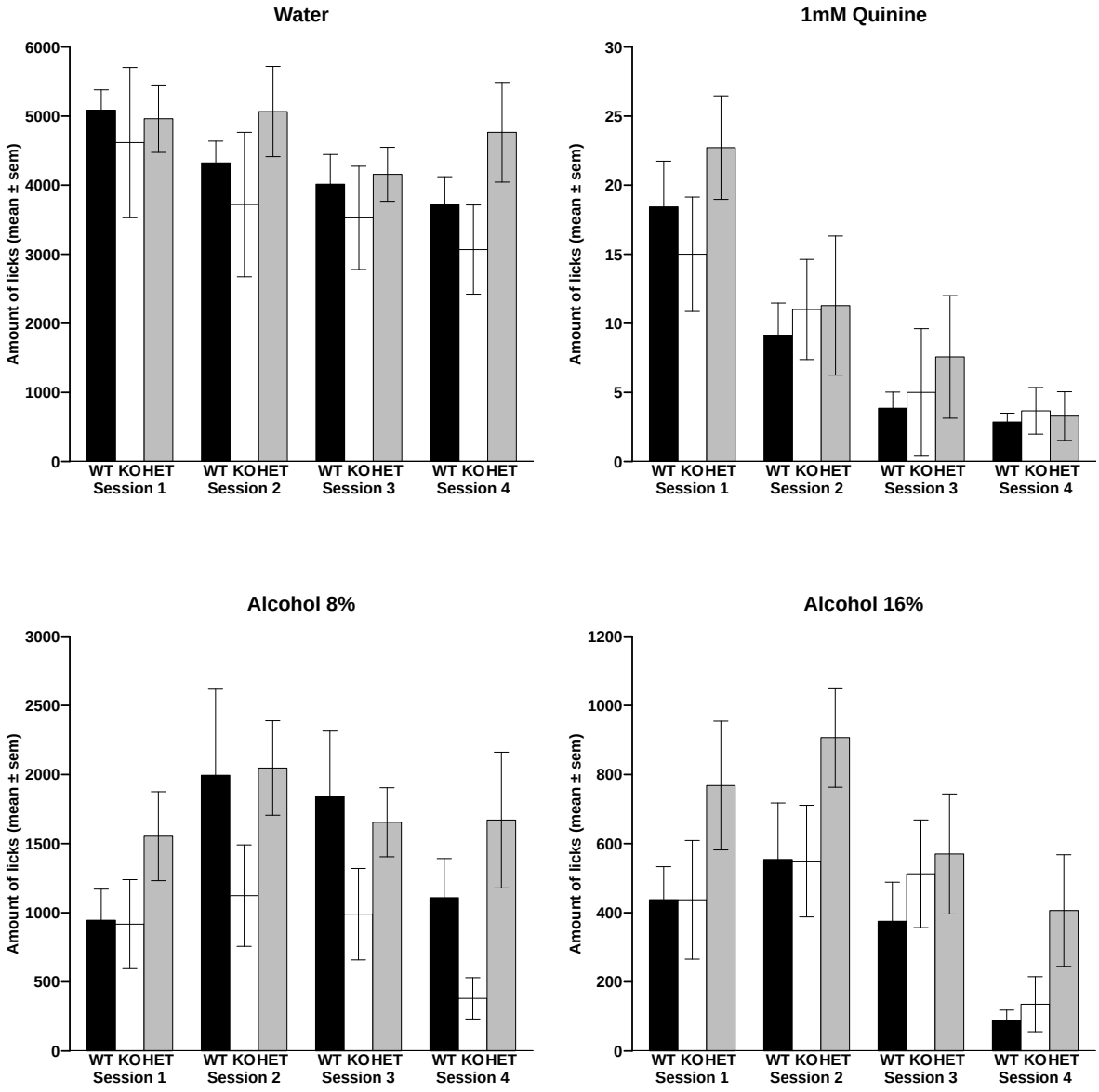
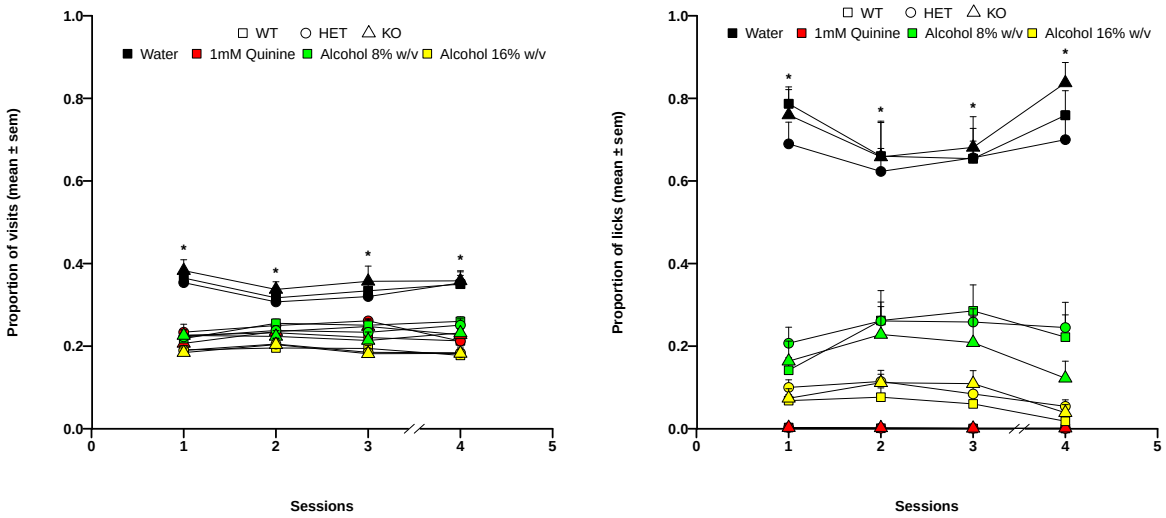


Figure 4.22: **Intellicages®: Detailed liquid consumption behaviors in females.** Female mice were exposed in the IntelliCages for 3 testing sessions (session 1 to 3) before experiencing alcohol abstinence for two weeks in their home cage (session 4). The amounts of licks of WT (black; n=7), HET (grey; n=7) and ppHcrt KO mice (white; n=6) were assessed at the four different corners: (a). water, (b). 1mM quinine, (c). alcohol 8% and (d). alcohol 16%). The data are represented as the mean ± SEM.

Proportion of visits and licks

We previously reported that cocaine sensitization was similar among each genotype after normalization of the data. In order to check whether the slight differences observed here were correlated to the total activity in the IntelliCage, the numbers of visits observed in each corner were related to the total number of visits performed during each session (figure 4.23).

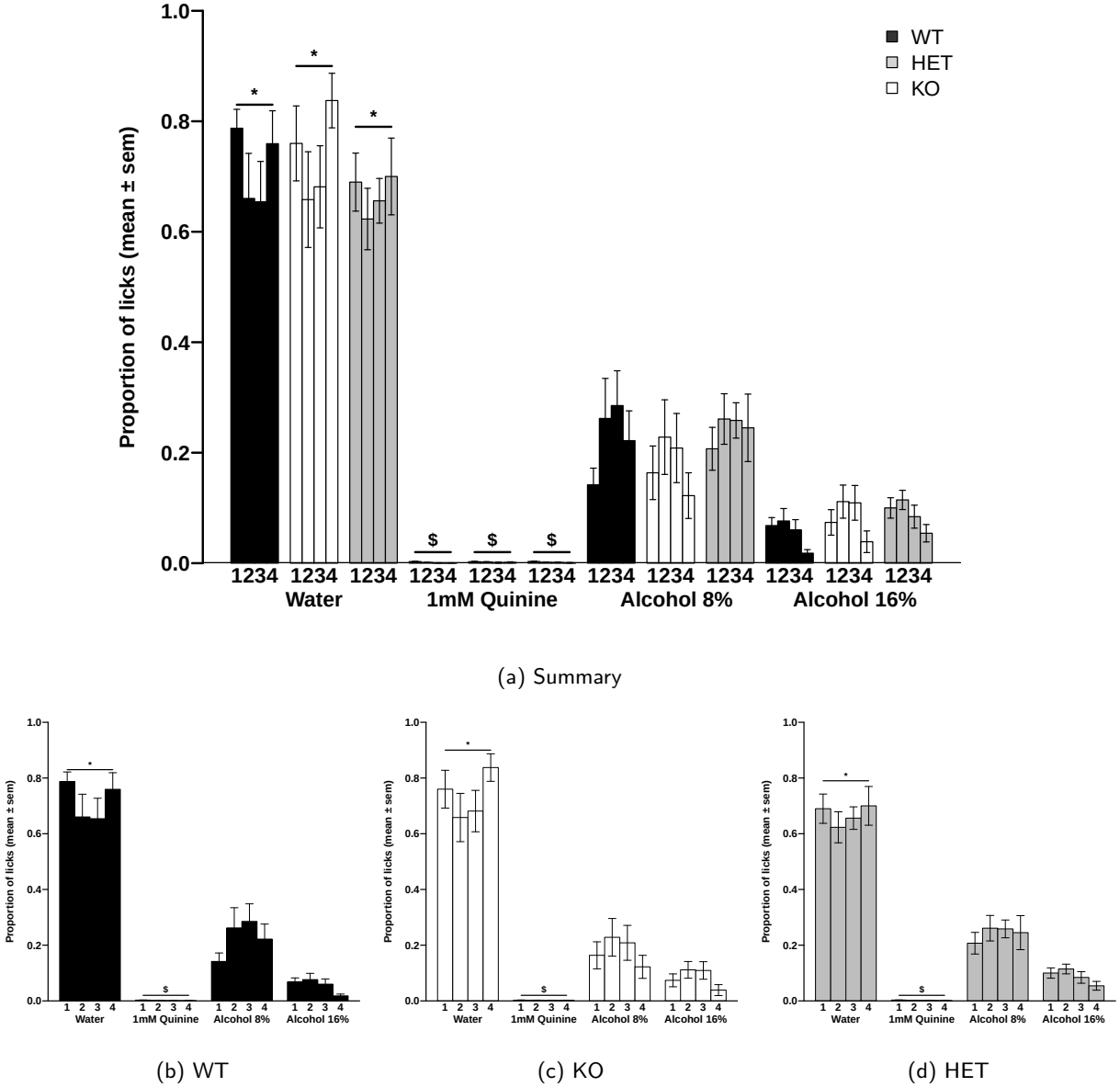


(a) Visits (b) Licks

Figure 4.23: **IntelliCages®: Proportion of visits and licks in females.** Female mice were exposed in the IntelliCages for 3 testing sessions (session 1 to 3) before experiencing alcohol abstinence for two weeks in their home cage (session 4). The proportions of visits (a) and licks (b) of WT (square; n=7), HET (circle; n=7) and ppHcrt KO mice (triangle; n=6) were assessed at the different corners, where water (black), 1mM quinine (red), alcohol 8% (green) and alcohol 16% (yellow) were provided, are represented as the mean ± SEM. Asterisk denote a p-value<0.05.

The proportion of visits demonstrated that ppHcrt KO mice did exhibit similar exploratory behavior relative to WT and HET over time in each corner ($F_{(2,272)}$(genotypes)=0.03, p-value>0.05) (figure 4.23a). The corner associated with water was significantly preferred compared to the other corners ($F_{(3,272)}$(liquid solutions)=239.7, p-value<0.001) and the proportion of visits remained stable in each corner. Surprisingly, the corner paired with alcohol 16% was the least visited corner. During the challenge session (session 4), animals did not exhibit any difference in proportion of visits compared to session 3, meaning that no relapse for alcohol-seeking was observed following two weeks of alcohol abstinence. All animals kept exhibiting a preference for the water corner and an aversion for the corner paired with alcohol 16%. The proportion of visits remained stable in the corners paired with alcohol 8% and 1mM quinine ($F_{(3,272)}$(sessions)=0.08, p-value>0.05).

The number of licks was also normalized with the total number of licks of the four corners (figures 4.23b, 4.24 and 4.25). When the data were normalized, no statistical differences in liquid consumption were observed between the different genetic background ($F_{(2,272)}$(genotypes)=1.3, p-value>0.05). However, the nature of the liquid solutions influenced the licking behavior, all animals preferring water over the three others liquid solutions ($F_{(3,272)}$(liquid solutions)=783.3, p-value<0.001) (figure 4.24). And the proportion of licks for 1mM quinine was significantly lower compared to the other solution from session 1 to session 3. During the challenge session, the proportion of licks was not statistically different from the other sessions for water, quinine and alcohol (8% and 16%), demonstrating no relapse for alcohol (figure 4.25).



(a) Summary

(b) WT (c) KO (d) HET

Figure 4.24: **Intellicages®: Liquid consumption behaviors in females.** Female mice were exposed in the IntelliCages for 3 testing sessions (session 1 to 3) before experiencing alcohol abstinence for two weeks in their home cage (session 4). The proportions of licks of WT (black; n=7), HET (grey; n=7) and ppHcrt KO mice (white; n=6) were assessed at the four different corners (water, 1mM quinine, alcohol 8% and alcohol 16%). The data are represented as the mean ± SEM. Tukey post hoc test: * p-value<0.05 versus the other liquid solutions (preference) and $ p-value<0.05 versus the other liquid solutions (aversion).

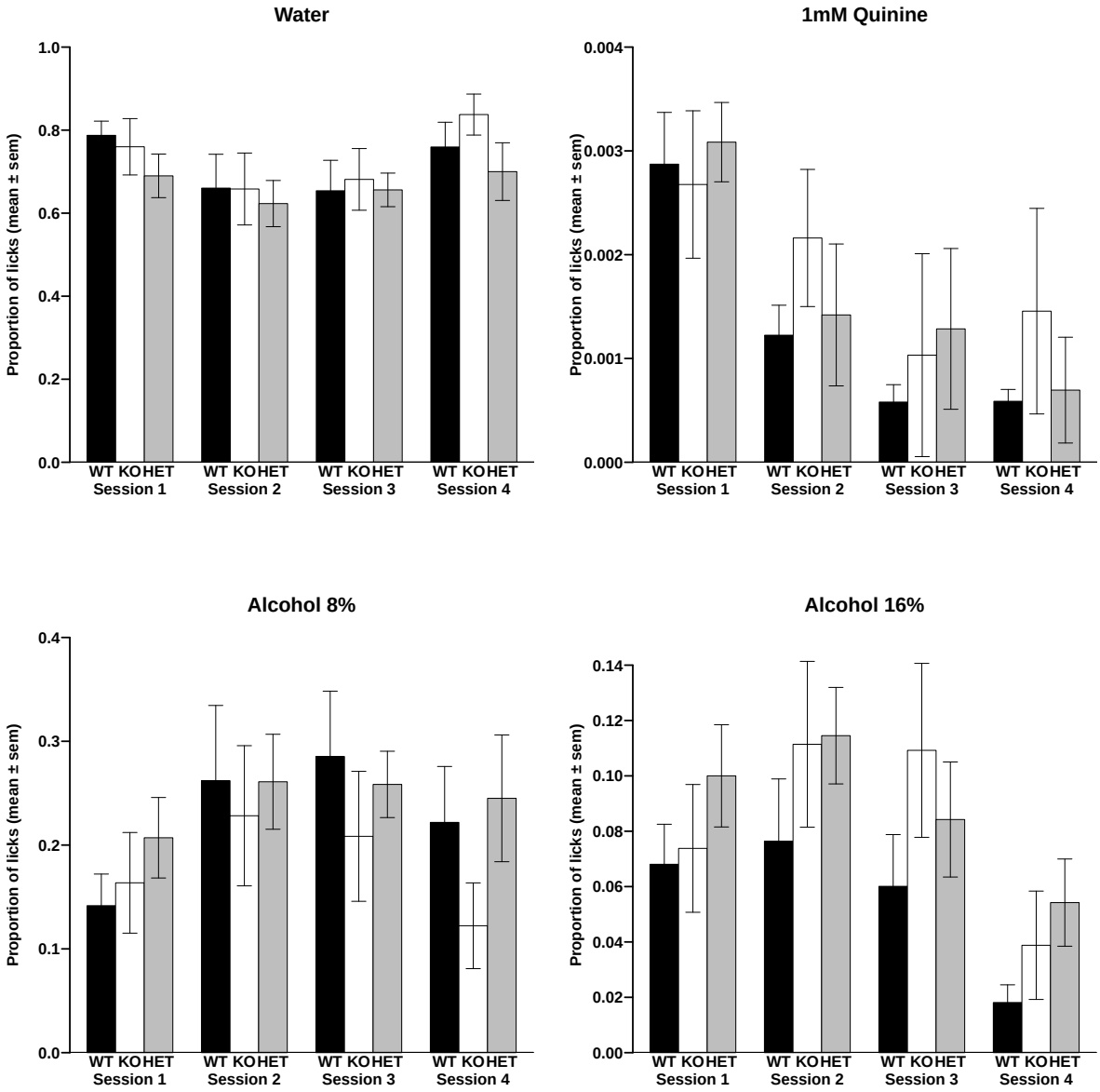
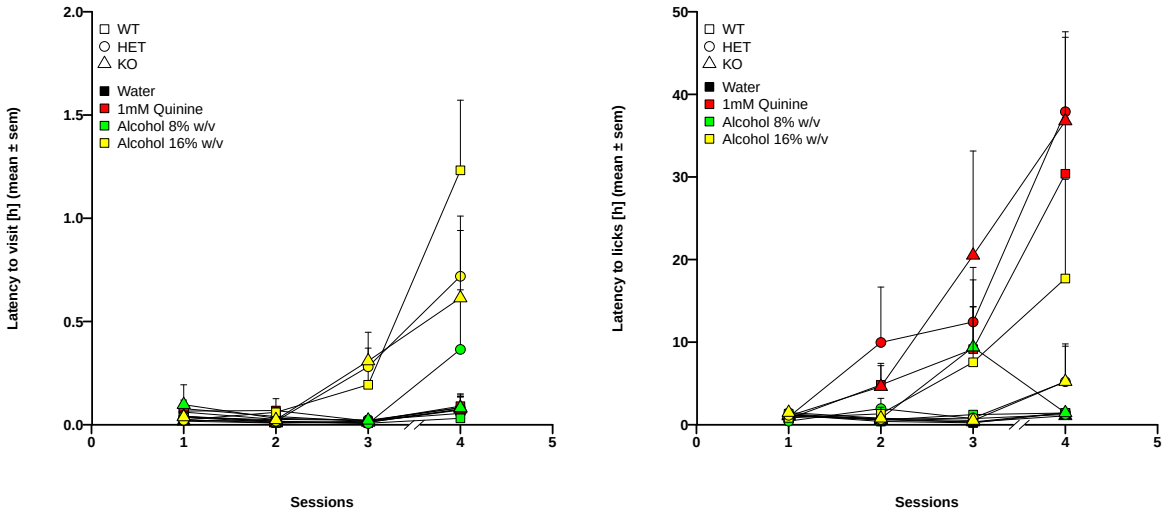


Figure 4.25: **Intellicages®: Detailed liquid consumption behaviors in females.** Female mice were exposed in the IntelliCages for 3 testing sessions (session 1 to 3) before experiencing alcohol abstinence for two weeks in their home cage (session 4). The proportions of licks of WT (black; n=7), HET (grey; n=7) and ppHcrt KO mice (white; n=6) were assessed at the four different corners: (a). water, (b). 1mM quinine, (c). alcohol 8% and (d). alcohol 16%). The data are represented as the mean ± SEM.

The latency to first visit and lick

In order to check whether mice learned to discriminate between corners and the latency to first visit and first lick was assessed in the three testing sessions and during the last challenging session (session 4).



(a) Latency to first visits (b) Latency to first licks

Figure 4.26: **IntelliCages®: Latency to first visit and lick at the four corners.** Female mice were exposed in the IntelliCages for 3 testing sessions (session 1 to 3) before experiencing alcohol abstinence for two weeks in their home cage (session 4). WT (square; n=7), HET (circle; n=7) and ppHcrt KO mice (triangle; n=6) latency to first visit (a) and lick (b) were measured in the different corners where water (black), 1mM quinine (red), alcohol 8% (green) and alcohol 16% (yellow) were provided. The data are represented as the mean ± SEM.

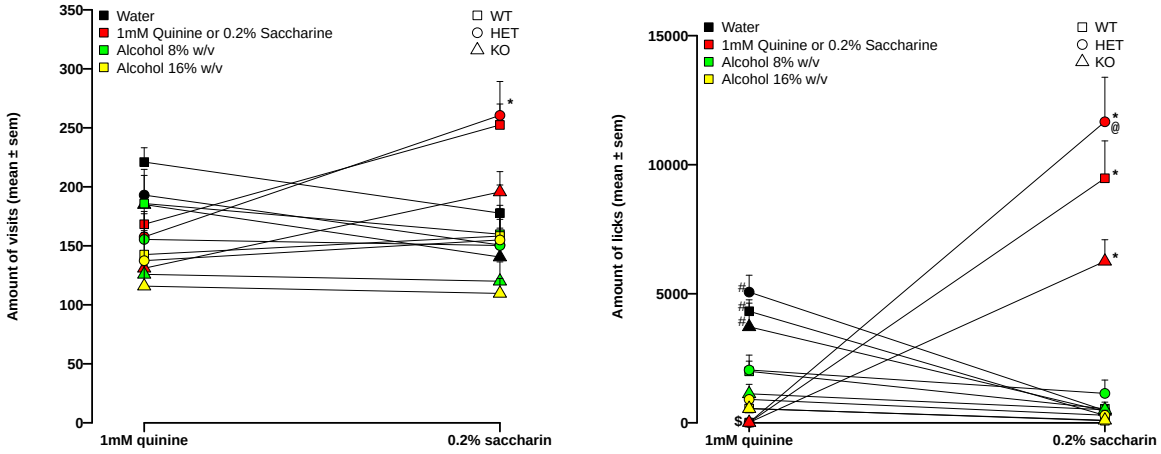
Animals readily explored each corner during the first two hours systemically (figure 4.26a). The latency to **first visit** was similar in all genotype from session 1 to session 3 for all corners ($F_{(2,272)}$(genotypes)=0.4, p-value>0.05). All mice displayed low latencies to first explore corners paired with water, quinine, alcohol 8% and an increased latency for first exploring the corner of alcohol 16%. During the challenge session, the latency to first visit the corner paired with water and quinine was similar to previous sessions. Only HET animals exhibited higher latency to first visit alcohol 8% (0.36 ± 0.3h) compared to ppHcrt KO (0.08 ± 0.05h) and WT mice (0.03 ± 0.01h). The latencies to first visit the corner paired with alcohol 16% reached the highest values indicating that animals manifested a real aversion for the corner paired with alcohol 16%. Statistical analysis confirmed that the latency to first visit corners was highly dependent on the liquid solutions available ($F_{(3,272)}$(liquid solutions)=22.2, p-value<0.001).

The latency to **first lick** ranged from the few minutes to few hours depending on the liquid solutions ($F_{(3,272)}$(liquid solutions)=31.8, p-value<0.001), but was not influenced by the genetic background ($F_{(2,272)}$(genotypes)=0.8, p-value>0.05). During the procedure, the latencies to first lick water reached a few minutes, whereas the latency to first lick the quinine solution reached hours (figure 4.26b). The latency to lick alcohol 8% and alcohol 16% was similar in session 1 and 2 for all genotypes. On session 3, the latency to first lick alcohol 8% and 16% increased in ppHcrt KO and WT mice, respectively. During the challenge session, the latency to first lick water and alcohol 8% was similar in all genotype. WT, KO and HET mice showed increased latencies to first lick alcohol 16%. Animals of all genotypes did exhibit a robust aversion for the quinine taste even after two weeks of passive extinction. The development of preference or aversion was maintained or reinforced during the entire procedure since a significant sessions effect ($F_{(3,272)}$(sessions)=31.8, p-value<0.001) was observed. Reduced number of animals per group explains the high variability of the latency to first lick. Further, a few animals only

visited the corners without licking quinine or alcohol 16%, whereas other mice chose to taste every solution, even in very limited amounts.

Introduction of a new liquid solution in the IC

The liquid solutions provided in the IntelliCages influenced the exploratory pattern and the liquid consumption behaviors of females. All mice displayed an aversion for bitter taste. We then investigated their sensitivity for sweet taste. During session of 3 consecutive days, quinine was replaced by 0.2% saccharin and the total number of visits and licks were again monitored (figure 4.27).



(a) Amount of visits

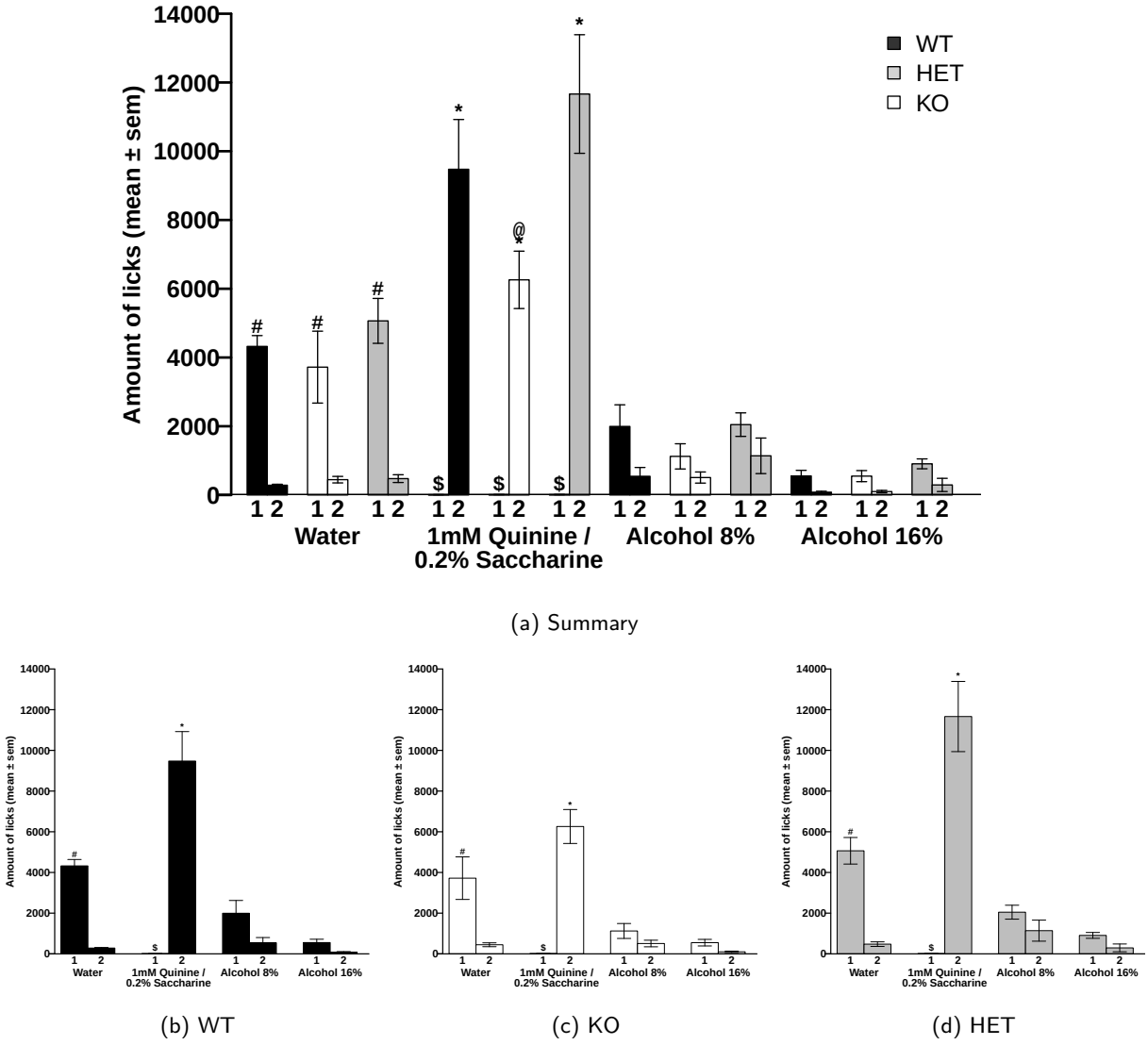
(b) Amount of licks

Figure 4.27: **IntelliCages®: Comparison between quinine and saccharine.** Female mice were challenged for both 1mM quinine and 0.2% saccharine in one week interval. The total number of visits (a) and licks (b) of WT (square; n=7), HET (circle; n=7) and ppHcrt KO mice (triangle; n=6) were measured in the different corners where water (black), 1mM quinine or 0.2% saccharine (red), alcohol 8% (green) and alcohol 16% (yellow) were provided. The data are represented as the mean ± SEM. # p-value<0.05 versus alcohol 8%, alcohol 16%, 1mM quinine (quinine session), water and 0.2% saccharine (saccharine session). $ p-value<0.05 versus water, alcohol 8% (quinine session). * p-value<0.05 versus water, alcohol 8%, alcohol 16% and 1mM quinine (saccharine and quinine sessions). @ p-value<0.05 HET versus KO (saccharin session).

Not surprisingly, replacement of quinine by saccharine shifted the amount of visits from the water corner to the saccharine corner ($F_{(3,136)}$(liquid solutions)=11.3, p-value<0.001) (figure 4.27a). Only WT and HET mice significantly visited more frequently the corner paired with saccharine, whereas ppHcrt KO did not. A significant genotype effect ($F_{(2,136)}$(genotypes)=9.8, p-value<0.001) was observed. Consequently, the number of visit in the water corner was reduced in all genotypes relative to the session where quinine was present.

When quinine was available in one of the four corners, animals did show a clear preference for the corner associated with water (figure 4.27a), and mainly drank water than alcohol (8% and 16%) (figure 4.27b), with a particular aversion for quinine. In addition, the presence of saccharin in the IntelliCages blunted consumption of both alcohol solutions (8% and 16%) and even water (figure 4.28). However, all genotype did exhibit a residual consumption of alcohol 8%

that was similar to alcohol intake observed when quinine was available (figure 4.29). Noteworthy, consumption behavior was significantly influenced by genotypes effect ($F_{(2,136)}$(genotypes)=7.9, p-value<0.001); KO mice consume significantly less liquid compared to WT and HET littermates (figure 4.29).



(a) Summary

(b) WT (c) KO (d) HET

Figure 4.28: **Intellicages®: Liquid consumption behaviors in females.** Female mice were challenged for both 1mM quinine (1) and 0.2% saccharine (2) in one week interval. The total number of licks of WT (black; n=7), HET (grey; n=7) and ppHcrt KO mice (white; n=6) was measured in the different corners where water, 1mM quinine or 0.2% saccharine, alcohol 8% and alcohol 16% were provided. The data are represented as the mean ± SEM. # p-value<0.05 versus alcohol 8%, alcohol 16%, 1mM quinine (quinine session), water and 0.2% saccharine (saccharine session). $ p-value<0.05 versus water, alcohol 8% (quinine session). * p-value<0.05 versus water, alcohol 8%, alcohol 16% and 1mM quinine (saccharine and quinine sessions). @ p-value<0.05 HET versus KO (saccharin session).

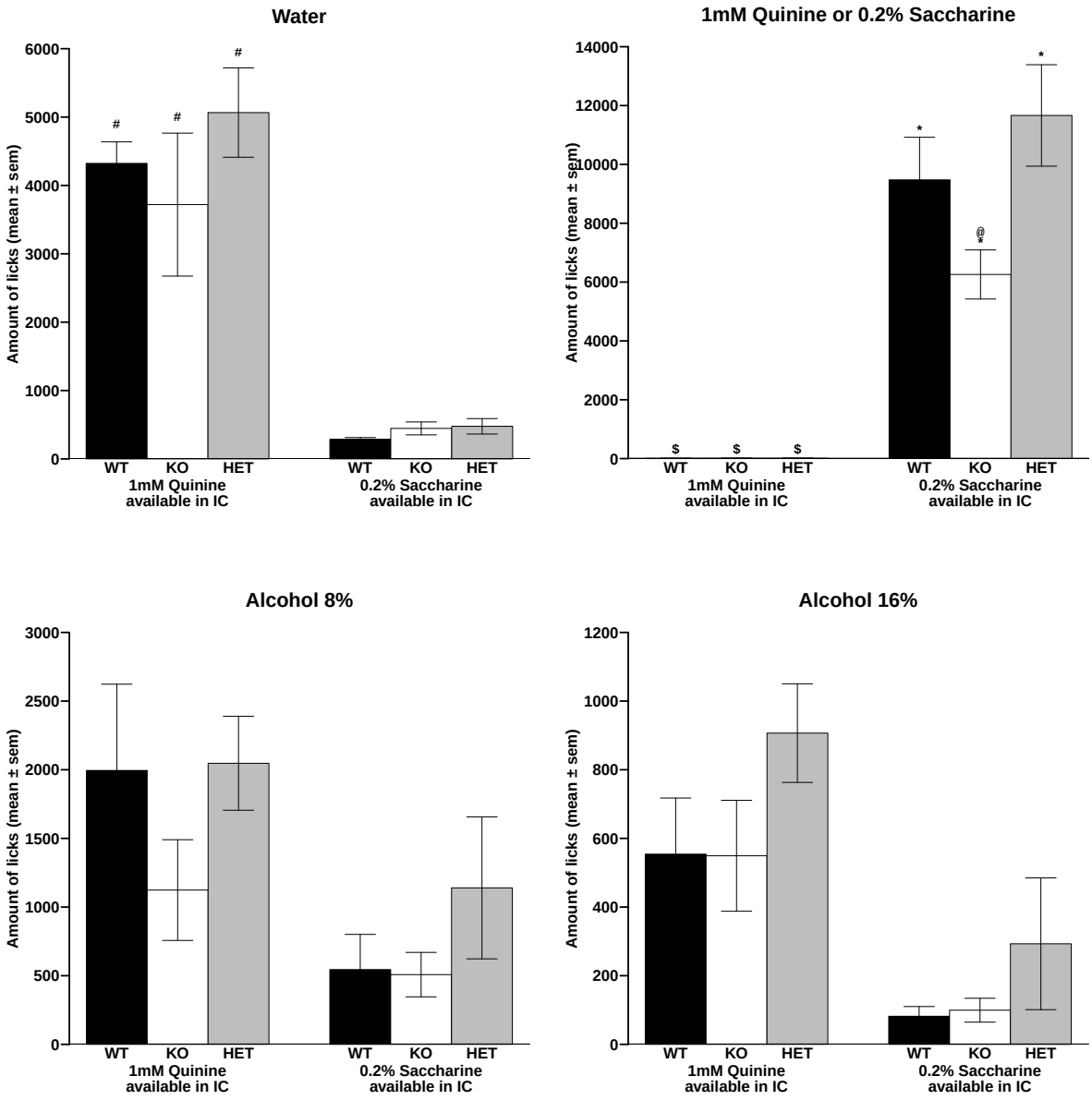
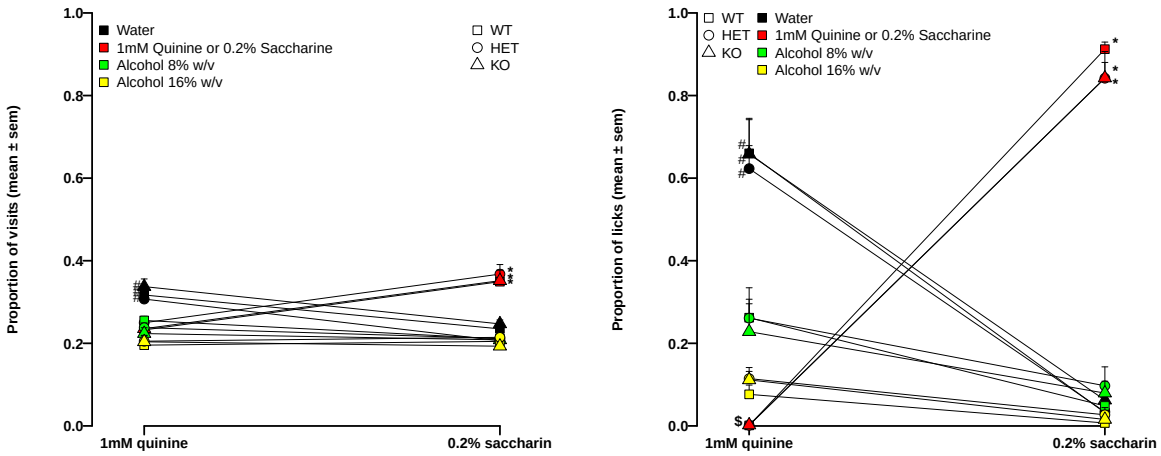


Figure 4.29: **Intellicages®: Detailed liquid consumption behaviors in females.** Female mice were challenged for both 1mM quinine (1) and 0.2% saccharine (2) in one week interval. The total number of licks of WT (black; n=7), HET (grey; n=7) and ppHcrt KO mice (white; n=6) was measured in the different corners where water, 1mM quinine or 0.2% saccharine, alcohol 8% and alcohol 16% were provided. The data are represented as the mean ± SEM. # p-value<0.05 versus alcohol 8%, alcohol 16%, 1mM quinine (quinine session), water and 0.2% saccharine (saccharine session). $ p-value<0.05 versus water, alcohol 8% (quinine session). * p-value<0.05 versus water, alcohol 8%, alcohol 16% and 1mM quinine (saccharine and quinine sessions). @ p-value<0.05 HET versus KO (saccharin session).

After normalization of previous data collected in the IntelliCages®, we observed an attenuation of the differences between WT, HET and ppHcrt KO animals. Here, again the amounts of visits and licks in each corner were reported to the total number of visits and licks, respectively, for each genotype.



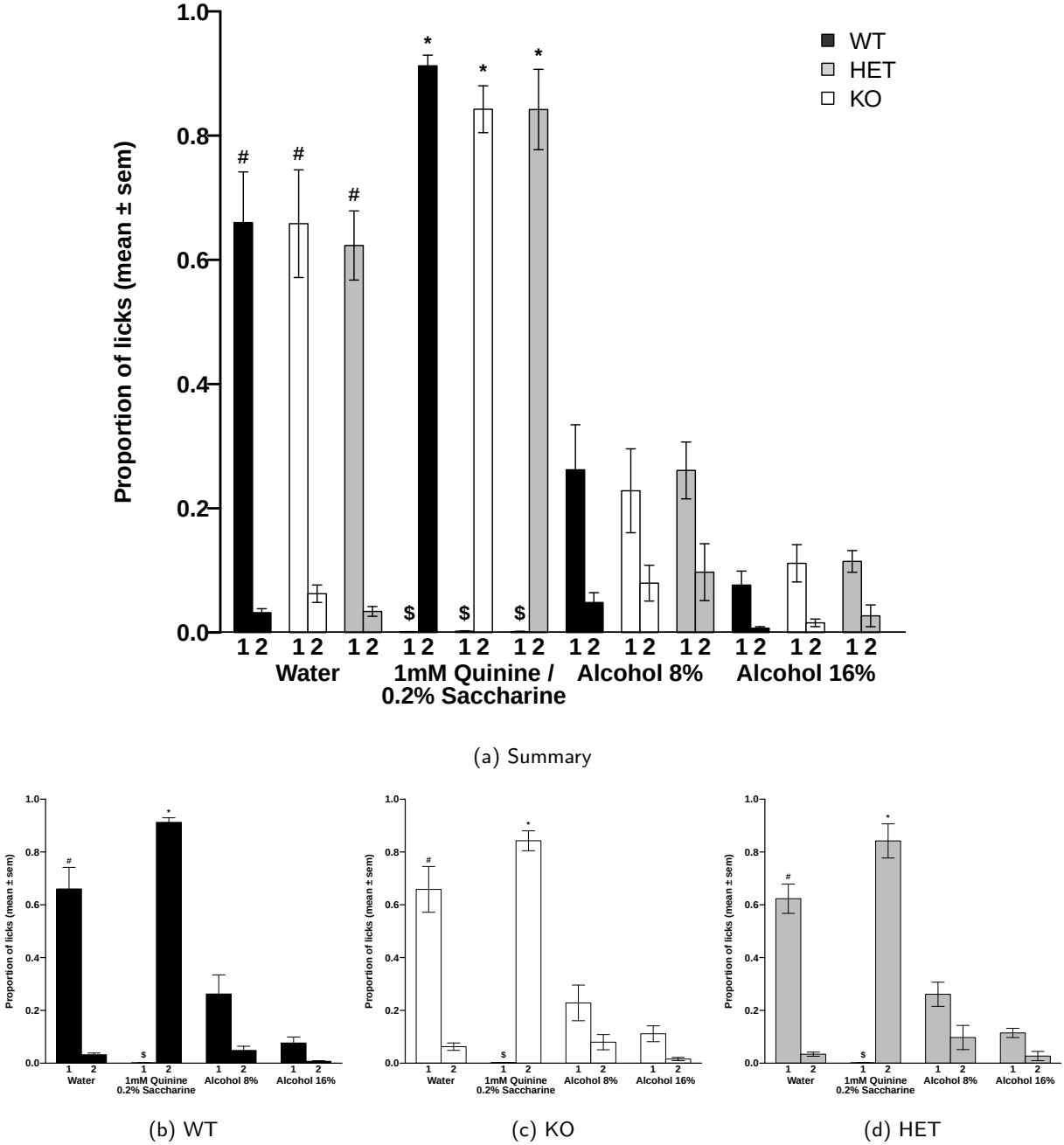
(a) Proportion of visits

(b) Proportion of licks

Figure 4.30: **IntelliCages®: Comparison between quinine and saccharine.** Female mice were challenged for both 1mM quinine and 0.2% saccharine in one week interval. The proportions of visits (A) and licks (B) of WT (square; n=7), HET (circle; n=7) and ppHcrt KO mice (triangle; n=6) were measured in the different corners where water (black), 1mM quinine or 0.2% saccharine (red), alcohol 8% w/v (green) and alcohol 16% w/v (yellow) were provided. The data are represented as the mean ± SEM. # p-value<0.05 versus alcohol 8%, alcohol 16%, 1mM quinine (quinine session), water and 0.2% saccharine (saccharine session). $ p-value<0.05 versus water, alcohol 8% (quinine session). * p-value<0.05 versus water, alcohol 8%, alcohol 16% and 1mM quinine (saccharine and quinine sessions).

The proportion of visits was significantly higher for the water corner compared to other corners in presence of quinine (figure 4.30a), an observation that was not that clear cut on figure 4.27a. After replacement of quinine by saccharine, all animals exhibited a significant preference for the corner paired with saccharine relative to the other corners ($F_{(3,136)}$(liquid solutions)=53.5, p-value<0.001). The proportion of visits in the water corner was decreased in favor of the saccharine corner. Interestingly, no genotypes effect was observed; ppHcrt KO, HET and WT exhibited similar exploration pattern ($F_{(2,136)}$(genotypes)=5.5e-17, p-value>0.05).

All females displayed a strong preference for water relative to alcohol solutions (8% and 16%) and a robust aversion for quinine ($F_{(3,136)}$(liquid solutions)=116.0, p-value<0.001) (figure 4.31). All mice did drink alcohol 8% and to a lower extent alcohol 16% (figure 4.32). In the presence of saccharine, all animals significantly exhibited a preference for the sweetened solution and drastically reduced the proportion of licks for water, alcohol 8% and 16%. Interestingly, all genotypes did exhibit similar consumption behavior, no statistical differences with our sample size were observed between genotypes ($F_{(2,136)}$(genotypes)=0.06, p-value>0.05), once the licking behavior in one corner was compared to the total number of licks (all corners included).



(a) Summary

(b) WT

(c) KO

(d) HET

Figure 4.31: **Intellicages®: Liquid consumption behaviors in females.** Female mice were challenged for both 1mM quinine and 0.2% saccharine in one week interval. The proportions of licks of WT (black; n=7), HET (grey; n=7) and ppHcrt KO mice (white; n=6) were measured in the different corners where water, 1mM quinine or 0.2% saccharine, alcohol 8% and alcohol 16% were provided. The data are represented as the mean ± SEM. # p-value<0.05 versus alcohol 8%, alcohol 16%, 1mM quinine (quinine session), water and 0.2% saccharine (saccharine session). $ p-value<0.05 versus water, alcohol 8% (quinine session). * p-value<0.05 versus water, alcohol 8%, alcohol 16% and 1mM quinine (saccharine and quinine sessions).

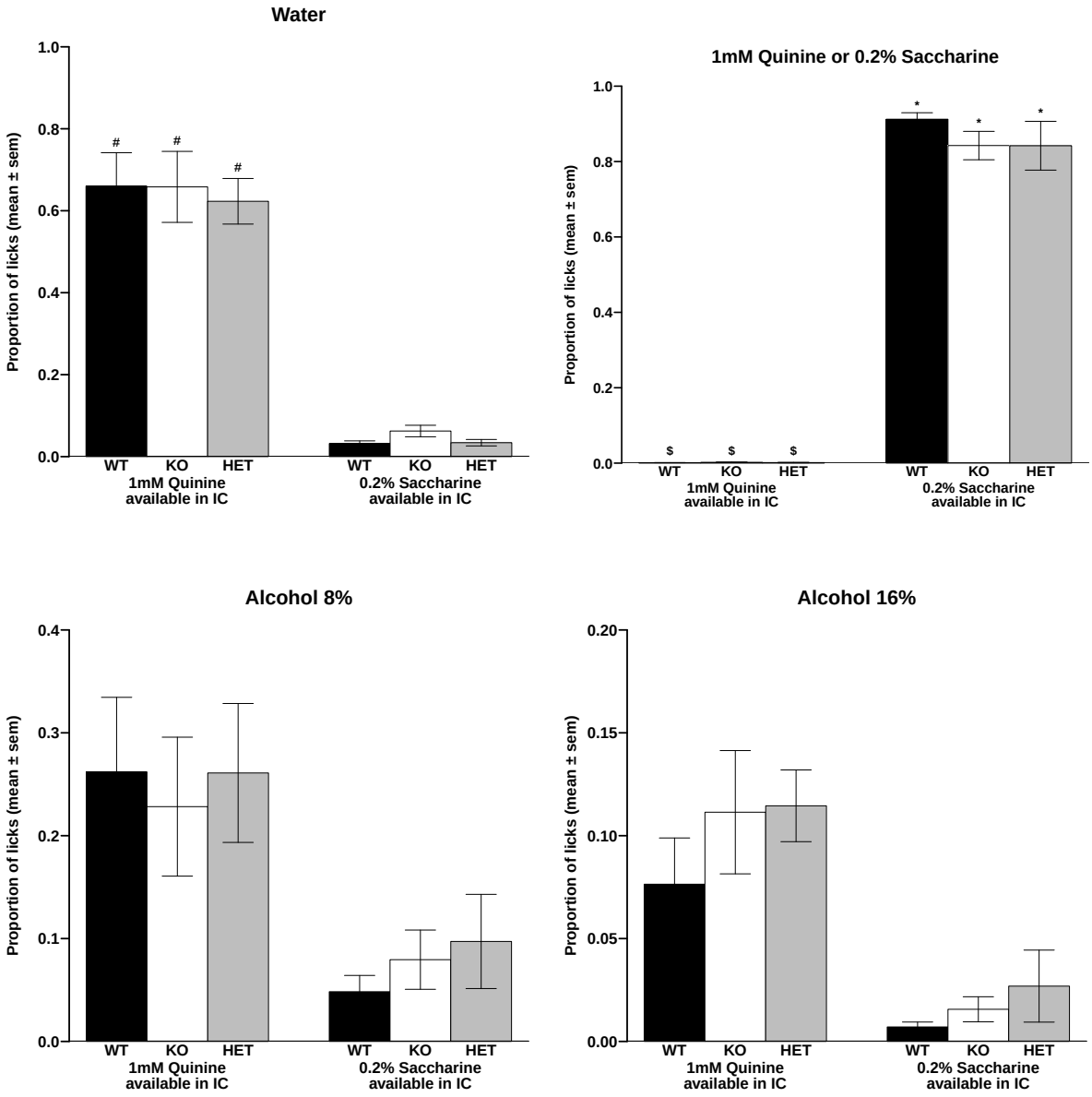


Figure 4.32: **Intellicages®: Detailed liquid consumption behaviors in females.** Female mice were challenged for both 1mM quinine and 0.2% saccharine in one week interval. The proportions of licks of WT (square; n=7), HET (circle; n=7) and ppHcrt KO mice (triangle; n=6) were measured in the different corners where (a) water, (b) 1mM quinine or 0.2% saccharine, Â© alcohol 8% and (d) alcohol 16% were provided. The data are represented as the mean ± SEM. # p-value<0.05 versus alcohol 8%, alcohol 16%, 1mM quinine (quinine session), water and 0.2% saccharine (saccharine session). $ p-value<0.05 versus water, alcohol 8% (quinine session). * p-value<0.05 versus water, alcohol 8%, alcohol 16% and 1mM quinine (saccharine and quinine sessions).

5 Discussion

The hypothalamus is an important mediator of reward-related and motivational behaviors. LH lesions led to a reduced motivation for pleasurable stimuli and electrical intracranial self-stimulation of the LH is powerfully rewarding for humans and for rodents. The identification of novel neuropeptidergic pathways originating from the LH, such as Hcrt, has given new insights in the function of LH neurons. Growing evidence shows that Hcrt have implications in drug-related behaviors. However, only a few reports used constitutive ppHcrt KO mice to study addictive behaviors. This current report aimed at better understanding the functional role of the hypocretin system in alcohol- and cocaine-related behaviors. Several experimental procedures were used to assess alcohol- and cocaine-related behaviors in WT, HET and ppHcrt KO mice from both gender.

5.1 Behavioral sensitization

5.1.1 Behavioral sensitization in males

Constitutive ppHcrt KO mice and their littermates were first tested using the classical locomotor sensitization paradigm, which assesses the pharmacological property of psychostimulant drugs. In the present work, we observed that WT, HET and KO males exhibited a cocaine-induced locomotor sensitization in an openfield. However, the ppHcrt KO mice showed a significant delayed and attenuated cocaine-induced behavioral sensitization compared to WT mice, which is in agreement with previous observations reported either with HcrtR1 antagonist (SB-334867) or with constitutive ppHcrt KO mice treated with morphine, methamphetamine, methylphenidate and MDMA [204, 217]. Interestingly, HET mice, which express reduced Hcrt-1 and Hcrt-2 peptide levels, exhibited an intermediate behavioral sensitization pattern. To our knowledge, this is the first study assessing behavioral sensitization in HET animals. These data suggest that when preprohypocretin is downregulated or absent or when the hypocretin receptor 1 is blocked, animals display an impaired drug-induced locomotor response to psychostimulants and opioids [200, 204, 218]. However, we observed that when the distance traveled within the first 15 min following cocaine treatment was normalized to the 15 min of habituation, the differences between KO, HET and WT were minimized. Indeed, all mice did demonstrate similar amplitude in behavioral sensitization following daily cocaine treatment, except following the first cocaine session during which a significant difference was noticed between KO and WT mice. Sharf et al. [216] and Georgescu et al. [279] already reported very limited difference between KO and WT mice following chronic intermittent morphine treatment.

Constitutive ppHcrt KO males exhibit a reduced cocaine-induced behavioral sensitization. In the literature, a few transgenic mice exhibit hypoactivity and reduced behavioral sensitization following repeated psychostimulant or opioid exposures. Dopamine deficient mice, for example,

also display an attenuated locomotor sensitization [49, 50]. In addition, pharmacological treatment with D1 or D2 receptor antagonists in WT animals did suppress locomotor response to psychostimulant and opioid drugs [280]. It has been demonstrated that the hypocretin system anatomically and functionally interacts with the dopaminergic system [197]. And impairment of the dopamine neurotransmission leads to dyskinesis. Hypocretin containing neurons send strong projections to the VTA which, in turn, sends projections to the NAcc [78] playing a key role in the striato-nigro striatal spiraling connection [211] regulating the locomotor activity. Activation of the NAcc is critical for the development of behavioral sensitization. Cocaine blocks the dopamine, noradrenalin and serotonin transporters thus enhancing monoaminergic concentrations in the synaptic cleft [281, 282]. Increasing DA release in the NAcc induces the activation of D1 dopamine receptors responsible for long term modification in this region and therefore the development of sensitization [47]. It has been observed that hypocretin neurons are highly active during locomotor activity [283]. Direct application of the hypocretins into the VTA induces DA release in the VTA, but also in the PFC and NAcc [180, 204]. Blockade of the HcrtR1 in the VTA or constitutive hypocretin deficient mice showed reduced DA release [217, 227]. In addition, constitutive ppHcrt KO mice express low DA ratio and elevated serotonin metabolites and ratio in the medial PFC compared to WT [217]. In addition, administration of hypocretins induces hyperlocomotion in rodents and pretreatment with D1 or D2 dopamine receptor antagonists [204], but not serotonin receptor antagonists [284], abolished the hypocretin-induced locomotor activity. Since hypocretin producing neurons interact anatomically and functionally with the mesolimbic structures and since constitutive ppHcrt KO mice express low level of DA in the medial PFC, it is possible that hypocretins deficiency lead to impaired DA system.

Drugs of abuse not only act through the dopaminergic system, but also through the glutamatergic system. NMDA receptor-deficient mice do also exhibit attenuated cocaine-induced locomotor activity [42, 285, 286]. In addition, blockade of NMDARs and AMPARs blunted drug-induced hyperlocomotion in WT animals. Cocaine treatments induce long term changes in glutamatergic transmission in the VTA through the induction of NMDAR-dependent LTP which is thought to modify AMPAR function and number [39]. It has been reported that *in vitro* application of hypocretins in the VTA induces changes in glutamate transmission and especially at the receptor level through NMDARs and AMPARs [200, 201]. Treatments with Hcrt-1 and/or Hcrt-2 peptides potentiate NMDARs EPSCs current and stimulate subunits reorganization and translocation to the synapses [200]. Hcrt-1, but not Hcrt-2, treatment also modulates late phase AMPARs expression and function which facilitates cocaine-mediated responses [200, 201]. Cocaine treatments upregulate cell surface expression of AMPARs [287] and upregulate the expression levels of HcrtR2, but not those of HcrtR1, in NAcc [288]. And application of Hcrt-1 in the striatum also mediates an increase in AMPARs cell surface expression [212]. Hence, most likely Hcrt deficiency alters glutamate receptors expressions and thus behavioral sensitization.

Behavioral sensitization is the result of multiple concomitant actions in distinct brain regions occurring from the very first cocaine use [39]. Low expression levels or Hcrt deficiency might alter drug-induced response through the dopaminergic and glutamatergic systems. Both systems

target the NAcc and concomitant activation of the dopamine and glutamate signaling pathways induce neuronal plasticity. It receives strong input from the VTA and, in turn, sends strong projections to hypocretin producing neurons which innervate the VTA. Together these observations suggests that the VTA, the NAcc and the LH interact closely. In addition, intact dopaminergic, glutamatergic and hypocretinergic systems are necessary to trigger normal drug-induced hyperlocomotion. Constitutive ppHcrt KO mice did also exhibit delayed behavioral sensitization regarding the distance traveled in the one hour testing session, suggesting that the processes are partially independent of hypocretins, but hypocretins facilitate its development and expression from the first cocaine exposure. The hypocretin system seems to be a relay between the VTA and the NAcc for promoting potentiation of drug-induced response. The impairment in basal locomotor activity and the development of sensitization induced by the first cocaine treatment observed in rodents treated with pharmacological antagonist of the hypocretin receptor 1 or in constitutive ppHcrt KO might occur through the concomitant impairment of DA release and neuronal plasticity [289].

5.1.2 Behavioral sensitization in females

In openfield

The hypocretinergic system is age- and gender-dependent [62]. It has been observed that WT females have higher levels of preprohypocretin mRNA and peptides compared to males, and also higher hypocretin receptors mRNA levels [62, 84, 290]. In addition, gender differences were already reported in behavioral sensitization to psychostimulants [291].

In order to determine whether gender could affect cocaine-related behaviors, KO, HET and WT females were also tested in an openfield for assessing locomotor sensitization. Surprisingly, we observed that KO, HET and WT exhibited similar cocaine-induced behavioral sensitization. In agreement with previous results with morphine [216], our observations suggest that cocaine-induced behavioral sensitization is hypocretin-independent in females since their absence did not alter behavioral response to chronic treatment with psychostimulant. We hypothesized previously that the basal locomotor activity and the attenuated drug-induced hyperlocomotion in males could be due to an alteration of the DA and glutamate systems. However, this hypothesis does not match with the results obtained in females, as no overt difference was observed between genotypes, even following the first cocaine injection. In addition, comparison between males and females data did not highlight any significant gender effect. Nevertheless, it has been reported that dopamine level is also gender-dependent and female hormones modulate DA activity in the NAcc and the striatum [292]. One possible hypothesis is that estrogen and progesterone, two hormones also present in the hypothalamus, might compensate the lack of hypocretins and thus maintain the appropriate basal levels of dopamine. However, Mori et al. [217] only look at DA levels in males. So far, no data were found in the literature with regards to monoamines expression in ppHcrt KO females only.

Neuropeptides involved in feeding behavior have also the potency to facilitate drug-induced behavioral sensitization. Furthermore, numerous reports indicated that leptin or insulin did

influence drugs of abuse consumption [293]. Interestingly, ppHcrt KO females have higher leptins levels relative to ppHcrt KO males and WT animals [294]. And it has been observed that leptin facilitates psychostimulant behavioral sensitization through activation of the mesolimbic circuit [295, 296]. Leptin-deficient mice exhibit blunted locomotor sensitization to psychostimulant, however when rescued with leptin infusions, animals exhibited normal locomotor sensitization [297]. KO females might compensate the lack of hypocretins with higher levels of leptin and maintain the appropriate neuronal activity for normal behavioral response to psychostimulants.

Little was reported on hypocretin deficient females and many reports did not mention any major differences between males and females. Here, we reported that, in contrast to males, hypocretins are not necessary in female mice for the development and expression of cocaine-induced locomotor sensitization.

In running wheel

Animals tested in a large circular arena might be stressed and might produce cross sensitization between stress and psychostimulant responses. Thus, the hyperlocomotion observed in KO females following cocaine exposure might not only result from the activation of the dopamine circuit only, but also from the brain stress pathways [298].

Other groups of KO, HET and WT littermates were tested in smaller cages equipped with running wheels. Wheel running is an alternative measurement of voluntary locomotor activity. Moreover, it has been reported that wheel running augments drug-induced sensitization [299–301]. In this paradigm, we observed that the distance traveled by hypocretin deficient mice was shorter compared to HET and WT animals during the entire procedure. However, following repeated psychostimulant exposures, ppHcrt KO mice did exhibited behavioral sensitization similar to that observed in HET and WT mice. This observation suggests that ppHcrt KO mice might display locomotor deficiency. Although no statistical difference was observed, KO females tended to exhibit more difficulty to adjust movements to speed in the running wheel compared to HET and WT animals.

5.1.3 Coordination deficiency at high speed in ppHcrt KO females

KO mice, HET and WT littermates were tested on rotarod in order to assess movement coordination. We observed that ppHcrt KO and HET mice exhibited coordination deficiency at high speed compared to WT. This result was consistent with results obtained with the running wheel paradigm; both experiments demonstrated impaired locomotor activity in mutant mice. These observations were masked by locomotor sensitization in the openfield. These results suggest that hypocretin peptides are necessary for an efficient movement coordination. Hypocretin deficiency may triggered lower dopamine release level in the ventral part of the striatum which might induce a weaker response of the motor cortex and therefore may impair motor control. In addition, it has been reported that running wheel promotes wakefulness and cataplexy attacks in ppHcrt KO

mice [302] possibly responsible for the reduced distance traveled in comparison with HET and WT animals. Positive emotions may trigger cataplexy in mice, just as in narcoleptic patients. Cocaine treatment may induce positive affects in rodents. Ultrasonic vocalizations provide insight into the affective state of animals. 22-kHz calls occur in the presence of aversive events, while 50-kHz calls are linked with anticipatory and appetitive outcomes. Ultrasonic vocalizations likely coincides with cocaine self-administration and 50-kHz frequency calls were observed in rats trained to self-administer high dose of cocaine [303]. Noteworthy, most of patients with narcolepsy-cataplexy are treated with psychostimulants, such as amphetamine or amphetamine-like drugs. Psychostimulants are wake-promoting substances used for the treatment of excessive daytime sleepiness in narcoleptic patients and have minimal effect on cataplexy [304]. In the present report, ppHcrt KO mice were treated with 15mg/kg of cocaine which may produce positive emotions. Since positive emotions and wheel running promotes cataplexy attacks in ppHcrt KO and cocaine treatments did not reduce cataplexy symptoms [302, 305], it is most likely that cocaine may induce several cataplexy attacks in running wheels and thus supports the reduced distance traveled in KO mice.

5.2 Cocaine rewarding properties in ppHcrt KO mice

The rewarding and reinforcing properties of cocaine in ppHcrt KO, HET and WT mice was then assessed with the help of the conditioned place preference paradigm, which provides indirect measurement of drug seeking behaviors. It has been reported that females and males differentially express preprohypocretin mRNA and peptides, as well as different hypocretin receptor levels [62, 84, 290]. However, we observed that all mice, whatever their gender and genotype, did exhibit similar conditioned place preference for the context paired with repeated cocaine injections, which is consistent with previous reports [216]. In line with the data obtained in the locomotor sensitization paradigm, we confirmed the reduced locomotor activity in male and female KO mice during the pre- and post-conditioning sessions. The preference for the environment previously paired with drug reward has been reported to persist for up to four weeks in WT rodents [276,277]. Here, we confirmed that both WT and HET animals (males and females) still displayed a significant place preference for the compartment paired with repeated cocaine administration, whereas ppHcrt KO mice stopped expressing a preference two weeks after the last cocaine injection. Hence the long term maintenance of the preference for the compartment previously paired with cocaine might depend on the hypocretin system.

This finding is in line with the attenuated morphine withdrawal observed in ppHcrt KO mice [279] or in WT mice treated with SB-334867 [306]. Morphine withdrawal produces acute activation of Hcrt cells and especially the Hcrt gene demonstrating the regulation of Hcrt neurons by chronic morphine and morphine withdrawal [279]. Hcrt action through HcrtR1 contributes to the expression of the withdrawal symptoms [306]. The attenuation of morphine withdrawal behaviors in ppHcrt KO mice or in SB-334867 treated WT animals implies that the Hcrt system serves an important functional role in modulating responses to drugs of abuse. The projections of Hcrt neurons and their interactions with dopaminergic systems suggest a potential pathway that may be implicated in Hcrt modulation of morphine withdrawal [104, 197]. Analysis of c-

Fos expression suggests that the NAcc shell may be a critical region in mediating this effect [306].

The normal development of conditioned place preference also depends on mesocortical circuitry. The association between the context and the effect induced by psychostimulant administration is mediated by the activation of mesolimbic brain structures [196]. Positive or negative sensory stimuli induced by drugs of abuse treatment affect the amygdala, the hippocampus and the PFC which all mediate the association between the context and drugs of abuse treatment contingency. All these sites are important in the development and the maintenance of conditioned place preference. Lesions of the amygdala or the PFC affect the reinstatement of extinguished preference for the context previously associated with repeated cocaine administration [307, 308]. On the other hand, lesions of the dorsal hippocampus alter the acquisition and expression, but not the consolidation of cocaine conditioned place preference [309]. As previously mentioned the hypocretin system is closely connected to mesolimbic structures; it sends strong projections and receives dense afferents from the amygdala, the cerebral cortex and, to a lower extent, from the hipppocamus and the VTA. Hypocretin producing neurons might act as a regulatory relay facilitating the synchronization of different neuronal populations in order to promote the appropriate response, for instance triggering phasic firing of dopamine neurons known to play a key role in behavioral conditioning [47, 48]. The lack of Hcrt may not alter the development of CPP, since KO mice showed normal place preference, but hypocretin deficiency may attenuate the long-term maintenance of the conditioned preference since KO mice stopped expressing the place preference after a two weeks of passive extinction. Hence, the association between the context and the rewarding properties of cocaine may not have been that robust in KO mice compared to HET and WT littermates. This hypothesis is consistent with recent findings according to which narcoleptic patients failed to exhibit any increased activity in the amygdala in response to conditioned stimuli [310]. In addition, it has been reported that rats treated with the HcrtR1 antagonist SB-334867 have impaired cue and context driven drug seeking behavior [233]. Since context driven relapse to drug seeking requires intact dorsolateral striatum [311], and since the dorsal striatum is crucial for drug seeking habits [312], the deficiency of hypocretins might affect the functioning of the dorsal striatum by interfering with inputs from the amygdala, the hippocampus and the PFC.

Noteworthy, dopamine is not the only monoaminergic system that could mediate conditioned place preference. Indeed dopamine deficient mice display normal conditioned place preference possibly through serotonin signaling [313]. Constitutive ppHcrt KO mice showed reduced dopamine levels and higher basal serotonin ratio relative to WT [217]. The hypocretin system sends dense fibers into the raphe [73] considered to be the main source of serotoninergic neurons in the brain [314]. In addition, cocaine treatment inhibits the reuptake of serotonin leading to overstimulation of the NAcc through activation of serotonin receptors [281, 282]. 5-HT_{1A} receptors have a wide CNS distribution and contribute to the regulation of many behaviors, such as locomotor activity, feeding behaviors, grooming, anxiety, sleep/wake cycles and reward-related behaviors. 5-HT_{1A} receptor agonists facilitate behavioral sensitization to psychostimulant drugs, whereas antagonists attenuate its expression [315, 316]. Furthermore, 5-HT_{1A} receptors mediate

conditioned place preference [317]. Application of serotonin on hypocretin neurons induces hyperpolarization through 5-HT_{1A} receptors [318]. Hence, ppHcrt KO animals might compensate the reduced DA response to psychostimulant with an increased activation of the serotoninergic system.

Together, these observations suggest that in ppHcrt KO mice, the NAcc might integrate inappropriately dopamine and serotonin signaling and might induce inappropriate signaling through the serial dopamine striato-nigro-striatal connection, resulting in an altered activation of the dorsal striatum and an impaired transition from goal directed behavior to drug seeking habits. In brief, it seems that developmental neuronal adaptations occurring in ppHcrt KO mice may compensate the lack of hypocretins for promoting normal conditioned place preference, but not its maintenance over time. Further studies are needed to assess this hypothesis.

5.3 Drinking behaviors in ppHcrt KO female mice

Finally, we assessed the ability to drink alcohol in WT, HET and KO mice. The IntelliCage is a novel apparatus that permits individual monitoring in social groups (up to 12 mice). The IntelliCages could provide indirect measurement of circadian rhythm [319]. Thus, total numbers of visits, nosepokes and licks, as well as their respective duration, are collected over time. We were able to show that ppHcrt KO mice displayed similar circadian visiting pattern compared to HET and WT littermates with higher amounts of visit during the dark phase and lower amounts during the light phase. It has been reported that ppHcrt KO mice exhibit an impaired sleep/wake rhythm with narcoleptic-like phenotype and cataplexy attacks. We have not observed any difference in the amounts of visits between genotypes during the first two days of habituation. Licking pattern was also hourly assessed and showed a similar circadian rhythm. Noteworthy, ppHcrt KO mice did show significantly reduced amounts of licks for water compared to WT and HET animals. KO, HET and WT mice were then challenged with four different solutions, water, 1mM quinine, alcohol 8% and 16%. We report here a trend for reduced amounts of visits and licks in KO compared to WT and HET animals, confirming a hypoactive phenotype although our sample size did not permit the identification of any significant differences.

Similarly to the behavioral sensitization, when the data were standardized, the proportion of visits and licks in the KO group was identical to values observed in the WT group. Animals preferred visiting and licking at the water corner at the expense of other corners. The proportion of visits in the other corners was similar between genotypes. The corner associated with alcohol 16% was apparently aversive for all mice since they displayed very low amounts of visits similar to those observed for the corner associated with 1mM quinine. We also noticed that KO, HET and WT mice spontaneously drink alcohol 8%, since the proportion of licks for alcohol 8% was higher compared to quinine and alcohol 16%. Surprisingly, although all animals displayed a strong aversion for drinking quinine, they visited frequently for this corner compared to the alcohol 16% corner. These observations are consistent with data found in literature where mice avoid bitter taste and high alcohol concentrations.

It has been observed that the olfactory bulb receives dense fibers of hypocretin system [79] and the lack of hypocretins in human alter the olfactory sense which could be rescued by intranasal application of synthetic hypocretins [320]. The possibility that ppHcrt KO mice might have impaired gustatory sense is not supported here since the latency to first lick aversive liquid solutions (1mM quinine or alcohol 16%) increased over sessions suggesting that all genotypes learned to discriminate liquid solutions.

Following two weeks of alcohol abstinence, individuals of all genetic background, did not exhibit any rebound in alcohol drinking behavior. The proportion of licks for alcohol 8% tended to decrease in ppHcrt KO mice compared to WT and HET mice, but this difference was not statistically significant in our sample size. This latter observation could be related to the absence of persistent preference for the cocaine compartment after a two-week period of passive extinction in KO mice.

When quinine was replaced with saccharine, WT and HET, and to a much lower extent ppHcrt KO mice, drastically shifted their visits and licks activity toward the corner paired with saccharine. WT and HET and to a lesser extent KO mice preferred visiting and drinking at the saccharine corner rather than water and alcohol (8% and 16%). After standardization of the data, the proportion of visits and licks in the KO group was similar to the WT group. All animals preferred visiting and licking at the saccharine corner and strongly neglected the other corners. The proportion of visits and licks in the other corners was similar between genotypes. This support the idea that, in our sample size, KO mice exhibit a hypoactive phenotype which is responsible for the reduced sweet solution consumption.

The lateral hypothalamus has been implicated in alcohol seeking behaviors. A robust expression of hypocretins mRNA following acute alcohol exposure was observed, whereas chronic exposure suppresses hypocretin expression [278]. This suggests that the lack of hypocretin might impaired alcohol consumption. However, in the present study, spontaneous alcohol intake in KO, HET and WT mice was barely different suggesting that the hypocretin system is most likely involved in the operant conditioning associated with alcohol intake, rather than being involved in the regulation of alcohol intake *per se*.

Since the lack of hypocretin did not drastically alter spontaneous alcohol consumption, it suggests that alternative pathways might compensate the hypocretin deficiency. It is also notable that Hcrt cells release the endogenous opioid peptide dynorphin [321]. It remains to be determined whether the role of this neuropeptide has functional implications for alcohol consumption in constitutive ppHcrt KO. Further, the lateral hypothalamus also hosts the MCH system that does regulate alcohol consumption [267, 268, 272, 273] and interacts with Hcrt [274, 275]. When MCH is microinjected directly in the brain, it increases alcohol intake in a dose dependent manner and MCHR antagonists dose dependently suppress alcohol consumption [267, 268, 272, 273]. High alcohol intake reduces significantly MCH mRNA levels. An increase activity in the MCH

system might compensate Hcrt deficiency and might promote spontaneous alcohol drinking, but further studies are needed to assess this hypothesis.

5.4 Further considerations

We used constitutive ppHcrt KO mice, HET and WT littermates issued from HET crosses to assess reward related behaviors. Only the gene coding for the ppHcrt is disrupted in KO mice, therefore neurons are still present, but do not express the functional hypocretin peptides. The hypocretin-producing neurons do not only express hypocretins, but also other neuropeptides, such as dynorphin [321] or neuropeptide S [322,323], which are known to modulate reward seeking behaviors. Hypocretin-deficient mice show abundant prodynorphin mRNA in the LH [321]and compensations of the hypocretins deficiency is most likely occurring in this strain. The lack of hypocretin was also shown to interfere with the expression of peripheral peptides, such as leptin which is upregulated in ppHcrt KO mice [294]. The LH densely expresses leptin receptor and direct leptin application regulates gene expression in Hcrt neurons. In addition, the MCH system, the LH second neuronal population, directly interact with the Hcrt system [274, 275] and share several similarities. Both are required for the regulation of many physiological processes, such as arousal, feeding behaviors and reward-related behaviors. Since both neuronal populations trigger common outcomes, the MCH system may counterbalance the absence of hypocretin peptides in order to relay the necessary signaling to cortical structures to maintain the required level of arousal for orchestrating appropriate copying strategies and responses to environmental demands and basic metabolic needs. These suggest that constitutive ppHcrt KO mice may have developed several adaptations for counterbalancing various dysfunction in metabolic or cognitive processes.

6 Conclusion & Perspective

The present work emphasized the role of the hypocretin system in the regulation of reward seeking behaviors. We used transgenic mice model issued from HET crosses challenged them with cocaine and alcohol using classical paradigms.

Considering rough data, it is evident that Hcrt deficient males and to a lower extent females displayed a hypoactive phenotype which could be related to impaired mesolimbic structures. This could account for delayed locomotor sensitization compared to WT littermates. Nevertheless, after data standardization, ppHcrt KO still exhibited rewarding effect suggesting that these mice may have undergone major neurocircuitry adaptations leading to normal reward seeking behaviors.

Indeed, all mice exhibited a robust conditioned place preference for cocaine. Noteworthy, after two weeks of cocaine abstinence, KO males and females, no longer displayed preference for the compartment previously paired with repeated exposures to cocaine, whereas WT and HET mice continued expressing the place preference. This observation suggests that the development of behavioral conditioning is independent of hypocretins, but the maintenance of such conditioning may require an intact Hcrt system. Impaired connectivity or reduced strength signaling between the amygdala, the hippocampus and the PFC may be occurring in Hcrt deficient mice.

The ppHcrt KO mice exhibited a circadian rhythm regarding visits and licks in the IntelliCages. When four different liquid solutions were provided, we observed a reduced trend in ppHcrt KO mice considering the amount of visits and licks relative to HET and WT. After data standardization, all mice did exhibit similar exploratory and drinking behaviors with a strong preference for water and aversion for quinine. Following two weeks of alcohol abstinence, ppHcrt KO mice, HET and WT littermates did not display any rebound for alcohol drinking. No difference was observed between genotypes regarding the proportion of visits and licks for a sweet solution. However, the ppHcrt KO mice hypoactivity did affect the total number of visits and licks to the saccharine corner.

Here, we report that only cocaine-related behaviors are partially impaired in Hcrt deficient mice. In contrast, KO mice barely displayed any behavioral changes but a trend for reducing sweet and alcoholic beverages. The ppHcrt KO line may compensate the absence of hypocretins by other neuropeptides. To date, no deep investigations were performed on the molecular content of hypocretins neurons neither in WT nor in ppHcrt KO mice. In literature, little is known regarding neuronal plasticity in ppHcrt KO mice following repeated exposures to psychostimulant drugs. Thus, molecular investigations (microdialysis, qPCR, microarray) are necessary to further discuss cellular and molecular adaptations in these mice.

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