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Hcrtr1 and 2 signaling differentially regulates depression-like behaviors

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Abstract

The orexin/hypocretin system has the potential to significantly modulate affect, based on both the neuroanatomical projection patterns of these neurons and on the sites of orexin receptor expression. However, there is little data supporting the role of specific orexin receptors in the modulation of depression-like behavior. Here we report behavioral profiling of mice after genetic or pharmacologic inhibition of hcrtr1 and 2 receptor signaling. Hcrtr1 null mice displayed a significant reduction in behavioral despair in the forced swim test and tail suspension test. Wild-type mice treated with the hcrtr1 antagonist SB-334867 also displayed a similar reduction in behavioral despair. No difference in anxiety-like behavior was noted following hcrtr1 deletion. In contrast, hcrtr2-null mice displayed an increase in behavioral despair with no effect on measures of anxiety. These studies suggest that the balance of orexin action at either the hcrtr1 or the hcrtr2 receptor produces an anti-depressant or pro-depressant like effect, depending on the receptor subtype activated.

Keywords

Orexin; Depression; Anxiety

1. Introduction

Orexin (also known as hypocretin) neurons exist as a small cluster of cells found exclusively in the perifornical area, dorsal medial hypothalamus and lateral hypothalamus [1,2]. Orexin neurons secrete the neuropeptides orexin-A and B, synthesized from the common precursor pre-pro-orexin [2,3]. These neuromodulatory peptides signal through two G-protein coupled receptors, orexin receptor 1 and orexin receptor 2 (Hcrtr1 and Hcrtr2), with orexin-A showing equal affinity for both receptors while orexin-B demonstrates a higher affinity for Hcrtr2 [2]. Orexin signaling has been implicated in processes as diverse as sleep-wake transitioning [4], the control of food intake [5], and autonomic output [6]; acting to match

the activity of homeostatic circuits with the appropriate level of vigilance. Interestingly, although orexin neurons innervate most areas of the brain, there is minimal co-expression of the two orexin receptors throughout structures of the CNS [7], suggesting that little redundancy exists in the orexinergic modulation of behavior. For example, while *hcrtr1* exhibits high levels of expression in the locus coeruleus, *hcrtr2* expression is seen in histaminergic, sleep-state regulatory tuberomammillary neurons in the absence of *hcrtr1* expression [7]. Consequently, while *Hcrtr1* receptor deletion produces no measurable effect on sleep wake cycling, *hcrtr2* deletion produces an increase in waking during the rest phase or light cycle coupled with an inability to maintain wakefulness during the active phase or dark cycle [4]. *Hcrtr1* signaling, meanwhile, has been implicated in the re-instatement of cocaine seeking and the development of anhedonia [8]. This divergence in orexin receptor function likely extends to the modulation of mood, particularly depression, as orexin receptors are differentially expressed in many regions traditionally associated with the modulation of depression-like behavior including the cerebral cortex, hippocampus, dorsal raphe, and ventral tegmental area [7]. Although little data is available on the role of orexin in the acute modulation of depression, in a model of drug-induced depression, clomipramine induces an elevation in orexin-A and B levels along with an induction of a depressive-like state [9], suggesting orexin acts to negatively modulate affect. To date, however, the role of specific receptors in the regulation of depression-like behavior has not been investigated. To begin to explore the role of both *hcrtr1* and 2 receptors in the regulation of mood, specifically depression, we performed behavioral tests on mice lacking either the *hcrtr1* or *hcrtr2* receptor. We employed two novel mouse lines that have been engineered with a removable transcription-blocking cassette inserted into either the *hcrtr1* or 2 genes, arresting the production of the receptor. Subsequent exposure to Cre recombinase allows for the re-expression of receptor in select CNS nuclei, while maintaining the non-recombinase expressing neurons null. In this report, we present an initial characterization of these receptor null mice, illustrating that *hcrtr1* and 2 receptor signaling differentially regulates depression-like behaviors.

2. Materials and methods

2.1. Animal husbandry

All animal procedures were conducted in accordance with UT Southwestern's Institutional Animal Care and Use Committee guidelines and those of the Association for Assessment and Accreditation of Laboratory Animal Care. Mice were housed individually on a 12 h light/dark cycle with free access to food and water. Adult male mice (8–10 weeks of age) were used for all studies. *Hcrtr1* and 2 null mice were backcrossed for 10 generations onto C57BL/6. Mice heterozygous for the *hcrtr1* or 2 null allele were bred to generate wild-type and *hcrtr1* or 2 null littermates for all behavioral testing conducted within our mouse housing facility.

2.2. Generation of mice

Hcrtr2-null mice were generated by inserting a loxP-flanked transcriptional and translational blocking cassette as described previously [10,11]. These mice have been demonstrated to be functionally null for the *hcrtr2* receptor [11]. Similarly, *hcrtr1* null mice were produced by the insertion of a transcription and translational blocking cassette between exons 3 and 4 of the *hcrtr1* gene. Mice were subsequently shown to be null for the *hcrtr1* by *in situ* hybridization, as the receptor was shown to be absent from previously described sites of elevated receptor expression (Supplemental Fig. 1) and from all other CNS nuclei (not shown).

2.3. In situ hybridization

In situ hybridization was performed as described previously [12], using probes generated from mouse *hcrtr1* cDNA. The probe sequence spanned exons 4–8 and covered sequence unique to *hcrtr1*, with minimal similarity to *hcrtr2*. Probe validation was performed on wild-type mouse tissue, exhibiting an expression pattern similar to that reported for rat *hcrtr1* receptor expression (data not shown). Expression was strongest in the locus coeruleus (Supplemental Fig. 1).

2.4. Behavioral testing

Mice of each genotype were initially subjected to tests of anxiety, first the elevated plus maze followed by the light dark assay the next day. Seven days after these tests, mice were tested using the porsolt forced swim paradigm. An independent group of mice was then employed in the tail suspension test. For the orexin receptor antagonist studies, wild-type mice were either tested in the porsolt forced swim test or tail suspension test. All testing was performed at the same time of day, from 12 p.m. to 3 p.m. The number of animals used in each experiment is specified in figure legends.

2.5. Forced swim test

The Porsolt forced swim test was performed according to protocols employed in prior published studies [13]. Briefly, mice were placed in 4 L Pyrex beakers filled with 3 L of 24 ± 1 °C water for 6 min. Swimming activity was recorded on videotape and scored manually by two trained and blinded observers. After 2 min of acclimatization, the final 4 min of the test were scored. Immobility time was defined as time spent without any motion other than a single limb paddling to maintain flotation. Latency to immobility was scored as the amount of time (after the 2 min acclimatization period) that elapsed before the first period of immobility.

2.6. SB-334867 injections

Eight to ten week old C57Bl6/j wild-type mice were injected I.P. with either the *hcrtr1* receptor antagonist SB-334867 (10 mg/kg) (Tocris, Ellisville, MI) dissolved in a 50% (w/v) 2-hydroxypropyl-beta cyclodextrin (Sigma, St. Louis, MI) saline solution or control vehicle solution 30 min before exposure to either the TST or FST. This particular dose and route of administration has been demonstrated to modulate behavior through actions in the CNS [14].

2.7. Tail suspension test

Mice were attached from the tail to a force transducer (Med Associates, St. Albans, VT) suspended from the bench in a plexiglass chamber. Time spent immobile was subsequently scored for 6 min [15].

2.8. Elevated plus maze

Anxiety-like behavior was measured using an elevated plus maze apparatus according to published procedures [16]. The 12 cm × 50 cm maze was elevated 55 cm from the floor in a low-light environment. Time in the open arm was measured during a 5 min period. Scoring was done automatically using a videotracking system (Noldus Ethovision, Noldus Information Technology, Sterling, VA).

2.9. Light dark box

The procedure was performed according to a previously published procedure from our institute [17]. The dark–light two chamber apparatus (Med Associates) was made of black and white Plexiglass, with each chamber measuring 25 cm × 26 cm × 25 cm. The dark

chamber was not illuminated, while the light side was illuminated by a fluorescent lamp. Mouse movement within each chamber was measured by photocell beam breaks. Mice were placed in the dark chamber for 2 min. The divider separating the 2 chambers was then removed and the mice were allowed to freely investigate both chambers for 10 min.

2.10. Locomotor activity

Mice were placed in fresh cages and locomotor activity was measured using photo cell beams linked to computer data acquisition software (San Diego Instruments, San Diego, CA) as described previously [13]. Both locomotion in the *X* and *Y* planes were summated to generate a measure of total locomotor activity. Counts are presented, as number of beam breaks binned into 15 min intervals.

2.11. Statistical analysis

Data are reported as the mean \pm SEM for the specified number of animals. Graph-Pad Prism 5 software (GraphPad Software Inc., San Diego, CA) was used to perform all statistical analyses. Two-tailed Student's *t*-test was used in the analysis of all data unless otherwise described in figure legends. Significance was defined as $p < 0.05$.

3. Results

We first examined whether orexin receptor signaling modulated depression-like behavior using the forced swim test (FST), a measure of behavioral despair [18]. *Hcrtr1* null mice displayed a significant reduction in time immobile and in latency to first bout of immobility (both measures of despair [19,20]) in the FST, consistent with reduced depression-like behavior (Fig. 1A and B). Because genetic deletion can produce developmental compensation, we next sought to confirm our findings by treating wild-type adult mice with the *hcrtr1* antagonist SB-334867. As was observed in the *Hcrtr1* knockout, pharmacologic inhibition of *hcrtr1* also resulted in a significant reduction in immobility and latency to first bout of immobility in the FST (Fig. 1C and D). We then extended this initial observation using a complimentary test of behavioral despair, the tail suspension test (TST). Consistent with the FST result, inhibition of *hcrtr1* signaling by genetic (Fig. 1E) or pharmacologic (Fig. 1F) methods also reduced immobility in the TST.

Because depression and anxiety are often co-morbid, we next analyzed *hcrtr1* null mice in two measures of anxiety: the elevated plus maze (Fig. 2A) and the light-dark box (Fig. 2B). Deletion of *hcrtr1* had no effect in either test indicating that the effect of *hcrtr1* inhibition on depression-like behaviors was dissociable from anxiety-like responses.

Unlike *hcrtr1* null mice, mice lacking orexin ligand display no phenotype in the FST [13]. This finding suggests that *hcrtr2* signaling may oppose the actions of orexin receptor 1 on mood regulation such that loss of signaling at both receptors (as is the case in the ligand deletion) would result in the absence of any phenotype. We subsequently tested this hypothesis by examining depression-like behaviors in *hcrtr2*-null mice. Compared to wild-type littermates, *hcrtr2* deletion produced a significant increase in total time spent immobile in the FST (Fig. 2C) and TST (Fig. 2D), although latency to first bout of immobility in the FST was not significantly different from control (not shown). Furthermore, in examining the role of *hcrtr2* in anxiety like behaviors, similar to *hcrtr1*, no effect was observed in the elevated plus maze or light/dark tests (Fig. 2E and F), again demonstrating the specificity of the orexin effect in the modulation of depression like behavior. Unlike *hcrtr1*, published reports of compounds that produce selective *hcrtr2* antagonism in vivo in mice have yet to be described. Thus, we did not pursue a pharmacological study of the effect of *Hcrtr2* disruption on behavior.

Finally, since differences in locomotor activity may confound the interpretation of behavioral tests, we determined whether deletion of *hcrtr1* or *hcrtr2* affected locomotor activity in a novel environment. Deletion of either receptor had no effect on locomotion during a 9 h trial suggesting that differences in locomotor activity did not contribute to the observed behavioral phenotypes (Fig. 3A and B). At no point was there any difference observed in locomotor activity between the null *hcrtr1* and 2 mice and control animals (Fig. 3A and B), as initial and total exploratory activity was identical between nulls and wildtypes. This is not surprising, as the sleep state transition phenotype observed in the *Hcrtr2* null animals is most evident during the dark phase, while all behavioral testing was performed during the light phase [4]. Lastly, the effects of the orexin receptor antagonist SB-334867 on locomotor activity were not analyzed, as this compound has yet to demonstrate any effect on locomotion [21,22].

4. Discussion

Our results indicate that *hcrtr1* and 2 signaling oppose one another in the regulation of depression-like behaviors. More specifically, these data suggest that *hcrtr1* and *hcrtr2* signaling may act to counterbalance each other in regions of the brain that regulate antidepressant-like responses such as in the hippocampus, ventral tegmental area, or prefrontal cortex [23]. We postulate that states that favor activation of orexin neurons projecting to regions that express *hcrtr1*, such as occurs during highly stressful situations [24], may create a negative affective state that worsens depression-like symptoms. The finding that orexin receptor signaling may promote a negative emotional state is consistent with previous reports demonstrating that orexin-A peptide promotes the development of anhedonia, increasing intracranial self-stimulation thresholds [8], while re-instatement of drug seeking is blocked by the *hcrtr1* antagonist SB-334867 [8]. Our data is also in agreement with a significant number of studies demonstrating a role for *hcrtr1* in the modulation of reward dependent behavior. The enhancement of cocaine [25] and morphine place preference [26,27] is dependent on *hcrtr1* activation, while operant responding for palatable food also requires functional *hcrtr1* receptor signaling [28]. We contend that a negative emotional state may be produced by *hcrtr1* signaling, acting to enhance both the conditioning to and responding for a particular reward.

Furthermore, our data also illustrate how a negative emotional state along with the development of anhedonia (based on other reports [8]) may be enhanced, through disruption of *hcrtr2* signaling, leaving *hcrtr1* transmission intact. For example, states that favor activation of *hcrtr2* expressing neurons, such as calorie restriction, may promote antidepressant-like responses [13]. An important prediction of this hypothesis is that unique *hcrtr1* and *hcrtr2* neural circuits exist with potentially distinct patterns of innervation and activation by different stimuli. The existence of unique circuits would allow for the orexin system to integrate various inputs into coordinated behavioral and physiological responses. Interestingly, recent data highlight the differential regulation of orexin receptor expression in the medial prefrontal cortex. In this particular example, the authors describe a shift in orexin receptor expression, with enhanced *hcrtr1* expression accompanying a trend in the reduction of *hcrtr2* expression [29]. In this case, the change in orexin receptor expression is expected to drive operant responding following exposure to social stress. Our data compliment these findings, as we demonstrate that changes in orexin receptor expression modulate depression like behavior.

Testing this hypothesis, that selective activation of orexin receptor subtypes can produce distinct effects on behavior, can subsequently be examined using the mouse model systems described for the first time in this report. Using cre recombinase delivered either virally or expressed from a transgene, restoration of either *hcrtr1* or 2 may be accomplished in select

brain nuclei. These receptor-restored mice could then be analyzed for depression and anxiety-like behavior, permitting a mapping of the sites of orexin receptor expression that are responsible for the modulation of affect.

The transcriptionally blocked orexin receptor mice described here are a valuable tool in the investigation of orexinergic modulation of behavior. While developmental compensation, due to embryonic gene deletion, is often a concern when analyzing genetic mutations, there is no evidence, at least in terms of the effect of orexin on depression like behavior, that deletion of orexin receptors has produced such compensation in the reactivatable *hcrt1* and 2 lines. While deficits in sleep state transitioning are clearly evident in the transcriptionally blocked *hcrt2* null [11], data presented in this report demonstrates that the *hcrt1* selective antagonist SB-334867 mimics the effect of the *hcrt1* deletion on depression like behavior.

The studies presented here are the first to describe the effect of *hcrt1* and 2 receptor-specific modulation of depression like behavior. Interestingly, in limited literature describing orexin action in modulation of depression like behavior, chronic *hcrt1* receptor antagonism was shown to enhance the presentation of depression-like behavior [30,31]. Our data is then highly relevant to the discussion of the role of orexin receptor-acting compounds in the treatment of depression; that there may be significant differences in the modulation of affect between the acute and chronic actions of orexin receptor targeted therapeutics. Alternately, the use of the non-selective *hcrt* receptor agonist, orexin-A, along with the *hcrt1* selective antagonist at potentially non-selective doses in vivo, may result in the non-specific targeting of both *hcrt1* and 2. This would make it difficult to discern any differences in the modulation of depression like behavior by either *hcrt1* or 2.

Our data suggests that modulation of depression like behavior depends on the balance between *hcrt1* and 2 receptor signaling, thus the use of non-selective *hcrt1* and 2 agonists and antagonists at non-selective doses would not be expected to be able to discern the true roles of both receptors in the modulation of behavior.

Finally, considering that the orexin system has become an attractive target for the development of novel pharmacologic treatments for a variety of neuropsychiatric disorders including narcolepsy, insomnia, and drug addiction, careful monitoring of behavioral responses to these medications will be required to determine the potential for both novel applications as well as potentially unwanted side effects, based on the conclusions drawn from our studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.bbr.2011.02.044.

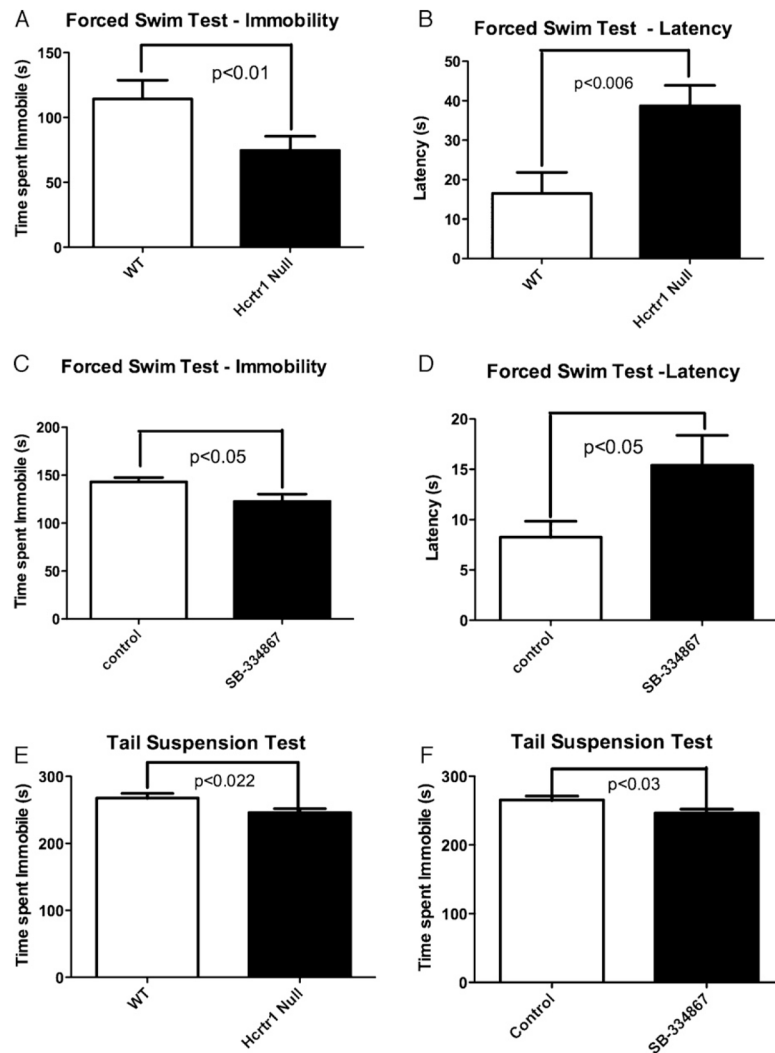


Fig. 1. Disruption of hcrt1 reduces behavioral despair

Eight to ten week old male mice were tested in mouse models of depression, the porsolt forced swim test and tail suspension test. (A) Time spent immobile in the FST. (B) Latency to immobility in the FST. (Data presented as mean \pm SEM, Student's *t*-test, $N = 10-15$ /group.) (C) Time spent immobile and latency to immobility. (D) After SB-334867 (10 mg/kg) injection. (Data presented as mean \pm SEM, Student's *t*-test, $N = 18$ /group.) (E) Time spent immobile in the TST. (Data presented as mean \pm SEM, Student's *t*-test, $n = 17-19$ /group.) (F) Time spent immobile in the TST after injection of vehicle or SB-334867 (10 mg/kg). Data presented as mean \pm SEM, Student's *t*-test, $n = 18$ /group.

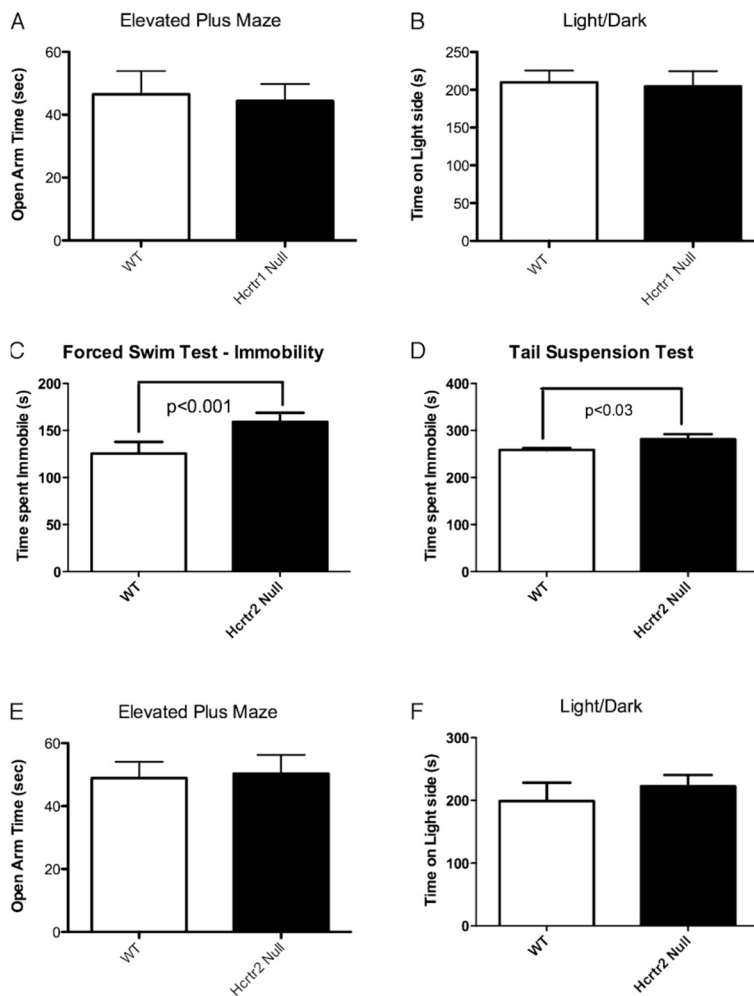


Fig. 2. Hcrtr1 and 2 deletions have no effect on measures of anxiety like behavior while hcrtr2 deletion enhances depression like behavior
 (A and E) Time spent in the open arm of the EPM. (Data presented as mean ± SEM, Student's *t*-test, *n* = 14–20/group.) (B and F) Time spent on the light side of light/dark box. (Student's *t*-test, *n* = 10–15/group.) (C and D) Time spent immobile in the FST and time spent immobile in the TST. (Data presented as mean ± SEM, Student's *t*-test, *n* = 7–10/group.)

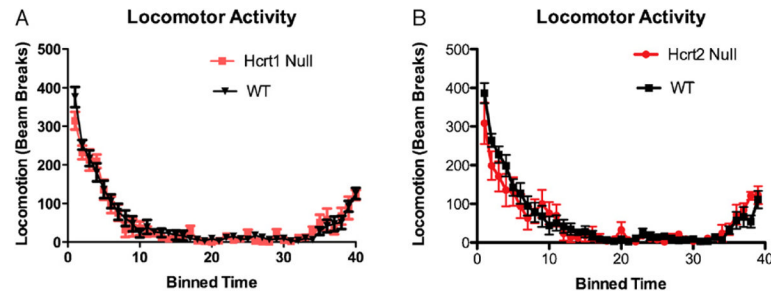


Fig. 3. Disruption of *hcrt1* and *2* does not affect locomotor activity

A and B individually housed mice were placed in a novel cage and then tested for locomotor activity. No difference was found between wild-type and (A) *Hcrt1r*-null (two way ANOVA with repeated measures, $p > 0.05$, $n = 13$ wild-type, $n = 9$ *Hcrt1*-null) or (B) *Hcrt2*-null mice (two way ANOVA with repeated measures, $p > 0.05$, $n = 13$ wild-type, $n = 7$ *Hcrt2*-null). Data are presented as mean \pm SEM.