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Hypocretin/Orexin deficiency decreases cocaine abuse liability

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Key words: Hypocretin, orexin, motivation, craving, cocaine, saccharine, relapse

Abstract

Compelling evidence indicates that hypocretin/orexin signaling regulates arousal, stress and reward-seeking behaviors. However, most studies on drug reward-related processes have so far described the effects of pharmacological blockers disrupting hypocretin/orexin transmission. We report here an extensive study on cocaine-related behaviors in hypocretin/orexin-deficient mice (KO) and their heterozygous (HET) and wildtype (WT) littermates. We evaluated behavioral sensitization following repeated administrations and preference for an environment repeatedly paired with cocaine injections (15 mg/kg). Mice were also trained to self-administer cocaine (0.5-1.5 mg/kg/infusion). Our observations show that whereas all mice exhibited quite similar responses to acute administration of cocaine, only Hcrt KO mice exhibited reduced cocaine-seeking behaviors following a period of abstinence or extinction, and reduced cocaine incubation craving. Further, if the present findings confirm that Hcrt deficient mice may display a hypoactive phenotype, possibly linked to a reduced alertness concomitant to a decreased exploration of their environment, hypocretin/orexin defiency did not cause any attentional deficit. We thus report that innate disruption of hypocretin/orexin signaling moderately alters cocaine reward but significantly reduces long-term affective dependence that may explain the lack of relapse for cocaine seeking seen in Hcrt KO mice. Overall, with blunted cocaine intake at the highest concentration and reduced responsiveness to cocaine cues after prolonged abstinence, our findings suggest that hypocretin deficient mice may display signs of resilience to cocaine addiction.

Key words: Hypocretin, orexin, motivation, cocaine, saccharine, relapse

Highlights:

- Hypocretin deficiency moderately alters cocaine and saccharine reward
- Disruption of Hcrt signaling does not alter responsiveness to cue, but rather decreases the underlying positive affective state that drives motivated behaviors and invigorate the organism to overcome efforts required to get access to reward.
- Hypocretin deficient mice may display signs of resilience to cocaine abuse

Introduction

Disruption of the hypocretin/orexin (Hcrt) transmission has been originally shown to cause destabilization of the boundaries between sleep states in dogs (Lin et al., 1999), in mice (Chemelli et al., 1999) and in human narcoleptic patients (Nishino et al., 2000). These observations contributed to acknowledge that narcolepsy is most likely related to ongoing loss of Hcrt neurons (Sakurai, 2007; Sutcliffe and de Lecea, 2002). Converging evidence have also established that the Hcrt system controls sleep and wakefulness through multiple interactions with brain structures involved in the regulation of stress, reward, and energy homeostasis (Berridge et al., 2010; Tsujino and Sakurai, 2009). Accordingly, Hcrt is believed to elicit appropriate levels of arousal to engage exploratory and goal-oriented behaviors depending on physiological needs, therefore driving motivation for natural reward seeking. Increasing evidence also suggests that upon chronic drug intoxication, the Hcrt system may serve for triggering drug-oriented behaviors at the expense of former basic need priorities (Boutrel et al., 2013).

Hcrt neurons arise exclusively from the lateral hypothalamus (LH), a brain structure known for mediating the integration and processing of basic needs, and project to the entire brain(Baldo et al., 2003; Peyron et al., 1998). Hcrt neurons receive abundant input from the limbic system and project to all the major components of the extended amygdala (Schmitt et al., 2011), a brain region considered to connect the basal forebrain to the classical reward systems of the LH via the medial forebrain bundle reward system. For the past few years, Hcrt signaling has been shown to be critically involved in drug reward seeking behaviors (Bentzley and Aston-Jones, 2015; Borgland et al., 2006; Boutrel et al., 2005; Espana et al., 2011; Espana et al., 2010; Flores et al., 2013; Harris et al., 2005; Hollander et al., 2008; Lawrence et al., 2006; Martin-Fardon et al., 2016; Martin-Fardon and Weiss, 2014a, b; Matzeu et al., 2016; Narita et al., 2006; Rao et al., 2013; Schmeichel et al., 2017; Thompson and Borgland, 2011). Most of these reports mainly characterized the pharmacological properties of the Hypocretin receptor 1 (Hcrtr-1) antagonist, SB 334867, and all these studies

converged toward a role of Hcrtr-1 signaling in cocaine reinforcing properties, but one (Riday et al., 2012). Meanwhile, two remarkable studies using Hcrtr-1 (-/-) mice reported a significant reduction of cocaine intake and an impaired cue-dependent fear memory formation (Hollander et al., 2012; Soya et al., 2013), and the implication of hypocretin signaling in the regulation cocaine reinforcing properties was recently confirmed using a dual receptor antagonist (Gentile et al., 2017a; Gentile et al., 2017b). Overall, these observations support past findings claiming that disruption of Hcrt signaling prevented footshock- and cue-induced reinstatement of previously extinguished cocaine seeking behaviors (Boutrel et al., 2005; Smith et al., 2009; Wang et al., 2009). Hcrt transmission has been suggested to selectively regulate "relapse" like behaviors in abstinent rats, without playing critical role in the reinforcing effects of cocaine that maintains ongoing drug-taking behavior.

The commonly used Hcrtr-1 antagonist SB 334867 presents low affinity for 5-HT_{2B} and 5-HT_{2C} receptors (Duxon et al., 2001), but most importantly administration of SB 334867 induces sedative effects at the highest doses (Rodgers et al., 2001), further questioning the pharmacological approaches using this compound (McElhinny et al., 2012). Meanwhile, only a few studies have reported drug-seeking behaviors in hypocretin-deficient (Hcrt KO) mice, the original mouse model described by Chemelli and colleagues in 1999 that has its own limits as well given the compensatory mechanisms most likely occurring during *in utero* development in absence of Hcrt peptides. To date, it has been shown that Hcrt KO mice display diminished signs of precipitated opiate withdrawal (Georgescu et al., 2003) and that they do not exhibit morphine-induced place preference (Narita et al., 2006), although this latter observation was not replicated recently (Sharf et al., 2010). Therefore, our aim was to extensively investigate cocaine related behaviors in Hcrt-deficient mice (KO), their heterozygous (HET) and wild type (WT) male and female littermates. Our results demonstrate that innate loss of hypocretin transmission does not impair the acute reinforcing properties of cocaine. However, with a blunted cocaine intake at the highest concentration and reduced cocaine-seeking behaviors after periods of abstinence, hypocretin deficient mice most likely display signs of resilience to cocaine addiction.

Materials and methods

Animals

We obtained hypocretin/orexin knockout mice (Chemelli et al., 1999) from Prof. M. Yanagisawa (HHMI, University of Texas Southwestern Medical Center, Dallas, USA). These mice have been backcrossed with C57BL/6J mice (Janvier Labs, Le Genest Saint Isle, France) for 10 generations. Our breeding pairs were maintained as heterozygous and crossed to obtain homozygous knockout (KO) mice, heterozygous (HET) and wildtype (WT) littermates. For genotype identification, genomic DNA extracted from ear biopsy was amplified using PCR amplification with a neomycine 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' shared by KO and WT mice (Mochizuki et al., 2004).

Mice used in this study were 10-18-weeks old, they were housed in a controlled environment (21 ± 1°C, humidity 50 ± 10%) and were kept under a reversed 12h light/dark cycle (lights off at 10 am). All experiments were performed in accordance with the Swiss Federal Act on Animal Protection and the Swiss Animal Ordinance and were approved by the cantonal veterinary office (authorization 1903 to B.B.).

Locomotor sensitization in open fields

Behavioral tests were performed during the dark cycle. Mice were habituated for 15-min in a large circular open field (diameter = 72.5 cm) before each 60-min testing session. Mice were tested for 7 consecutive days during which all animals were challenged with intraperitoneal (ip) injections of saline on day 1 and either with cocaine 15 mg/kg or with saline from day 2 to day 7. Locomotor activity was assessed with Ethovision 3.0 software (Noldus®, Wageningen, The Netherlands). To further compare the distance travelled by mice 15 min prior and 15 min after injections, a

normalization of the data was performed according to the following formula: (distance post injection minus distance prior injection)/(distance post injection plus distance prior injection). Negative values mean a decrease in the distance traveled during the first 15 min post injection compared to the 15 min prior injection. Positive values indicate an increase in the distance traveled during the first 15 min post injection compared to the 15min prior injection.

Conditioned Place Preference

Behavioral tests were performed during the dark cycle. 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. During habituation (preconditioning phase), all animals were allowed to freely explore both compartments for 20 min, and the time spent in each compartment was monitored with a video camera and analyzed simultaneously with the software Ethovision 3.0 (Noldus®, Wageningen, The Netherlands). Animals displaying >70% of the total time in one compartment were excluded. The conditioning phase was performed for 4 consecutive days, with two daily sessions. In the morning, animals were injected either with cocaine (15mg/kg ip) or saline and were confined to one compartment (randomly assigned at the beginning of the experiment) for a 20-min session. In the late afternoon, all animals were treated with saline and were confined in the other compartment for another 20-min session. During the test session, all animals were replaced in the CPP apparatus without injection, and the time spent exploring both compartment was measured. Mice were returned to the CPP apparatus for 20 min after a 2-week period of abstinence during which they remained in their home cages.

Saccharine self-administration acquisition, extinction and reinstatement

The experiments were conducted in mouse operant chambers (Med Associates, St Albans, VT, USA). Mice were trained to self-administer saccharine 0.2% liquid reward on a fixed ratio 1, time out 3 sec (FR1 TO3) schedule of reinforcement in the presence of an olfactory cue (apple aroma, Givaudan,

Dübendorf, Switzerland) during 30-min daily sessions. A single nose entry in the active nosepoke activated a liquid dipper equipped with a 0.01 ml cup and a light cue located inside the nosepoke. The liquid reward remained available for 3 sec once access to the liquid dipper had been detected with head entry detectors. Supplementary entries in the active nosepoke in the absence of head entry detection above the liquid dipper and entries in the inactive nosepoke were recorded but had no further consequence. After completion of the training phase, mice were trained to self-administer saccharine using a progressive-ratio schedule of reinforcement, which consisted of a systematic within-session increase in the ratio of responses required to earn one saccharine reward. Under this schedule, mice were required to progressively increase the number of active nosepokes between two successive rewards based on the progression sequence given by: response ratio (rounded to nearest integer) = $(5e^{(\text{reward}\times 0.2)}) - 5$(Richardson and Roberts, 1996). Hence, the progressive-ratio schedule followed the progression: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, etc. Mice were tested first under food restriction (they were maintained at 85% of their original weight), and tested again after 24h access to food *ad libitum* in their home cages.

Mice were then returned to the FR1 schedule of reinforcement for at least three successive days until stable levels of intake (≤ 25% variation of the mean responses for three consecutive sessions). Afterwards, mice underwent an extinction phase until completion of an extinction criterion (<30% of the mean responses obtained during the 3 days achieving the stabilization criteria across 3 consecutive extinction sessions). Animals were then tested for reinstatement of their nosepoking activities upon presentation of the olfactory and light cues, and the numbers of entries in the active and inactive nosepokes were recorded during 30-min sessions while no liquid reward was delivered.

For assessing the visual discrimination capacity, WT and KO mice were trained to respond to a cue-light inside the nosepoke to earn a 0.01ml of saccharine reward. A constant cue-light was randomly presented in one of the two nosepokes surrounding the dipper. After stable saccharine self-administration was achieved, the time presentation of the cue light was restricted to 1 sec, and

ultimately 0.5 sec. A single nose entry in the briefly illuminated nosepoke activated a liquid dipper equipped with a 0.01 ml of saccharine. The liquid reward remained available for 3 sec once access to the liquid dipper had been detected with head entry detectors. Supplementary entries in the active nosepoke in the absence of head entry detection above the liquid dipper were recorded as correct perseverative responses but had no further consequence. Supplementary entries in the non-illuminated nosepoke in the absence of head entry detection above the liquid dipper were recorded as incorrect perseverative responses. Access to saccharine reward (detected with head entry detectors) triggered the start of a 5-sec inter-trial-interval, before brief random illumination of one of the two nosepokes. Any nosepoke activity during these 5 sec was recorded as a premature response.

Jugular vein catheterization

An indwelling catheter was implanted into the right external jugular vein under oxygen/isoflurane vapor anesthesia (Thomsen and Caine, 2007). Briefly, a 7 cm length of silastic tubing (0.3 mm inner diameter, 0.6 mm outer diameter, Silastic, Dow Corning Corporation, Midland, MI, USA) was fitted to a 22-gauge steel cannula (PlasticsOne, Roanoke, VA, USA) that was bent at a right angle and then embedded in a cement disk (Dentalon Plus, Heraeus Kulzer, Germany) with an underlying nylon mesh. The catheter tubing was inserted 1.2 cm into the right jugular vein and delicately anchored to the vein. The catheter ran subcutaneously to the base located above the midscapular region. All incisions were sutured and coated with antibiotic ointment (Bepanthen® Plus, Bayer AG, Zurich, Switzerland). After surgery, mice were allowed 4-5 days to recover prior to initiation of self-administration sessions, during which 0.05 ml of 0.9% saline containing heparin (30 USP units/ml) was infused daily through the catheter to forestall clotting. Catheter patency was confirmed after completion of the experiment by the infusion of 0.02 to 0.03 ml of Etomidat-®Lipuro (B. Braun, Melsungen, Switzerland). Loss of muscle tone and clear signs of anesthesia within 2 sec of infusion indicated catheter patency. Only mice with a patent catheter were included in the final results.

Cocaine self-administration, extinction and reinstatement

The experiments were conducted in mouse operant chambers (Med Associates, St Albans, VT, USA), either using lever press in the de Lecea Lab (Suppl. Figure 2) or using nose pokes in the Boutrel lab (for all other recordings). Mice were trained to self-administer intravenous cocaine (obtained from Hänseler AG, Herisau, Switzerland) in the presence of an olfactory cue (apple aroma, Givaudan, Dübendorf, Switzerland) under a fixed ratio 1, timeout 20-s (FR1 TO20-s) schedule of reinforcement during daily 2-h sessions 7 days per week. A single nose entry in the active nose poke was paired with the illumination of a light cue located inside the nosepoke and the delivery of the reinforcer (1 mg/kg/infusion, 0.015 ml over 1 sec) through the tubing into the intravenous catheter by a Razel syringe pump. Entries in the active nosepoke during the TO period and entries in the inactive nosepoke were recorded but had no consequence. After mice established stable responding (see above) for the training dose (1mg/kg/infusion), the unit dose available for self-administration was varied to generate a dose-response function. Escalating doses of cocaine (0.5, 0.75, 1 and 1.5 mg/kg/infusion) were tested using a Latin-square design such that most of the mice received each dose. Each dose exposure was repeated twice, and the mean responses for each animal were calculated. Mice with a patent catheter were returned to the training dose for at least three successive days until stable levels of intake. Mice then underwent 2-h extinction sessions with saline infusion in absence of both the olfactory cue and the illuminated nosepoke until reaching the extinction criterion. The criterion was achieved when responses on the active hole were <30% of the mean responses obtained during the 3 days achieving the acquisition criteria across 3 consecutive extinction sessions. Tests for reinstatement were conducted under the same conditions used in the cocaine self-administration phase (olfactory cue and illuminated nosepoke) but saline replaced cocaine.

For assessing the expression of cocaine incubation craving, another group of WT and KO mice was trained for self-administering cocaine (1mg/kg/infusion) in similar conditions but each cocaine infusion was paired with three home cage flashlights. After stable cocaine self-administration was

achieved, mice were returned to the operant chambers to assess contextual cue-induced cocaine seeking after 1, 7, 14, 21 and 28 days of withdrawal. On each test day, mice were replaced into the same self-administration chambers, and cocaine cue-induced drug-seeking behavior was assessed under the same previously described conditions but cocaine was unavailable. Each session lasted 2 hours, as well.

Data analysis

Data shown are presented as the mean ± SEM. We first assessed the model assumptions for ANOVA (normality assessed with a Shapiro-Wilk test, and homogeneity of variances assessed with a Bartlett's test). Then data were subjected to one- and two-factor repeated measures analysis of variance (ANOVA) followed by Tukey's HSD post-hoc tests. Statistical analyses were performed using R software (version 2.15.1), and the level of significance was set at 0.05. (See supplementary information for extended analysis).

Results

Loss of Hcrt transmission only delays cocaine-induced behavioral sensitization

First exposure to cocaine treatment induced a significant increase in locomotor activity in WT and HET males, and to a much lesser extent in KO ones (Figure 1A), whereas the locomotor activity of mice treated with saline remained unchanged. WT, HET and KO mice were further challenged with 15mg/kg of cocaine for six more days in order to monitor the development and acquisition of cocaine sensitization. In contrast to the saline group, all mice exhibited a robust sensitization with chronic cocaine administration (WT, $F_{(1,110)}$=121.4, p-value<0.001; HET, $F_{(1,117)}$=69.0, p-value<0.001; KO, $F_{(1,117)}$=116.6, p-value<0.001) but KO males displayed delayed behavioral sensitization compared to WTs ($F_{(2,308)}$=36.7, p<0.001; Figure 1B). On day 7, all males exhibited similar locomotor activity (WT: 58389 ± 7359 cm, HET: 45834 ± 5483 cm and KO: 42303 ± 6106 cm, post-hoc test: p-value>0.05).

Noteworthy, we found that KO mice treated with saline tend to display a reduced locomotor activity compared to WT and HET animals, confirming previous reports(Hara et al., 2001; Mochizuki et al., 2004). We decided to compare the activity of cocaine- and saline-treated mice during the 15 min before drug injection and the 15 min after the injection in order to evaluate the amplitude of individual behavioral sensitizations. Saline-treated mice displayed negative amplitudes reflecting a decreased locomotor activity after the injection. In contrast, mice of the three genotypes exhibited positive behavioral sensitization amplitudes starting from day 2 (first cocaine administration) reflecting the increased locomotor activity after cocaine injection. Interestingly, WT, HET and KO mice displayed similar amplitude of behavioral sensitization, except on the first day of cocaine treatment during which KO mice responses remained significantly lowered ($F_{(2,308)}$ (genotypes)=7.0, $p<0.01$; Figure 1C). Thus, cocaine-induced behavioral sensitization was delayed in KO mice, but when comparing the amplitude of the sensitization using each mouse as its own control, we conclude that all mice responded likewise to chronic cocaine administration.

Unexpectedly, females of the three genotypes did exhibit identical responses to repeated cocaine injections in contrast to male mice ($F_{(2,560)}$ (sex x genotype)=9.88, $p<0.001$; Figure 1D-F). Considering that females might have been more sensitive than males to the anxiogenic environment of the open field (Bangasser et al., 2010), which could have explained the enhanced response to chronic cocaine administrations, we assessed cocaine-induced behavioral sensitization in individual cages equipped with running wheels (Suppl. Figure 1). Statistical analyses (see supplementary information) revealed a significant reduction in the total distance traveled by KO females at baseline (prior injection) compared to the performance displayed by HET and WT mice, but ultimately, WT, HET and KO females did exhibit similar amplitude of the cocaine-induced behavioral sensitization, which agrees with the data reported above.

Hcrt deficient mice display a robust cocaine-induced place preference

We tested then the rewarding effects of cocaine (15 mg/kg) on WT, HET and KO males and females using a conditioned place preference (CPP) paradigm. The CPP procedure was conducted in four phases: habituation (pretest), conditioning, test session (1st posttest) and a second posttest session after 2-weeks of abstinence (meaning two weeks after the last cocaine administration). All animals (males and females of the three genotypes) exhibited a marked preference for the compartment previously paired with cocaine administration on the 1st posttest session compared to the pretest, whereas saline treated animals did not prefer one compartment over the other [$F_{(1,186)}$(treatment)=39.2, $p<0.001$; $F_{(1,186)}$(session)=76.1, $p<0.001$; $F_{(1,186)}$(treatment x session)=61.5, $p<0.001$; Figure 2A and 2B]. Further, the rewarding effect of cocaine was independent of the genetic background ($F_{(2,186)}=0.3$, $p>0.05$) and the sex ($F_{(1,186)}=1.4$, $p>0.05$). After a two week-period of abstinence, all mice were returned to the CPP apparatus. WT and HET mice maintained their preference for the compartment previously paired with cocaine administrations whereas KO male and female mice stopped expressing such a preference [$F_{(2,174)}$(genotype x session)=0.02, $p<0.05$; Figure 2C and 2D].

The distance travelled by mice during the 20 min of the pretest and the posttest sessions were also monitored. In agreement with the behavioral sensitization data, only WT and HET animals exhibited an increased locomotor activity during the posttest session ($F_{(1,186)}=34.6$, $p<0.001$; Suppl. Figure 2A and 2B), although mice did not receive any cocaine injection on the test day (see supplementary information). Two weeks after the last cocaine injection, although WT and HET males and females persisted in preferring the former cocaine-paired compartment, all mice exhibited similar locomotor activity (Suppl. Figure 2C and 2D).

Hypocretin deficient mice manifest a blunted cocaine intake at the highest dose and a reduced cue-induced reinstatement of previously extinguished cocaine seeking behavior

After showing that WT, HET and KO mice exhibited similar preference for the rewarding properties of cocaine, we assessed cocaine self-administration in WT and KO males only. In a first series of experiments, WT and KO mice were trained to lever press for intravenously self-administering cocaine (0.7 mg/kg/infusion). We did not observe any difference either in a fixed- or a progressive-ratio schedule of reinforcement (Suppl. Figure 3). In a second series of tests, mice were trained to nose poke for self-administering cocaine (0.5-1.5 mg/kg/infusion). A two-factor ANOVA revealed significant main effects for cocaine dose ($F_{(3,235)}$=13.3, p<0.001) and genotype ($F_{(1,235)}$=10.3, p<0.01), with a significant dose x genotype interaction ($F_{(3,235)}$=2.75, p<0.05). Hence, although Hcrt deficient mice self-administered middle range doses of cocaine (0.5-1 mg/kg/infusion) reflecting a normal ability to acquire ongoing cocaine-taking behaviors compared to WT littermates, a Tukey's HSD post-hoc tests among means revealed that KO mice displayed a blunted cocaine intake at the highest dose (1.5 mg/kg/infusion) compared to their WT littermates (Figure 3A).

After a period of extinction (10.4 $\pm$ 0.9 versus 10.2 $\pm$ 0.5 sessions in WT and KO mice, respectively) during which mice gradually decreased their nosepoking behavior in absence of light and olfactory cues, we assessed the ability of these mice to reinstate their cocaine seeking behavior upon presentation of the light and olfactory cues. A two-way repeated measure ANOVA revealed significant main effects for genotype ($F_{(1,114)}$=17.24, p<0.001), phase ($F_{(2,114)}$=58.36, p<0.001) and genotype x phase ($F_{(2,114)}$=4.99, p<0.01). Tukey's HSD post-hoc tests revealed that WT mice significantly reinstated their previously extinguished cocaine-seeking behaviors whereas KO mice did not (Figure 3B). To assess whether Hcrt deficient mice may display signs of resilience to the long-term reinforcing properties of cocaine, we ran a cocaine incubation craving procedure in which mice were returned to the operant chambers every week under identical conditions (see materials and methods, and supplementary information). A two-way repeated measure ANOVA revealed significant

main effects for genotype ($F_{(1,95)}$=5.79, p<0.05) and session ($F_{(5,95)}$=12.97 p<0.001) and a significant genotype x session interaction ($F_{(5,95)}$=2.63, p<0.05). Tukey's HSD post-hoc tests revealed that whereas WT mice exhibited a significant increase in cocaine seeking behavior at Day 7, 14 and 21, KO mice showed a significant but blunted cocaine incubation craving after a 2-week period of withdrawal (Figure 3C).

It then appeared relevant to assess the acquisition, extinction and reinstatement of saccharine seeking behaviors in WT and KO mice since the Hcrt was shown to play a key role in the reinforcing properties of sweetened taste (Cason and Aston-Jones, 2013a, b; Ho and Berridge, 2013). We first trained another group of mice to self-administer saccharine 0.2% *per os* during the light and the dark phases since a recent report claimed that Hcrt deficient mice displayed reduced motivation for food reward specifically during the light period (McGregor et al., 2011). In our hands, food restricted Hcrt deficient mice exhibited a significantly reduced motivation (see Suppl. Information for statistical analyses) for saccharine reward in a progressive ratio schedule of reinforcement during both the light and dark period compared to WT mice (Suppl. Figure 4). Interestingly, when fed *ad libitum*, WT mice drastically reduced the amount of work required to earn liquid rewards whereas KO mice did not, confirming that Hcrt deficient mice could not provide supplementary effort despite starvation (Yamanaka et al., 2003). Another group of WT and KO mice (fed *ad libitum*) was then trained for self-administering saccharine in a fixed ratio 1 schedule until stable intake, and after a period of extinction, we assessed their ability to reinstate their saccharine seeking behavior upon presentation of the light and olfactory cues. Again, a two-way repeated measure ANOVA revealed significant main effects for genotype ($F_{(1,127)}$=45.68, p<0.001), phase ($F_{(2,127)}$=152.12, p<0.001) and genotype x phase ($F_{(2,127)}$=13.22, p<0.01). Post-hoc tests revealed that only WT mice significantly reinstated their previously extinguished saccharine-seeking behavior (Figure 4A), confirming KO mice deficits to reinstate reward seeking after a period of extinction. Since the absence of cue-induced reinstatement could have been explained by altered responsiveness to cue rather than alterations in underlying motivational states, we performed a visual discrimination task in WT and KO mice to

assess any putative attention deficits in Hcrt deficient mice. Both groups of mice were trained to respond to a briefly illuminated cue light (0.5 sec) inside the nosepokes to earn a 0.01ml of saccharine reward. This cue light was randomly presented between the two nosepokes. Whereas Hcrt deficient mice failed to reinstate a previously extinguished saccharine seeking behavior, their visual discrimination capacity was similar to that of WT mice (Figure 4B), further suggesting that disruption of Hcrt signaling does not alter responsiveness to cue, but rather decreases the underlying positive affective state that drives motivated behaviors.

Discussion

In the present work, we observed that WT, HET and KO males exhibited cocaine-induced locomotor sensitization in an open field. However, KO males showed a delayed and attenuated cocaine-induced behavioral sensitization compared to WT mice, which is in agreement with previous observations using the Hcrtr-1 antagonist SB-334867 in rodents (Borgland et al., 2006; Hutcheson et al., 2011; Quarta et al., 2010). Nonetheless, a comparison of the distance traveled during the first 15 min following cocaine treatment with the distance traveled during the 15 min of habituation shows that the differences between KO, HET and WT are largely reduced. Indeed, males of the three genotypes exhibited similar amplitude in behavioral sensitization following daily cocaine treatment, with the notable exception of the first cocaine session during which KO males displayed a reduced cocaine-induced hyperlocomotion. Unexpectedly, female mice of the three genotypes exhibited similar cocaine-induced behavioral sensitization. Could higher levels of leptin, as previously reported in female Hcrt KO mice (Fujiki et al., 2006), compensate for Hcrt deficiency and thus facilitate cocaine-induced behavioral sensitization (Fulton et al., 2006; Fulton et al., 2000) remains an open question that needs further investigation to be addressed. With the highest expression of Hcrtr-1 in the brain being the locus coeruleus (LC) (Hervieu et al., 2001), another plausible explanation would take into account the sexual dimorphism in LC dendritic morphology (Bangasser et al., 2011) to explain the

delayed behavioral sensitization in KO males. Indeed, an increased attenuation of basal and cocaine-induced locomotor activities were already reported after administration of SB 334867 in males compared to female rats (Zhou et al., 2012).

Interestingly, despite a reduced locomotor activity compared to WT and HET littermates in a cocaine-induced place preference paradigm, KO animals did exhibit a significant preference for the context previously paired with repeated drug injections, in line with a previous report (Sharf et al., 2010). Though, Hcrt KO mice have been shown to display disrupted dopamine signaling and attenuated dopamine responses to cocaine (Espana et al., 2010; Shaw et al., 2016), which contrasts with our findings. However, dopamine is not critical for developing cocaine-induced CPP since dopamine deficient mice display normal CPP possibly through serotonin signaling (Hnasko et al., 2007). Noteworthy, Hcrt KO mice exhibit reduced dopamine levels and a higher basal serotonin ratio relative to WT (Mori et al., 2010), which could explain the acquisition of the cocaine-induced CPP, and raises the question of compensatory mechanisms occurring during embryo development in Hcrt deficient mice, that most likely would be different from those occurring in Hcrt-1-R KO mice (Hollander et al., 2012). Our hypothesis is that the reduced dopamine response to cocaine, reported in Hcrt deficient mice by Espana and colleagues (2010), might not have significant consequences on the acquisition and expression but on the long-lasting responsiveness to cocaine cues. Indeed, the preference for an environment previously paired with cocaine reward has been accounted to persist for up to four weeks in rodents (Bardo et al., 1986; Mueller and Stewart, 2000). Here, we report that not only KO mice stopped expressing such a preference two weeks after the last cocaine injection, but they also exhibited a weakened cocaine incubation craving after a 2-week period of withdrawal, in striking contrast to WT animals. Hence, the Hcrt system might play a key role in the long-lasting conditioned responses to cocaine.

In agreement with the above-mentioned observations, we report comparable acquisition of self-administration behaviors for middle range doses of cocaine in WT and Hcrt deficient mice. However,

KO animals consumed significantly less cocaine than WT littermates at the highest dose tested, 1.5 mg/kg/infusion, suggesting a blunted cocaine intake in these mutants. Interestingly, a significant reduction of the motivation for saccharine reward was observed in KO mice compared to WT mice under food restriction during both the light and dark phases (Suppl. Figure 3). Further, when sated, only WT mice exhibited a significant decrease in the effort required for liquid reward suggesting that food restriction did not enhance motivation for saccharine in KO mice, notably during the dark period. Our observations contrast with those reported by McGregor and colleagues (2011), but are in line with the reduced alertness (Chemelli et al., 1999), the absence of fasting-induced increase in arousal and motor activity (Yamanaka et al., 2003), and the reduced sucrose intake reported in Hcrt deficient mice (Matsuo et al., 2011). Interestingly, when KO mice were off cocaine (meaning after a period of extinction), they displayed a decreased cue-induced reinstatement for reward seeking that was similar to the deficit they exhibited when challenged in a cue-induced reinstatement for saccharine seeking. These series of observation fit with the motivational activation hypothesis suggested by Mahler and colleagues, according to which Hcrt/Ox peptides facilitate reward seeking when this seeking is highly motivated by a physiological need and/or by a psychological need triggered by external cues or stressors (Mahler et al., 2014).

Ultimately, with WT and KO mice displaying similar visual discrimination capacities, we hypothesize that the constant hypoactive phenotype seen in the KO mice, that most likely is accountable for their reduced tendency to explore the environment, is probably due to a reduced motivational drive in absence of urge or basic needs. Our findings suggest that although the stimulant effects of cocaine compensate their apathy-like behavior, Hcrt deficient mice keep control: they take cocaine when available but do not exhibit significant/exaggerated efforts for cocaine seeking during prolonged abstinence, therefore displaying signs of reduced vulnerability towards cocaine addiction.

In sum, our findings provide clear evidence that innate loss of Hcrt peptides most likely does not decrease the acute reinforcing properties of cocaine (in contrast to Hcrt-1-R knock out reported by

Hollander and colleagues in 2012), but possibly decrease its long-term effects. Interestingly though, with a blunted cocaine intake at the highest concentration and reduced reinstatement of cocaine-seeking behaviors after periods of abstinence (either using an operant conditioning procedure or a conditioned place preference paradigm), Hcrt deficient mice seem to display signs of resilience to cocaine abuse. Our observations further support the complex role of the Hcrt/Ox signaling in the rewarding and motivational properties of drugs of abuse, with a system possibly contributing to invigorating the organism to seek for the drug, thus overcoming work and effort to perform to ultimately get access to the drug. In line with former observations reported in the literature, our data highlight the potential therapeutic utility of disrupting hypocretin/orexin transmission for alleviating symptoms of drug dependence, notably by reducing the urge for drug seeking during protracted abstinence.

Acknowledgments

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The authors report no conflict of interest.

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Legends to figures

Figure 1. WT, HET and KO mice developed cocaine induced sensitization. (A) Time course of cocaine-induced hyperlocomotion after the first cocaine administration (Day 2) in WT (square), HET (circle) and KO (triangle) males. Data represent the mean distance traveled (± SEM) in cm for 60 min (+10 min habituation) in 8-9 mice. (B) Locomotor activity was daily monitored after ip injection of cocaine (15 mg/kg, filled symbols) or saline (open symbols) in WT, HET and KO males. Data represent the mean distance traveled (± SEM) in cm for 1h in 8-9 mice. (C) Amplitude of the behavioral sensitization in WT, HET and KO males. Data represent the mean ratio (± SEM) defined as: (distance post injection minus distance prior injection)/(distance post injection plus distance prior injection). (D, E and F) are equivalent experiments to (A, B and C) performed in female mice. Tukey post hoc test: * p-value<0.05, ** p-value<0.01 and *** p-value<0.001 versus saline, # p-value<0.05, ## p-value<0.01 versus WT and $ p-value versus HET.

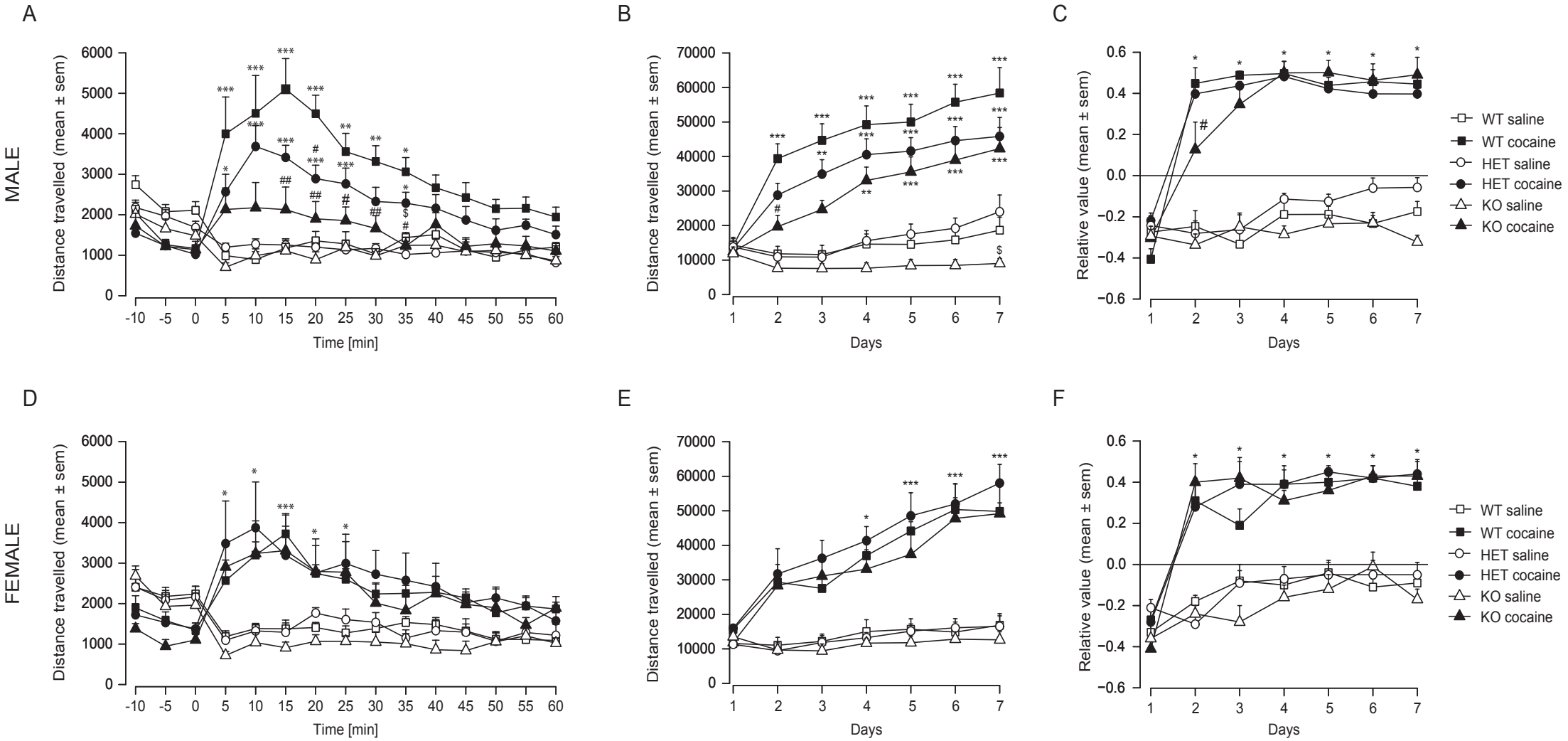
Figure 2. WT, HET and KO mice developed robust cocaine-induced conditioned place preference. Data represent mean time (± SEM) expressed in seconds spent in the drug paired compartment for WT, HET and KO animals (A, males and B, females) before (open boxes) and after (filled boxes) cocaine conditioning in 7-8 mice. (C, D) Data represent mean time (± SEM) expressed in seconds spent in the drug paired compartment for WT, HET and KO animals (E, males and F, females) before conditioning (open boxes) and after (filled boxes) a 2-week period of cocaine abstinence (passive extinction) in 7-8 mice. Tukey post hoc test: * p-value<0.05 and *** p-value<0.001 versus the respective pretest session, # p-value<0.05 versus WT.

Figure 3. KO mice exhibited reduced conditioned responses to cocaine cues. (A) Data represent mean cocaine intake (± SEM) expressed in mg/kg in WT (square) and Hcrt KO (triangle) male mice (n=7-8). Tukey post hoc test: * p-value<0.05, *** p-value<0.001 versus the 0.5 mg/kg dose in respective genotype and # p-value<0.05 versus the 1.5 mg/kg dose in WT mice. (B) Data represent mean (± SEM) number of nosepokes during cocaine acquisition (average of last three session), extinction and

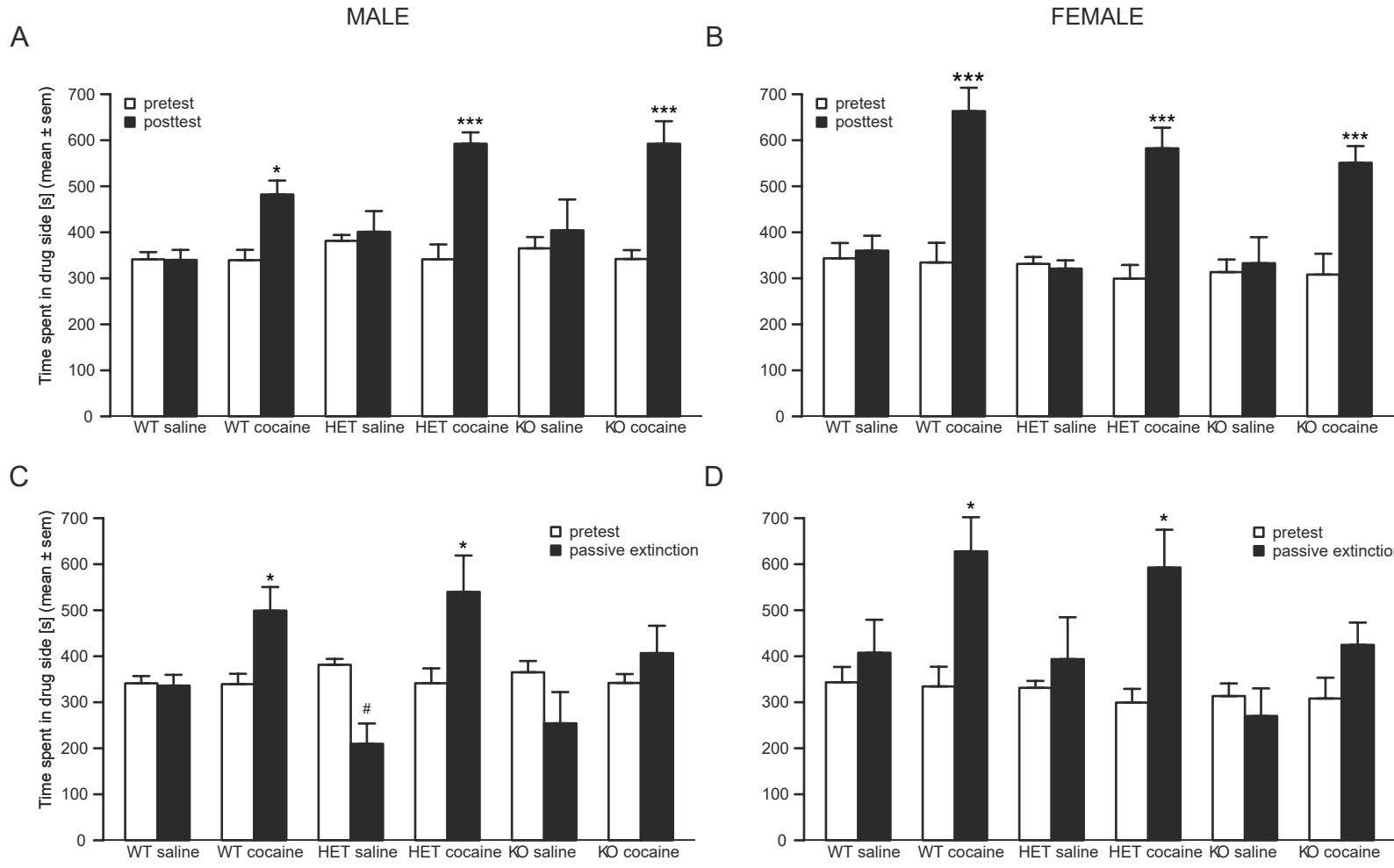
cue-induced reinstatement phases in WT (filled boxes) and KO (open boxes) male (n=7-8). Tukey post hoc test: *** p-value<0.001 versus the respective acquisition session, ## p-value<0.01 versus WT males and $$$ p-value<0.001 versus the respective extinction session. (C) Data represent mean (± SEM) number of nosepokes during cocaine acquisition (average of last three session) and during 1-28 days of withdrawal after last cocaine self-administration in WT (filled boxes, n=9) and KO (open boxes, n=12) animals. Tukey post hoc test: * p-value<0.05, ** p-value<0.01 and *** p-value<0.001 versus the respective acquisition session (cocaine 1 mg/kg), # p-value<0.05 and ## p-value<0.01 versus WT males and $$$ p-value<0.001 versus the respective extinction session.

Figure 4. KO mice failed to reinstate a previously extinguished saccharine seeking behavior following presentation of reward cues, but performed normally a visual discrimination task. Data represent mean number of nosepokes (± SEM) following presentation of reward cues (A) and mean number of correct, incorrect, premature and perseverative nosepokes (± SEM) (B) in WT (black boxes, n=8-10) and Hcrt KO (white boxes, n=6-9) mice. Tukey post hoc tests: *** p-value<0.001 versus respective acquisition session, ### p<0.01 versus WT acquisition session, $$ p-value<0.01 versus WT extinction session.

Steiner et al. Figure 1

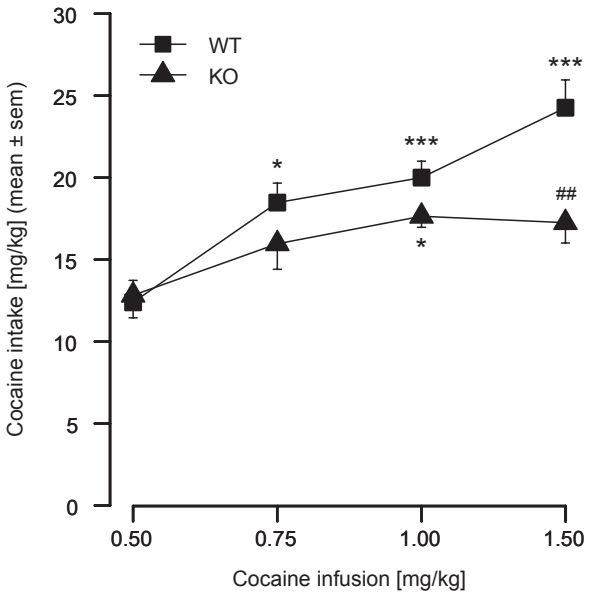


Steiner et al. Figure 2



Steiner et al. Figure 3

A



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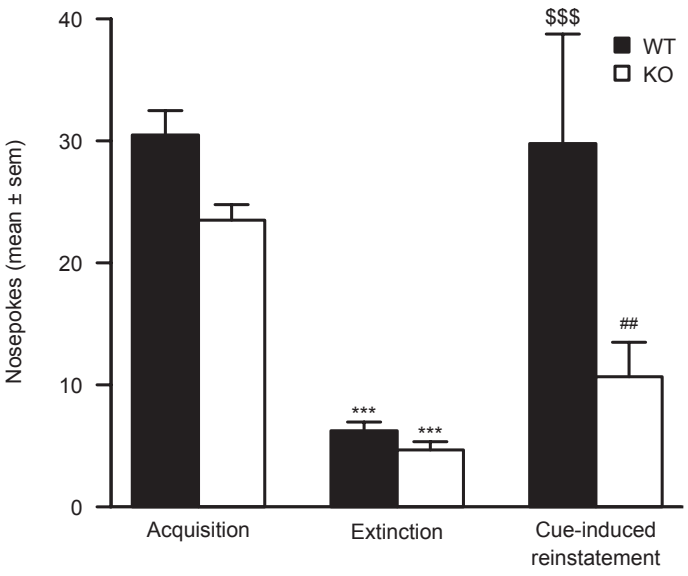

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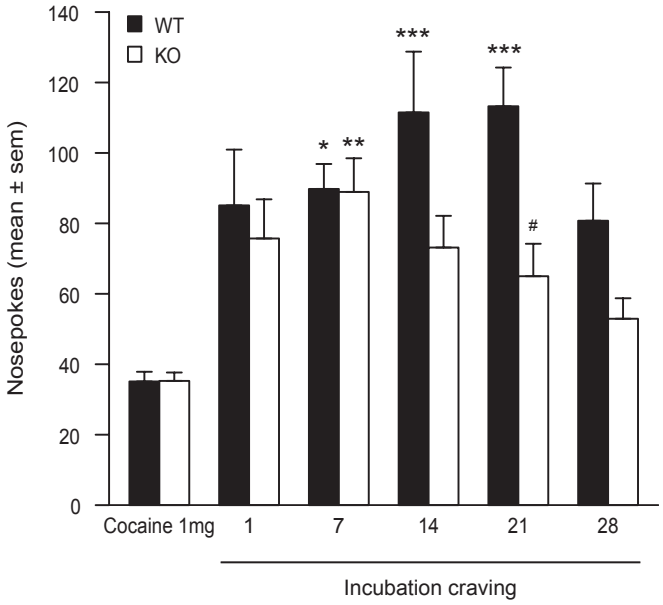
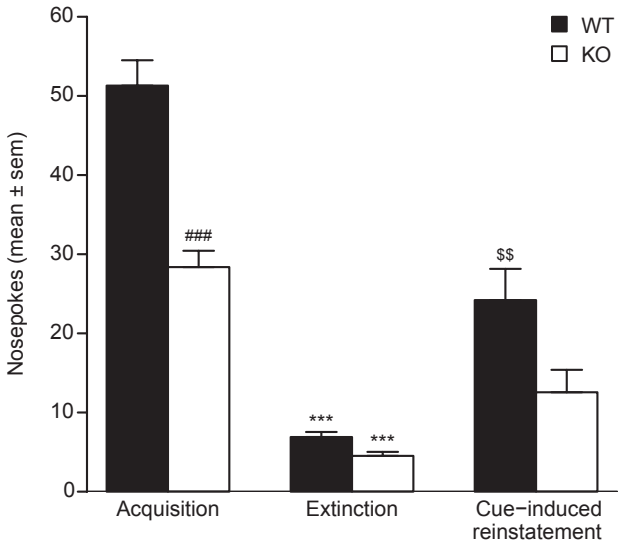


Steiner et al. Figure 4

A



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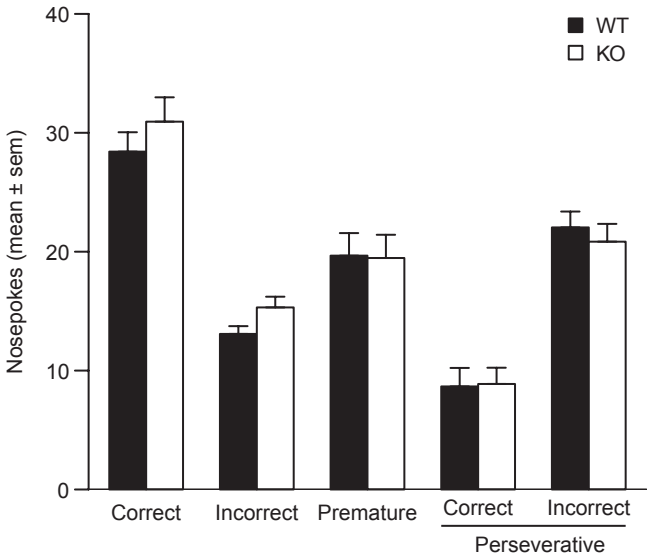

Supplementary information for

Hypocretin/Orexin deficiency decreases cocaine abuse liability

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This supplementary information contains further results, and Figure S1, Figure S2, Figure S3 and Figure S4.

Results

Behavioral sensitization in the running wheels

WT, HET and KO females were tested in running wheels, for 60 min after saline treatment, twice on day 1. From day 2 to day 7, mice were monitored first for 60 min after a saline injection, and second for 60 min following a 15 mg/kg ip cocaine administration. On day 1 (saline treatment, Suppl. Figure 1A), a two-way repeated measure ANOVA revealed significant main effects for genotype ($F_{(2,30)}$=3.45, p<0.05), with KO females displaying a reduced activity. Saline treatment did not modify locomotor activity compared to the pre-injection test ($F_{(1,30)}$=0.084, p>0.05) and the interaction was not significant as well ($F_{(2,30)}$=0.181, p>0.05). Post-hoc tests revealed that KO females treated with saline exhibited similar locomotor activity compared to both WT and HET mice treated with saline (Tukey HSD, p=0.085 and p=0.055, respectively). On the second day (first cocaine exposure, Suppl. Figure 1B), all mice exhibited similar locomotion after cocaine injection ($F_{(2,30)}$=2.860, p=0.073) and on the seventh day (sixth cocaine administration, Suppl. Figure 1C), all mice exhibited similar performances, with no significant effect for genotype ($F_{(2,30)}$=2.558, p=0.094) and no effect for cocaine treatment ($F_{(1,30)}$=3.303, p=0.079).

Increased locomotor activity during the posttest session of the conditioned place preference

We monitored the distance travelled by mice during the 20 min of the pretest (before cocaine conditioning) and the posttest (after cocaine conditioning) sessions. Although mice did not receive any cocaine administration on the posttest session, we report an increased locomotor activity after cocaine conditioning [$F_{(1,186)}$(treatment x session)=37.3, p<0.001] in WT animals [males: 4284 ± 208 cm (after cocaine conditioning) vs 3244 ± 157 cm (before cocaine conditioning); females: 4650 ± 134 cm (after) vs 3616 ± 336 cm (before), p<0.05 Tukey's HSD post-hoc tests]. Similarly, we report an increased locomotor activity in HET mice [males: 4248 ± 191 cm (after) vs 2813 ± 128 cm (before); females: 4555 ± 205 cm (after) vs 3450 ± 269 cm (before), p<0.05 Tukey's HSD post-hoc tests].

However, KO animals did not exhibit any increased locomotor activity after cocaine conditioning [males: 3203 ± 173 (after) vs 2876 ± 170 cm (before); females: 3799 ± 169 cm (after) vs 2869 ± 370 cm (before), p>0.05 Tukey's HSD post-hoc tests].

Saccharine seeking in males

WT and KO males were tested in a progressive ratio schedule of reinforcement for saccharine 0.2% during the light (quiet) and the dark (active) phases of their circadian cycle, once under food restriction and once sated (Suppl. Figure 4). A two-way repeated measure ANOVA revealed significant main effects for *genotype* ($F_{(1,80)}$=12.923, p<0.001), *food condition* ($F_{(1,80)}$=25.679, p<0.001) and a significant *genotype x food condition* interaction ($F_{(1,30)}$=4.072, p<0.05) proving that KO mice exhibited a reduced motivation for saccharine seeking under food restriction compared to WT animals.

When looking at WT mice behavior only, a two-way repeated measure ANOVA revealed significant main effects of *food condition* ($F_{(1,40)}$=29.640, p<0.001), but no effect of the *cycle* ($F_{(1,40)}$=1.527, p>0.05). Tukey's HSD post hoc tests revealed a significant reduction of saccharine seeking in sated WT mice (either during the light or the dark period).

When looking at KO mice behavior, a two-way repeated measure ANOVA failed to reveal any significant effects of *food condition* ($F_{(1,40)}$=4.032, p=0.0514), or *cycle* ($F_{(1,40)}$=0.153, p>0.05).

Cocaine incubation craving

Cocaine incubation craving is usually interpreted by comparing the first and last day of abstinence. Here, we compared nose poke responses during abstinence to nose poke responses during cocaine intake. We considered important to stress out that both WT and KO mice exhibited similar cocaine intake, and similar seeking behaviors on d1 and d7, and found intriguing that nose poking started decreasing on d14 in KO mice, while we observed the absence of place preference after a 2-week period of passive extinction. These observations suggest that cocaine maintains its reinforcing

properties in KO mice, acutely and shortly after abstinence, but not after a prolonged abstinence. Since incubation craving typically compares a delayed session to d1 of abstinence, we compared d1 and d21 in WT and KO mice. A two-way repeated measure ANOVA revealed a significant genotype x session interaction ($F_{(1,19)}$=4.997, $p<0.05$), supporting a reduced incubation craving in Hcrt KO mice.

Legends to supplementary figures

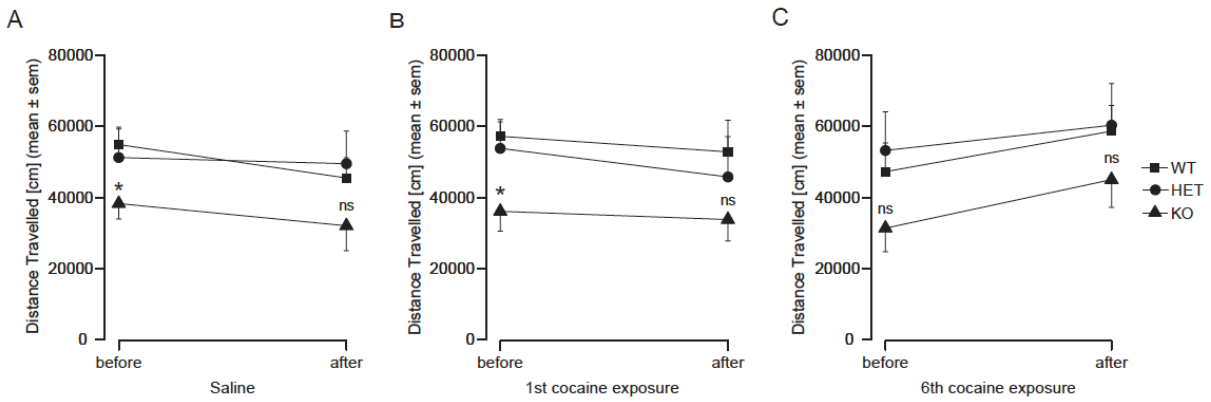
Suppl. figure 1. Locomotor sensitization of WT, HET and KO female mice assessed in running wheels. Data represent the mean distance traveled (± SEM) in cm for 60 min in 5-7 mice per group, (A) before and after saline injection, (B) before and after the first cocaine injection (15 mg/kg) and (C) before and after the last cocaine injection following chronic intermittent treatment with cocaine (15 mg/kg) in WT (square), HET (circle) and KO (triangle) mice. Tukey post hoc tests: * p-value<0.05 compared to WT mice; ns, non-significant compared to WT mice.

Suppl. figure 2. WT, HET and KO mice developed robust cocaine-induced conditioned place preference. Data represent mean distance (± SEM) expressed in cm traveled by WT, HET and KO mice (A, males and B, females) before (open boxes) and after (filled boxes) cocaine conditioning in 7-8 mice. (C, D) Data represent mean distance (± SEM) expressed in cm traveled by WT, HET and KO mice (C, males and D, females) before conditioning (open boxes) and after (filled boxes) a 2-week period of cocaine abstinence (passive extinction) in 7-8 mice.

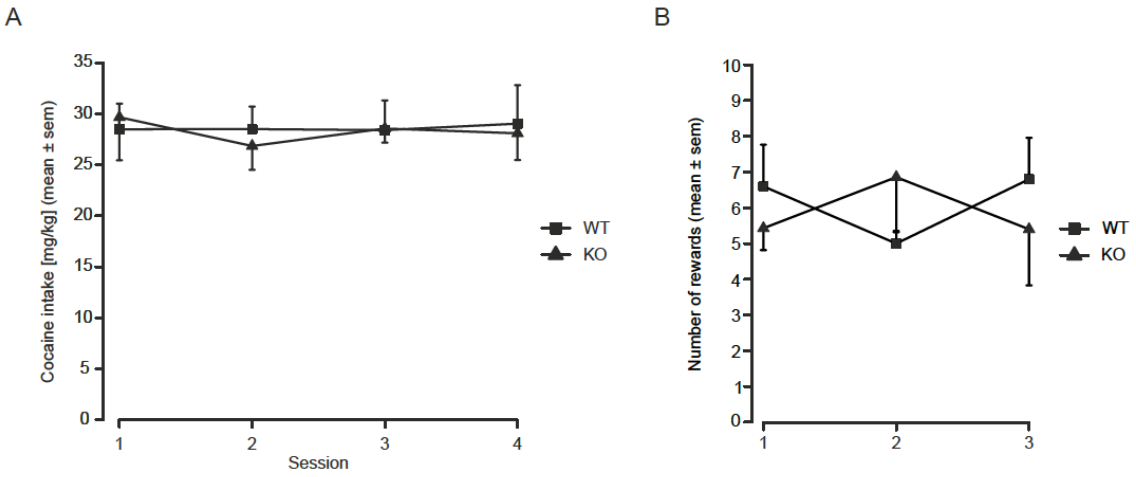
Suppl. figure 3. WT and KO mice exhibited similar pattern of cocaine intake (0.7 mg/kg/infusion). (A) Data represent mean cocaine intake (± SEM) expressed in mg/kg in WT (square) and Hcrt KO (triangle) male mice (n=7-8) using a fixed ratio 1 time out 20 sec schedule. (B) Data represent mean number of cumulated lever presses (± SEM) and equivalent earned rewards using a progressive ratio schedule of reinforcement (n=6-7).

Suppl. figure 4. Food restricted KO mice displayed reduced motivation for saccharine in a progressive ratio schedule of reinforcement. Data represent mean number of collected rewards (± SEM) during the dark (A) and the light (B) phase in WT (black boxes) and KO (white boxes) mice. Tukey post hoc tests: * p-value<0.05, ** p-value<0.01, *** p-value<0.001 versus food restricted WT (satiety effect) and # p-value<0.05 versus food restricted WT mice (genotype effect).

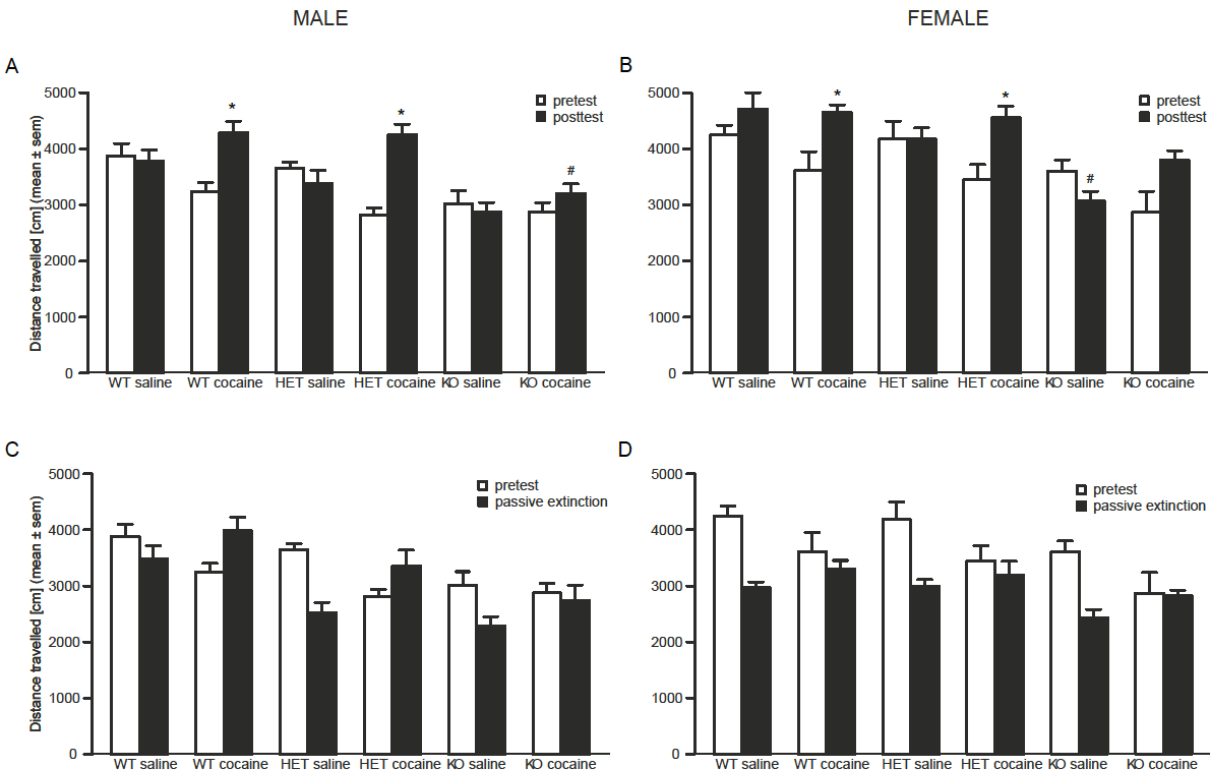
Steiner et al., Supplementary Figure 1



Steiner et al., Supplementary Figure 2



Steiner et al., Supplementary Figure 3



Steiner et al., Supplementary Figure 4