

Cerebrospinal fluid hypocretin (orexin) levels are elevated by play but are not raised by exercise and its associated heart rate, blood pressure, respiration or body temperature changes

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ABSTRACT

Hypocretin (Hcrt) has been implicated in the control of motor activity and in respiration and cardiovascular changes. Loss of Hcrt in narcolepsy is linked to sleepiness and to cataplexy, a sudden loss of muscle tone which is triggered by sudden strong emotions. In the current study, we have compared the effects of treadmill running to yard play on cerebrospinal fluid (CSF) Hcrt level in normal dogs. We find that treadmill locomotion, at a wide range of speeds, does not increase Hcrt level beyond baseline, whereas yard play produces a substantial increase in Hcrt, even though both activities produce comparable increases in heart rate, respiration and body temperature. We conclude that motor and cardiovascular changes are not sufficient to elevate CSF levels of Hcrt and we hypothesize that the emotional aspects of yard play account for the observed increase in Hcrt.

Key words

REM sleep • Breathing canine

Introduction

Loss of hypocretin (Hcrt) neurons in humans (Peyron et al., 2000; Thannickal et al., 2000), mice (Chemelli et al., 1999) and rats (Beuckmann et al., 2004; Zhang et al., 2007) or a mutation of the Hcrt-2 receptor in dogs (Lin et al., 1999) causes narcolepsy with cataplexy. Although the link between narcolepsy and hypocretin deficiency is well established, the normal function of hypocretin is unclear. We found that Hcrt levels in the cerebrospinal fluid (CSF) are greatly elevated in both narcoleptic and normal Doberman pinchers after their daily exercise/play period in a large yard (Wu et al., 2002)

and in cats during active waking periods maintained by human interaction (Kiyashchenko et al., 2002). During these periods, the electroencephalogram was activated and the amplitude of the electromyogram (EMG) and locomotor activity was greatly increased relative to that in baseline waking periods. It is not clear, however, if it is the motor activity per se or other changes correlated with motor activity during active waking induced the increase in Hcrt level. Studies in rodents, mostly mice, have indicated that Hcrt is crucial for the regulation of respiration (Zhang et al., 2005; Zhang et al., 2009), coordination of respiration with behavior (Corcoran et al., 2010; Kuwaki, 2010) and for long term facilitation

of respiration (Terada et al., 2008). These studies have used ICV injection of Hcrt, Hcrt receptor 1 antagonists and Hcrt ataxin mutant and Hcrt knock out (KO) mice.

Other studies in rodents have concluded that Hcrt neurons have a major role in the regulation of blood pressure (BP) and heart rate (HR). Intracisternal Hcrt administration increases HR and BP (Hirota et al., 2003; Jochem et al., 2006; Huang et al., 2010) and microinjections into the nucleus of the solitary tract elicit dose dependent changes in HR and BP. Stimulation of the hypothalamic perifornical region in Hcrt-ataxin mice with depleted Hcrt produced smaller and shorter lasting increases in HR and BP than in control mice (Zhang et al., 2006). Hcrt KO mice were found to have elevated BP during sleep but normal waking BP in one study (Bastianini et al., 2011) but lower BP in another study (Kayaba et al., 2003), and reduced BP and HR responsiveness to stress (Kuwaki, 2011). Ataxin-Hcrt mutant mice were found to have lower BP than control mice in all sleep and quiet waking states (Schwimmer et al., 2010). Another study found a short term, but no long term, role of Hcrt in BP regulation (Lin et al., 2002). Administration of a Hcrt receptor 1 antagonist was found to decrease BP and HR (Hirota et al., 2003; Guo et al., 2010). Hcrt-deficient human narcoleptics were found to have elevated HR and increased BP variability (Fronczek et al., 2008). HR has been found to greatly decrease in both canine and human cataplexy (Siegel et al., 1989; Donadio et al., 2008).

Hcrt has also been implicated in thermoregulation. Administration of an Hcrt-r1 antagonist increased brown adipose tissue temperature. Hcrt KO mice have elevated body temperature during sleep (Mochizuki et al., 2006). In apparent contrast, injection of Hcrt-1 into the diagonal band of Broca increases body temperature (Monda et al., 2004).

A limitation of these studies is that many involved manipulation of the Hcrt system using systemic, ICV or local injection of Hcrt agonists or antagonists. Such manipulations are not likely to mimic the normal pattern of synaptic release of Hcrt. Another approach employed, was the analysis of cardio-respiratory changes in Hcrt KO mice or, in the case of humans, narcoleptics with a longterm absence of Hcrt. In these conditions, brain reorganization in response to the absence of Hcrt during development

or after degenerative changes may alter cardiovascular and respiratory regulation. None of these studies monitored the levels of Hcrt in normal animals to provide a direct measurement of its release during behaviors that alter respiration or HR.

To clarify the underlying relations between exercise, emotion, respiration, cardiovascular changes and Hcrt elevation, we measured CSF Hcrt-1 level in normal Doberman pincher dogs allowed to play in a yard or trained to exercise on a treadmill. We varied the speed of the treadmill from zero m/min to the maximum rate that each dog was capable of following, to see if Hcrt-1 level changes with the intensity of locomotion. We measured the respiration, HR, BP and body temperature changes.

Methods

All procedures were approved by the Institutional Animal Care and Use Committees of the University of California, Los Angeles and the Veterans Administration Greater Los Angeles Healthcare System.

Treadmill exercise

Four normal Doberman pinschers, all male, between 2 and 4.4 years old, were used. The large cisterna magna of the dog makes it easy to extract sufficient volumes of CSF for radioimmunoassay. Furthermore, dogs can be readily trained to run at high speeds on a treadmill. The dogs were trained in steps to first walk and then run on a treadmill (Jog A Dog, Ottawa Lake, MI, USA). The maximum speed each dog was capable of walking or running was determined. The treadmill had a speed adjustable from 0 to 268 m/min.

The experiments were done between 8:00 AM and 1:00 PM. Each dog was run at the same time of a day within this period. First, they were taken to a cage (3 m x 3 m x 3 m) one hour before the start of treadmill procedure. Then, they were placed on a treadmill. On experimental days the dogs were subjected to one of four conditions: 1) standing on the treadmill for 30 min without walking or running, 2) walking for 30 min at a speed of 25 m/min, 3) walking at an average speed of 65 m/min, alternating between 50 and 80 m/min every min for 30 min, and 4) brisk walking and running at an average speed of 105 m/

min for 30 min, alternating the speed among 80, 105 and 135 m/min every min). We varied running speeds within each speed group because we found that dogs performed more consistently if we varied treadmill speeds, whereas some balked and got off the treadmill when the higher speeds were maintained for as long as 30 min.

CSF was extracted on average 45 min after the end of each exercise condition (range 30-60 min). Three replications were done for each condition. Respiration was measured by visual observation of the chest and mouth. Rectal temperature, HR, BP and respiration were measured two times at a 2.5 min interval immediately before and after all conditions and the averages of the two replications for each subject were used for analysis. BP and HR were measured using an oscillometric non-invasive BP monitor (SurgiVet Model V60046, SurgiVet Inc. Waukesha, WI, USA) with a cuff on the limb. Systolic, diastolic and mean arterial pressure (MAP) measurements were recorded.

Yard play

Four normal Doberman pinschers, three male and one female, age between 2.4 and 5 years old, were used. Dogs were brought to a cage (3 m x 3 m x 3 m) in the laboratory one hour before the yard play/exercise. Then they were released and encouraged to run and play in an outdoor exercise area (26.7 m x 17.7 m) for 30 min with two other dogs. The exercise period occurred between 8:00 AM and 11:00 AM. No food was available during exercise or treadmill studies. The animals were brought back to the laboratory after exercise for CSF collection. For the baseline, no-exercise, trials the animals remained in the cage in a quiet waking state without the intervening yard activity. The animals were continuously monitored to assure that they remained awake for the entire 1.5 h period in all baseline and exercise conditions. Each dog was tested on one condition a week.

CSF collection

Two ml of cerebrospinal fluid (CSF) was collected from the cisterna magna using a 22 G spinal needle while the animal was under thiopental anesthesia (12.5 mg/kg, IV). The samples were frozen immediately on dry ice and stored in a -20°C freezer until analysis.

Hypocretin assay

The procedures for the assay for hypocretin have been described previously (Wu et al., 2002). Samples (0.5 ml) were acidified with 1% trifluoroacetic acid (TFA) and loaded onto a C18 SEP-Column (Waters, Milford, MA). The peptide was eluted with 1% TFA/40% acetonitrile. The eluant was then dried and resuspended in radioimmunoassay (RIA) buffer. The solid-phase RIA (Maidment and Evans, 1991) provided an IC₅₀ 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 (Cat. #RK-003-30; Belmont, CA).

Data analysis

All data are presented as mean \pm SEM. CSF Hcrt-1 levels after the treadmill conditions were compared with their corresponding values taken under baseline conditions. The dependent t-test was used to test for statistical analysis. Because all three measures of BP are highly correlated, only the analysis of MAP is presented. Data from the treadmill experiments were subjected to either t-test with Bonferroni correction or analysis of variance (ANOVA) followed by Tukey post-hoc comparisons. All samples used in Fig. 1A were analyzed together. Similarly all samples used in Fig. 1B were analyzed together. All RIA analyses were done blind to the animals exercise/play condition.

Results

Treadmill and yard play exercise

There was no significant change in CSF Hcrt-1 levels after treadmill exercise across different speeds as compared to the no-exercise control condition. The mean level in the exercise conditions were within $2.7 \pm 0.9\%$ of baseline levels (Fig. 1A). On the other hand, 30 min of playing in the yard significantly increased Hcrt levels in the CSF by 17% as compared to the baseline condition (Fig. 1B). At these animals' maximum treadmill speeds, HR and body temperature were significantly elevated but BP was not significantly changed (Fig. 2). Similarly, both HR and temperature were significantly elevated after 30 min of yard play, but BP did not change significantly (Fig. 2). Respiration rate, which was 37 breaths/minute after being on the treadmill at 0 m/

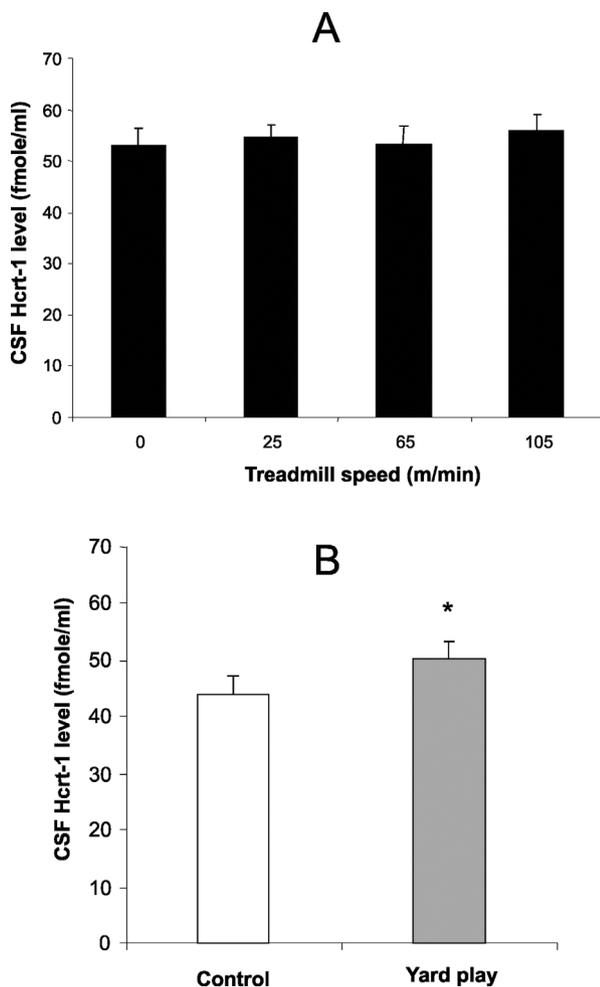


Fig. 1. - (A) CSF Hcr-1 levels were not significantly elevated after maximal speed treadmill exercise (105 m/min) compared to level when standing without moving on the treadmill (0) or at intermediate levels of exercise. (B). Hcr-1 levels were significantly elevated after 30 min of yard play. * $P < 0.02$, t-test comparing to control condition.

min for 30 min, increased by 9% after 30 min in the 25 m/min condition, 48% after the 65 m/min condition and 293% after the 105 m/min condition.

Discussion

In a previous study we reported that Hcr-1 levels in dogs were significantly increased by yard play (Wu et al., 2002), suggesting that the hypocretin system might be activated by motor activity. In the present study, however, we found that intense locomotor activity alone on a treadmill did not change

CSF Hcr-1 level compared to the control condition of simply standing on the treadmill, despite major changes in activity level, respiration, HR and body temperature. Clearly, changes in Hcr-1 release, as assessed by CSF level, are not required to mediate these phenomena. This contrasts with prior studies implicating Hcr-1 in mediating cardiovascular changes. However, these prior conclusions were all based on manipulations of the Hcr-1 system or examination of mutants rather than recordings of Hcr-1 release. Our results do not rule out the possibility of smaller changes that might not be detectable in the CSF. But they indicate that the magnitude of any such changes are significantly less than those produced by yard play. Furthermore our results show that great differences in locomotor speed, and the associated correlated changes in respiratory and cardiovascular activity during treadmill locomotion, produce no detectable change in CSF Hcr-1 level.

The emotional excitement that comes with social interaction with other dogs in the yard contrasts with the monotonous physical movement on the treadmill. Hypocretin neurons are heavily innervated by the amygdala and cholinergic arousal-related basal forebrain (Sakurai et al., 2005; Yoshida et al., 2006). They are activated by anticipation of positive reinforcements, such as food and opiate drugs (Boutrel et al., 2005; Harris et al., 2005; Mileykovskiy et al., 2005; Borgland et al., 2006), and during spontaneous wheel running (Anaclet et al., 2009; Furlong et al., 2009).

Heightened, and most often positive, emotions trigger cataplexy in Hcr-1-deficient narcoleptic humans and animals (Guilleminault, 1976; Lin et al., 1999; Mitler et al., 1976). In humans, laughter is the most common trigger. During the yard play period, the dogs were free to engage in any activity, including digging holes in the grass, playing with toys and interacting with other dogs. When cages were opened for the daily exercise period, the dogs would run to the yard. In contrast, they generally had to be coaxed to go to the treadmill room. This behavior suggests that they enjoyed the free play more than the treadmill locomotion. However, once they were on the treadmill, they ran and elevated autonomic measures to levels comparable to those achieved during the yard play.

In conclusion, the present experiments show that motor activity and the associated autonomic and

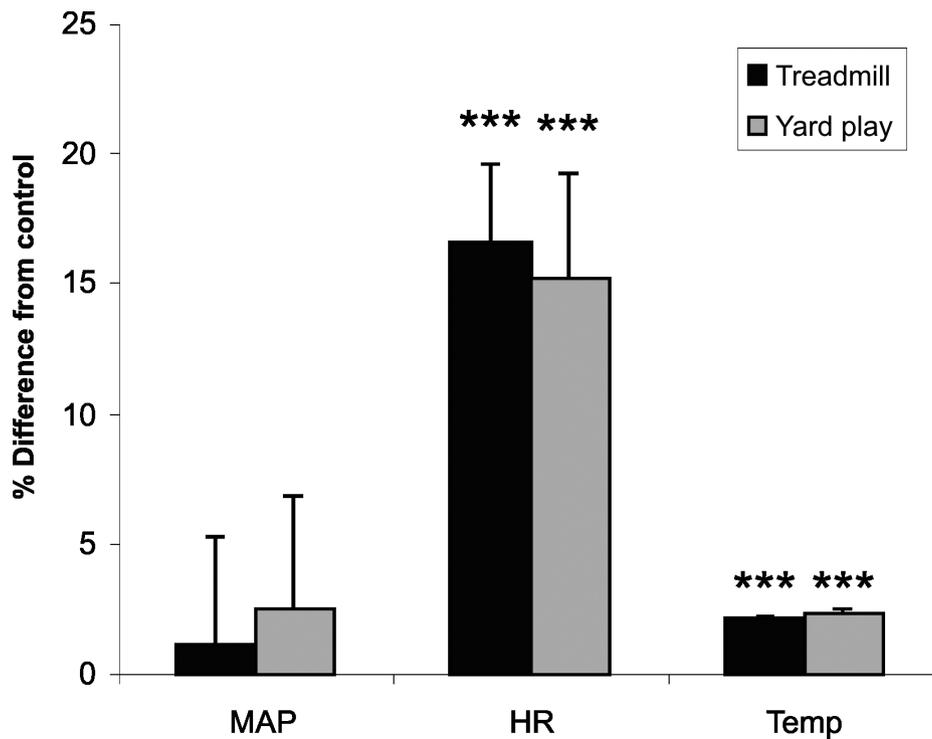


Fig. 2. - Exercise and play conditions significantly increased heart rate and body temperature in both treadmill and yard play conditions, but only increased heart level in the yard play condition (Fig. 1). Percent changes of the mean arterial blood pressure (MAP), heart rate (HR) and core body temperature (Temp) from control condition after 30 min of treadmill exercise at individual animal's maximum speed (Treadmill) or after 30 min of play in the yard (Yard play). *** $P < 0.0001$, t -test. Baseline BP (mmHg) was 117.9 ± 5.6 for treadmill, 120.4 ± 3.1 for yard play; HR (beats/min) baseline was 107.8 ± 5.0 for treadmill, 115.5 ± 3.3 for yard, Temp ($^{\circ}\text{C}$) baseline was 38.3 ± 0.1 for treadmill, 38.4 ± 0.1 for yard play.

respiratory changes do not alter heart level. We hypothesize that activity during positive emotions, of the sort that the dogs experienced during play behavior, is responsible for the increased heart release we observed.

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