Brief Communications

Role of the Hypocretin (Orexin) Receptor 2 (Hcrt-r2) in the Regulation of Hypocretin Level and Cataplexy

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Hypocretin receptor-2 (Hcrt-r2)-mutated dogs exhibit all the major symptoms of human narcolepsy and respond to drugs that increase or decrease cataplexy as do narcoleptic humans; yet, unlike narcoleptic humans, the narcoleptic dogs have normal hypocretin levels. We find that drugs that reduce or increase cataplexy in the narcoleptic dogs, greatly increase and decrease, respectively, hypocretin levels in normal dogs. The effects of these drugs on heart rate and blood pressure, which were considerable, were not correlated with their effects on cataplexy. Administration of these drugs to Hcrt-r2-mutated dogs produced indistinguishable changes in heart rate and blood pressure, indicating that neither central nor peripheral Hcrt-r2 is required for these cardiovascular effects. However, in contrast to the marked Hcrt level changes in the normal dogs, these drugs did not alter hypocretin levels in the Hcrt-r2 mutants. We conclude that Hcrt-r2 is a vital element in a feedback loop integrating Hcrt, acetylcholine, and norepinephrine function. In the absence of functional Hcrt-r2, Hcrt levels are not affected by monoaminergic and cholinergic drugs, despite the strong modulation of cataplexy by these drugs. Conversely, strong transient reductions of Hcrt level by these drugs do not produce episodes of cataplexy in normal dogs. The Hcrt-r2 mutation causes drug-induced cataplexy by virtue of its long-term effect on the functioning of other brain systems, rather than by increasing the magnitude of phasic changes in Hcrt level. A similar mechanism may be operative in spontaneous cataplexy in narcoleptic dogs as well as in narcoleptic humans.

Introduction

Genetically narcoleptic dogs have nonfunctional hypocretin-2 receptors (Hcrt-r2s), but normal hypocretin-1 receptors (Hcrt-r1s) (Lin et al., 1999). They have normal numbers of Hcrt neurons and normal CSF levels of Hcrt (Thannickal et al., 2000a; John et al., 2004b), yet they have severe cataplexy and sleepiness. Cataplexy and sleepiness in the narcoleptic dog resembles that of human narcoleptics, who on average have lost 90% of their Hcrt cells and have greatly reduced CSF levels of Hcrt (Nishino et al., 2000; Peyron et al., 2000; Thannickal et al., 2000b) but are thought to have structurally normal Hcrt-r1 and Hcrt-r2 (Mishima et al., 2008). The effect of drugs on narcoleptic dogs strikingly parallels their effect on symptoms in human narcoleptics, despite the different dysfunctions of the Hcrt system (Nishino and Mignot, 1997). The cause of the pharmacologic and behavioral similarity between narcoleptic dogs and narcoleptic humans is unclear. More generally, the specific roles of the Hcrt receptors in the regulation of Hcrt dynamics and narcoleptic symptoms are unknown.

Received Jan. 21, 2011; revised Feb. 25, 2011; accepted March 6, 2011.

Author contributions: M.-F.W. and J.M.S. designed research; M.-F.W., R.N., N.T.M., and H.A.L. performed research; N.T.M. and H.A.L. analyzed data; M.-F.W. and J.M.S. wrote the paper.

Supported by National Institutes of Health Grants NS14610 and MH64109, and the Medical Research Service of the Department of Veterans Affairs.

The author declare no competing financial interests.

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DOI:10.1523/JNEUROSCI.0365-11.2011

 0270-6474/11/316305-06$15.00/0

We investigated the effect on Hcrt levels of drugs modulating cataplexy and sleepiness in breed-matched normal and narcoleptic dogs. We also measured the effect of the drugs on heart rate (HR) and blood pressure (BP) in both types of dogs. We found that drugs that increase cataplexy in narcoleptics (prazosin and physostigmine) lowered Hcrt levels in normal dogs. Drugs that decrease cataplexy in narcoleptics (methamphetamine, labetalol, and phenylephrine) increased Hcrt levels in normal dogs. These changes appeared to indicate that the symptom effects are determined by drug-induced changes in Hcrt level. However, surprisingly, none of these changes in Hcrt levels were seen with administration of the same drugs to Hcrt-r2 mutant dogs, despite their strong modulation of symptoms. Our results suggest a vital role of Hcrt-r2 in feedback regulation of Hcrt release by drugs affecting cholinergic and aminergic systems. They also demonstrate that the symptoms of narcolepsy triggered and prevented by these drugs are not mediated by phasic changes in Hcrt level in the Hcrt-r2 mutant dog.

Materials and Methods

The subjects were four normal Doberman pinschers and four narcoleptic Doberman pinschers. The normals were 1.5–3.1-year-old males, and the narcoleptics were one male and three females 0.9–8.2 years old. No age or sex differences were seen in the results. The dogs were adapted to standing in a sling for 2 h/d for a period of 2 weeks. On the experimental day, the dog rested in the sling, and BP and HR were measured every 2.5 min for the entire 2 h period using an oscillometric noninvasive BP monitor (SurgiVet model V60046, SurgiVet Inc.) with a cuff on the tail or a limb. Systolic, diastolic, and mean arterial pressure (MAP) measurements were recorded. HR was recorded polygraphically with electrodes placed on the chest and limbs. Respiration was measured in the normal

dogs with a strain gauge placed around the chest. HR and respiration signals were filtered and amplified with a Tachograph preamplifier (model 7P4G EKG, Grass) and preamplifier (model 7P1F DC, Grass/Astro-Med) and digitized with Spike2 software (Cambridge Electronic Design). A 30 min baseline measurement was taken. All studies were completed between 10:00 A.M. and 2:00 P.M.

After the 30 min baseline period, dogs were given either saline (0.5 ml) or one of the following drugs intravenously: prazosin hydrochloride (α1 adrenergic antagonist; 40 μg/kg; Sigma); physostigmine salicylate (cholinesterase inhibitor; 50 μg/kg; Taylor Pharmaceuticals); labetalol hydrochloride (β and α1 adrenergic antagonist; 250 μg/kg; Bedford Laboratories); phenylephrine (α1 adrenergic agonist; 150 μg/kg; Sigma); or methamphetamine hydrochloride (monoamine release enhancer; 125 μg/kg; Sigma).

A second dose was given 30 min later (labetalol) or 45 min later (phenylephrine, methamphetamine, and physostigmine), except for prazosin, which was only given once because of its long half-life. Doses were those found optimal for triggering or preventing cataplexy in narcoleptic dogs (our data) (Nishino and Mignot, 1997). Resting in the sling for 2 h after saline injection served as a control. Three replications of each condition were performed. At least 1 week elapsed between trials in each dog. No food was given during trials. Administration of physostigmine or prazosin in the doses employed always produces cataplexy in the narcoleptic dogs without the need to present eliciting stimuli or food.

Cataplexy measurement. The dogs were continuously monitored, and defining symptoms of cataplexy, loss of neck, jaw, or limb support, were noted every 2.5 min at the time of blood pressure measurement. Cataplexy occurrence under drug conditions was compared with that after saline injections. Cataplexy never occurred in the normal dogs.

CSF collection after drug administration. CSF was taken from the cisterna magna 1.5 h after the initial dose (45 or 60 min after the second dose) under thiopental anesthesia (12.5 mg/kg, i.v.). This timing was determined based on a preliminary experiment that determined the time course (0, 30, 60, and 90 min) of Hcrt level with two drugs: labetalol and methamphetamine. It established that the peak level of Hcrt-1 in cisterna magna CSF occurred 60–90 min after drug administration at a time when the behavioral effects of all the drugs were pronounced. All assays were done blind to experimental conditions.

Hypocretin assay. CSF samples (0.5 ml) were acidified with 1% trifluoroacetic acid (TFA) and loaded onto a C18 SEP-Column (Waters). The peptide was eluted with 1% TFA/40% acetonitrile. The eluant was then dried and resuspended in RIA buffer. The solid-phase radioimmunoassay (Maidment and Evans, 1991) provided an IC_{50} of 2–3 fmol and a limit of detection of ~0.1 fmol. The Hcrt-1, iodinated Hcrt-1, and Hcrt-1 antiserum were obtained from Phoenix Pharmaceuticals (catalog #RK-003–30).

Data analysis. All data are presented as mean ± SEM. CSF Hcrt-1 levels after drug conditions were compared with their corresponding values obtained under control conditions. The dependent *t* test was used. Continuous recordings of BP and HR were averaged across repeated trials and after drug values were compared with those during the predrug baseline. Because all three measures of BP are highly correlated, only the analysis of MAP is presented.

Results

Normal dogs

Two drugs that increase cataplexy in the narcoleptic dog, prazosin and physostigmine, were administered to the normal dogs and found to reduce CSF Hcrt-1 level (Fig. 1). Prazosin, an α1 adrenergic antagonist, reduced CSF Hcrt-1 by 28.8% ($p < 0.02$, *t* test). Physostigmine, a cholinesterase inhibitor, also produced a substantial reduction in the CSF level of Hcrt-1 (22.1%, $p < 0.005$, *t* test). Despite the reduction in Hcrt level, neither drug produced any signs of cataplexy or weakness in the normal dogs. In a prior study, higher doses of physostigmine (0.1 mg/kg) or prazosin (0.6 mg/kg, p.o.) and the combination of both drugs administered to normal dogs never produced cataplexy (Mignot et al., 1993). Both prazosin and physostigmine increased HR

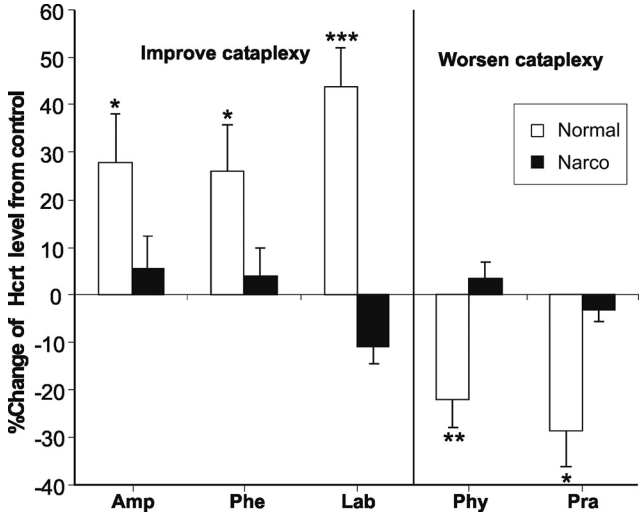

Figure 1. Changes in CSF Hcrt level after administration of drugs that affect cataplexy. Drugs that improve narcolepsy (left side) increase Hcrt levels in normal dogs (Normal), but do not affect Hcrt levels in Hcrt-r2-mutated narcoleptic dogs (Narco). Similarly, drugs that exacerbate cataplexy (right side) reduce Hcrt levels in normal dogs, but do not affect Hcrt levels in narcoleptic dogs. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, *t* test, compared with saline control. Amp, Methamphetamine; Phe, phenylephrine; Lab, labetalol; Phy, physostigmine; Pra, prazosin.

(14.7 and 14.3%, respectively; $p < 0.05$, *t* test). Prazosin significantly decreased BP (12.8%; $p < 0.01$, *t* test) (Figs. 2, 3), while physostigmine increased BP (16.5%; $p < 0.001$, *t* test) (Figs. 2, 3). Prazosin reduced respiratory rate by 28.9%, and physostigmine increased respiratory rate by 51.6% (both $p < 0.001$, *t* test) (Fig. 3).

Three drugs that decrease cataplexy were administered to the normal dogs (Fig. 1). Methamphetamine increased Hcrt-1 level (27.9%; $p < 0.05$, *t* test) (Fig. 1) and BP (14.8%; $p < 0.01$, *t* test) (Figs. 2, 3) without effect on HR (−0.2%, NS) (Figs. 2, 3). Phenylephrine, an α1 adrenergic agonist, produced a 26.1% increase in Hcrt-1 ($p < 0.05$, *t* test) (Fig. 1), increased BP (9.1%; $p < 0.05$, *t* test) (Figs. 2, 3), and decreased HR (23.2%; $p < 0.05$, *t* test) (Figs. 2, 3). Labetalol, an α1 and β adrenergic blocker, greatly increased the level of Hcrt-1 (43.9%; $p < 0.01$, *t* test) (Fig. 1), reduced BP (9.8%; $p < 0.001$, *t* test), and increased HR (12.6%; $p < 0.01$, *t* test) (Figs. 2, 3). Methamphetamine and phenylephrine both increased respiratory rate (40.8 and 25.7%; $p < 0.001$, *t* test), while labetalol had no significant effect on respiration (Fig. 3).

Thus, despite varying effects on BP and HR, all the above listed drugs that increased cataplexy in the narcoleptic dogs decreased Hcrt levels in normal dogs. Conversely, despite varying effects on BP and HR, all the drugs that decreased cataplexy in the narcoleptic dogs increased Hcrt in normal dogs. In prior work, we have shown that elevations of blood pressure can alter muscle tone in the decerebrate animal (Lai et al., 1987) and in the narcoleptic dog (Siegel et al., 1986, 1989). Hcrt activity has been shown to be correlated with blood pressure (Kuwaki et al., 2008). However, despite these relations, the effects of these drugs on cataplexy cannot be explained by the changes they elicited in BP or HR.

These results in normal dogs suggested to us that it was the effect of these drugs on Hcrt level rather than any effect on BP, HR, or other variables that was the common determinant of their effect on cataplexy.

Narcoleptic dogs

In the narcoleptic dogs, the same doses of drugs produced the same cardiovascular effects seen in normal dogs and humans

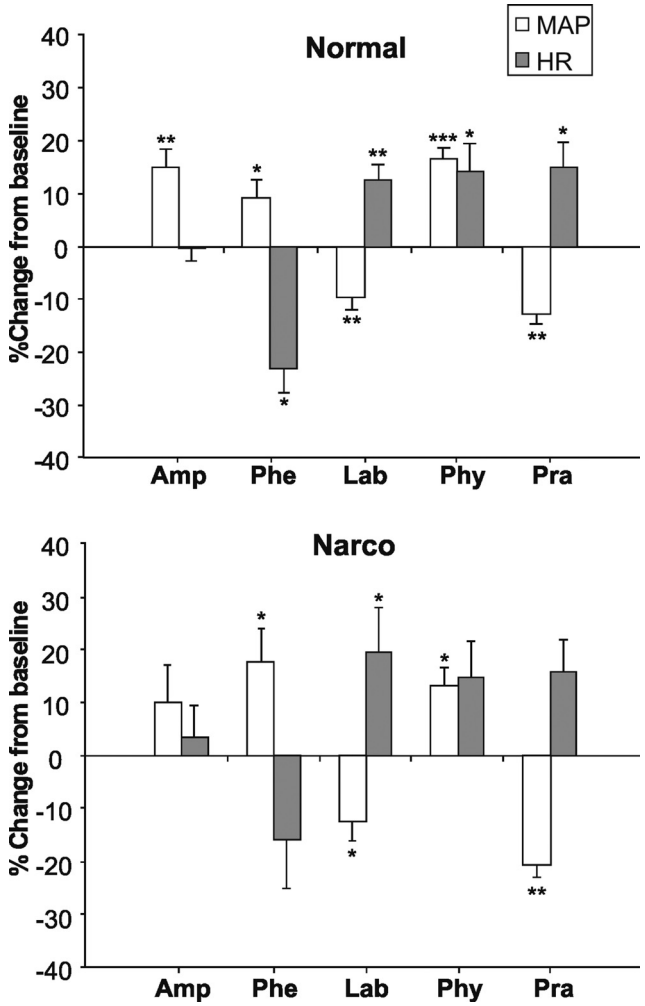

Figure 2. Blood pressure and heart rate changes after administration of drugs affecting cataplexy. Administered drugs had similar effects on MAP and HR in narcoleptic and normal dogs. Values are expressed as the percentage change from baseline (mean ± SEM). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, *t* test. Abbreviations as in Figure 1.

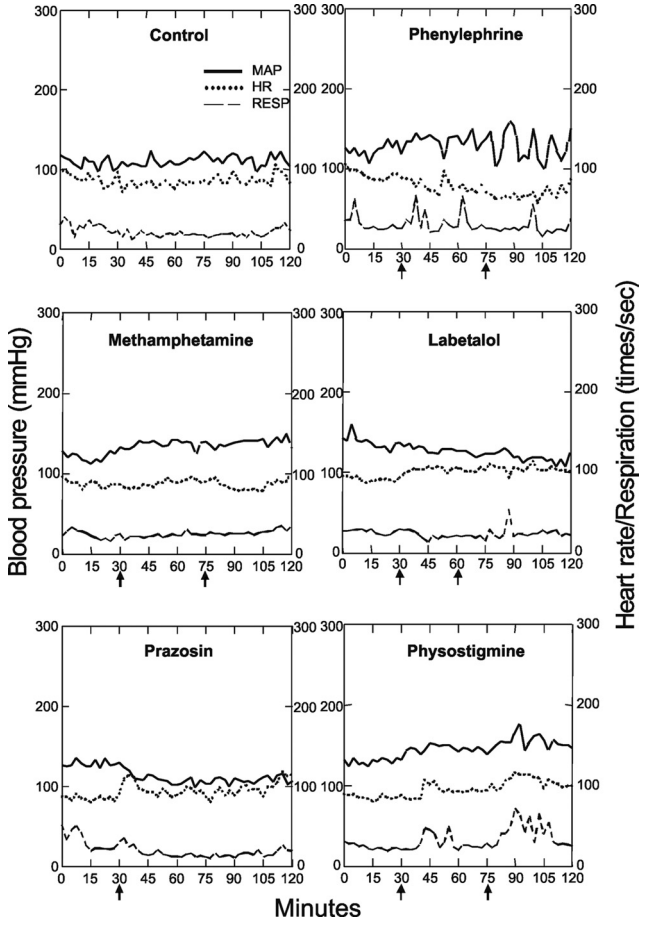

Figure 3. Time course of MAP, HR, and respiration (RES) before and after drug administration in normal dogs. Arrows indicate times of drug administration.

(Fig. 2). These drugs produced the expected changes in symptoms of narcolepsy in the dogs. Physostigmine and prazosin triggered strong cataplectic attacks, while methamphetamine, labetalol, and phenylephrine reduced drowsiness and prevented episodes of cataplexy, as previously reported (Fig. 4). However, none of these drugs produced significant changes in the level of Hcrt in the narcoleptic dogs (Fig. 1).

Discussion

The current results suggest a reinterpretation of the simplest explanation of the mechanisms responsible for narcolepsy and its unique symptom, cataplexy. Clearly, Hcrt deficiency is ultimately responsible for narcolepsy in humans (Peyron et al., 2000; Thannickal et al., 2000b). Just as clearly, the Hcrt-r2 mutation or the absence of Hcrt is responsible for these symptoms in genetically narcoleptic dogs and mice (Chemelli et al., 1999; Lin et al., 1999).

Relation between Hcrt release and symptoms of narcolepsy

However, this study, when considered in the context of some previously reported findings, indicates that the relation between Hcrt level and Hcrt receptor stimulation and the symptoms of narcolepsy is not always a direct one. Hcrt levels do not differ between normal dogs and Hcrt-r2 mutants (Thannickal et al., 2000a; John et al., 2004b). As we report here, decreased Hcrt levels produced by the administration of prazosin or physostigmine do not cause cataplexy in normal dogs. We know of no evidence that administration of these drugs alone or in combination elicits cataplexy at any dose in normal dogs (Mignot et al., 1993), normal humans, or in any other species.

The severity of cataplexy varies greatly between individual narcoleptic dogs with identical Hcrt-r2 mutations and Hcrt levels, and symptoms vary within each dog over the course of development. However, the onset and intensity of these symptoms does not correlate with Hcrt level over the developmental period (John et al., 2004b). Similarly, symptoms vary greatly over the lifespan of human narcoleptics and between human narcoleptics with indistinguishable Hcrt levels (Nishino et al., 2000). Acute pharmacological blockade of both Hcrt receptors should mimic the complete loss of Hcrt and produce the most severe cataplexy if phasic Hcrt receptor activation alone is responsible for preventing cataplexy. But this blockade does not cause cataplexy in normal humans, normal rats, or normal dogs (Brisbare-Roch et al., 2007).

Circuit for cataplexy

The current results show that the Hcrt-r2 mutation greatly diminishes the responsiveness of the Hcrt system to the tested drugs. These drugs are known to either activate or block aminergic or cholinergic receptors. They were selected because of their demonstrated modulation of cataplexy, at the doses used, in nar-

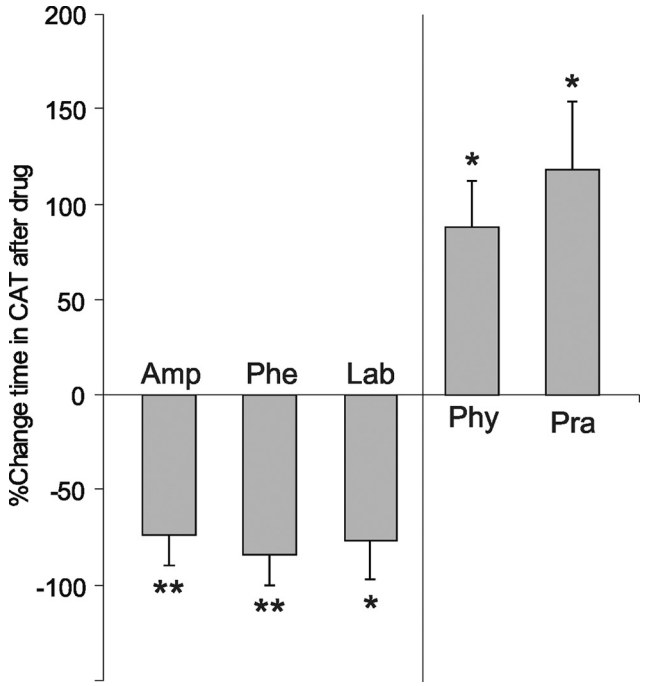

Figure 4. Changes in cataplexy occurrence in narcoleptic dogs after administration of drugs. Abbreviations as in Figure 1. Amp, Phe, and Lab reduced cataplexy, relative to saline control injections. Phy and Pra increased cataplexy. Hcrt levels did not change (see Fig. 1). Cataplexy was never seen in the normal dogs after administration of these drugs. $*p < 0.05$, $**p < 0.02$, *t* test.

coleptic dogs and humans. It is reasonable to hypothesize that the lack of change in Hcrt level with administration of these drugs in narcoleptic dogs is paralleled by reduced responsiveness of Hcrt neurons to the normal synaptic activation or inactivation of monoaminergic and cholinergic transmitter receptors. It might be expected that a change in the activity of these monoaminergic and cholinergic systems would occur during the sudden onset of strong emotions, which often triggers cataplexy (John et al., 2004a).

Because acetylcholine depolarizes Hcrt neurons *in vitro* and Hcrt depolarizes cholinergic neurons *in vitro* (Burlet et al., 2002; Yamanaka et al., 2003), the markedly decreased Hcrt level we see after physostigmine administration to normal dogs is unlikely to be a direct result of the action of this drug on Hcrt neurons. Instead, these drugs must be acting on non-Hcrt cholinoceptive cells. It is most likely that the reductions in Hcrt level we see are circuit effects, mediated at the Hcrt soma level by GABA or other transmitters. Conversely, the increased release of Hcrt after administration of drugs that decrease cataplexy may be mediated by actions on non-Hcrt cells in circuits that project to Hcrt cells and release glutamate or other excitatory transmitters upon them (Li et al., 2002). These circuit mechanisms are disrupted in Hcrt-r2 mutant narcoleptic dogs and perhaps in other narcoleptic animals.

Non-Hcrt changes in narcoleptic animals

Hcrt-r2 mutant narcoleptic dogs show a greatly elevated level of axonal degeneration early in development relative to breed-matched normals (Siegel et al., 1999). This degeneration peaks at 1–4 months of age and is maximal in limbic regions, which do not have Hcrt cell somas. It has been reported that the Hcrt-r2 mutation in the dog, the Hcrt-null mutation in the mouse, and the loss of Hcrt cells in human narcolepsy cause changes in the concentration of a number of receptor types, including receptors for dopamine and acetylcholine, and also cause abnormities in monoamine metabolism (Mefford et al., 1983; Kilduff et al., 1986; Fruhstorfer et al., 1989; Aldrich et al., 1993; Nishino et al., 1994; Nishino and Mignot, 1997; Mishima et al., 2008; Mori et al., 2010; Nishino and Sagawa, 2010). Mice lacking Hcrt receptors have elevated levels of choline acetyltransferase, vesicular acetylcholine transporter, and the high-affinity choline transporter in the laterodorsal tegmental nucleus (Kalogiannis et al., 2010). These changes are consistent with a prior report of increased numbers of immunohistochemically identified cholinergic cells in the laterodorsal tegmental nucleus of the narcoleptic dog (Nitz et al., 1995; but see Tafti et al., 1997).

The current results suggest that differences in aminergic and cholinergic receptor sensitivities and in the anatomy and physiology of limbic systems that result from the Hcrt receptor mutation, rather than phasic changes in Hcrt release, may be responsible for cataplectic attacks triggered by limbic excitation. Changes in Hcrt level are clearly not required for cataplectic attacks in the narcoleptic dogs. Our studies in normal dogs and the findings by others discussed above, including studies of the effects of Hcrt receptor blockade, show that reduction of Hcrt level or blockade of Hcrt action are not sufficient to induce cataplectic attacks in normal animals.

We hypothesize that in normal animals a positive feedback enhancement of Hcrt neuronal discharge, mediated by Hcrt-r2, reinforces Hcrt activity and the activity of locus ceruleus and other monoaminergic and glutamatergic cell groups receiving Hcrt input. The crucial Hcrt-r2 receptors for this feedback may be on Hcrt neurons, as it has been recently demonstrated in an *in vitro* study that such autoreceptors exist and are excitatory (Yamanaka et al., 2010). Alternatively, or in addition, crucial receptors may be on non-Hcrt cells, which produce an excitatory feedback excitation of Hcrt cells. The absence of this feedback alters the morphology of neurons in this circuit, with pathological results.

Evidence from neuronal activity

We have found that the activity of Hcrt neurons in normal rats is highly variable during waking. The complete cessation of Hcrt neuronal activity for extended periods of time ($\geq$1 min) does not produce cataplexy or any similar symptom in normal rats (Mileykovskiy et al., 2005). Human narcoleptics and Hcrt KO mice are not tonically weak. Rather, they are unable to maintain muscle tone when certain sudden-onset emotions are triggered. Locus coeruleus neurons have been shown to cease activity or transmitter release in tight correlation with loss of muscle tone in narcoleptic dogs and mice (Wu et al., 1999; Carter et al., 2010; McGregor and Siegel, 2010). At the same time, medial medullary inhibitory neurons become active (Siegel et al., 1991). The activity of these medullary neurons is tightly linked to the cessation of locus coeruleus discharge (Mileykovskiy et al., 2000). The locus coeruleus receives several excitatory inputs that might serve to maintain muscle tone during periods of emotional excitation (Luppi et al., 1995). The current results suggest that Hcrt dysfunction may not only prevent phasic Hcrt release onto locus ceruleus, but may also alter the activity of other locus ceruleus afferents and afferents to other components of the motor inhibitory system.

Clinical implications

Administration of Hcrt by intravenous or intracerebroventricular injection, or by genetic overexpression of Hcrt attenuates nar-

coleptic symptomatology (Mignot et al., 1993; John et al., 2000; Mieda et al., 2004; Deadwyler et al., 2007). However, since Hcrt excites virtually all neurons to which it is applied, these are relatively nonspecific manipulations that may cause deleterious side effects.

It seems unlikely, though not impossible, that a phasic reduction in Hcrt level is responsible for cataplectic or sleep attacks in narcoleptic humans who have on average lost 90% of their Hcrt neurons (Thannickal et al., 2000b; Gerashchenko et al., 2003). Instead, the current results suggest that the symptoms of narcolepsy may be caused by the loss of trophic influences of Hcrt, causing downstream changes in cholinergic, aminergic, and perhaps other yet-to-be-identified systems. The standard treatment for cataplexy is drugs that activate noradrenergic receptors (Nishino and Mignot, 1997), and it is possible that this treatment deals more directly and specifically with the immediate triggers of these symptoms than would be the case with acute Hcrt administration.

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