Control of cardiovascular variability during undisturbed wake-sleep behavior in hypocretin-deficient mice

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Silvani A, Bastianini S, Berteotti C, Lo Martire V, Zoccoli G. Control of cardiovascular variability during undisturbed wake-sleep behavior in hypocretin-deficient mice. Am J Physiol Regul Integr Comp Physiol 302: R958–R964, 2012. First published February 22, 2012; doi:10.1152/ajpregu.00668.2011.—The central neural mechanisms underlying differences in cardiovascular variability between wakefulness, non-rapid-eye-movement sleep (NREMS), and rapid-eye-movement sleep (REMS) remain poorly understood. These mechanisms may involve hypocretin (HCRT)/orexin signaling. HCRT signaling is linked to wake-sleep states, involved in central autonomic control, and impaired in narcoleptic patients. Thus, we investigated whether HCRT signaling plays a role in controlling cardiovascular variability during spontaneous behavior in HCRT-deficient mice.

HCRT-ataxin3 transgenic mice lacking HCRT neurons (TG), knock-out mice lacking HCRT peptides (KO), and wild-type controls (WT) were instrumented for long-term monitoring of cardiovascular fluctuations with an array of signal analysis techniques based on spontaneous cardiovascular fluctuations. Fluctuations of systolic blood pressure (SBP) and heart period (HP) were assessed with an array of signal analysis techniques, and coherent averaging of SBP surges. During NREMS, all mice had lower SBP variability, greater baroreflex contribution to HP control at low frequencies, and greater amplitude of the central autonomic and baroreflex changes in HP associated with SBP surges than during wakefulness. During REMS, all mice had higher SBP variability and depressed central autonomic and baroreflex HP controls relative to NREMS. HP variability during REMS was higher than during NREMS in WT only. TG and KO also had lower amplitude of the cardiac baroreflex response to SBP surges during REMS than WT. These results indicate that chronic lack of HCRT signaling may cause subtle alterations in the control of HP during spontaneous behavior. Conversely, the integrity of HCRT signaling is not necessary for the occurrence of physiological sleep-dependent changes in SBP variability.

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MATERIALS AND METHODS

The study protocol was approved by the Bologna University ethics committee on animal experimentation and complied with the National Institutes of Health “Guide for the Care and Use of Laboratory Animals”.

BLOOD PRESSURE AND HEART PERIOD (HP) undergo spontaneous fluctuations in physiological conditions. During wakefulness, cardiovascular variability is enhanced by central autonomic commands, which act on the heart and blood vessels and may modify the baroreflex operating point (10, 15, 20). Cardiovascular variability persists during non-rapid-eye-movement sleep (NREMS) and rapid-eye-movement sleep (REMS), even though these are conditions of quiescence and behavioral disengagement from the environment. NREMS entails reduced cardiovascular variability and prominence of baroreflex control compared with wakefulness, whereas cardiovascular variability rises again during REMS because of sleep-related central autonomic commands (28). To date, little is known on the central neural mechanisms that underlie these sleep-dependent changes in cardiovascular variability.

A possible candidate mechanism of sleep-dependent changes in cardiovascular variability is represented by hypocretin (HCRT, also called orexin) peptides, which are released by hypothalamic neurons that send widespread projections throughout the central nervous system (24). HCRT neurons are involved in multiple physiological functions, including wake-sleep state and autonomic control (24) and are functionally lost in patients with narcolepsy with cataplexy (7). Recent experimental studies have revealed that HCRT signaling plays a critical role in the central autonomic commands of the defense response (19, 35, 36) and some types of stress (13). These findings raise the question of whether HCRT neurons are also involved in moment-to-moment regulation of the cardiovascular system during spontaneous undisturbed behavior. HCRT neurons, which discharge maximally during active wakefulness, are almost silent during NREMS, and show occasional burst discharge during REMS (21). We have recently shown that HCRT-deficient mice have blunted differences in the mean values of blood pressure between wakefulness and the sleep states of NREMS and particularly of REMS (1). In that study, however, we only analyzed the average values of blood pressure and heart rate associated with each wake-sleep state. The hypothesis that HCRT signaling also plays a critical role in the control of the cardiovascular variability, which is superimposed upon the mean values of blood pressure and heart rate during each wake-sleep state, thus still awaits experimental evaluation.

In the present study, we tested this hypothesis by investigating whether the magnitude and the control of the variability of blood pressure and HP during wakefulness, NREMS, and REMS differ between wild-type control mice and mice lacking either the whole HCRT neurons (17), similarly to patients with narcolepsy-cataplexy (7), or solely the HCRT peptides released by these neurons (6). Mice were instrumented for long-term simultaneous determination of sleep and cardiovascular variables during undisturbed freely behaving conditions. The magnitude and control of variability in systolic blood pressure (SBP) and HP were assessed with an array of signal analysis techniques based on spontaneous cardiovascular fluctuations.
HCRT-deficient mouse models of narcolepsy. Experiments were performed on three groups of mice congenic (≥9 generations of backcrossing) to C57BL/6J (HCRT-ataxin3 transgenic mice, TG, n = 12; HCRT knockout mice, KO, n = 8; wild-type controls, WT, n = 15). All of the mice were males and age-matched (15.0 ± 0.3 wk old, mean ± SE). Mouse colonies were maintained at the Department of Human and General Physiology at the University of Bologna, Italy. Mice were housed under a 12:12-h light-dark cycle with ambient temperature set at 25°C and free access to water and food. This regimen was maintained throughout all experimental procedures.

TG were hemizygous for a transgenic construct coding for the human neurotoxin Ataxin-3 under the control of the HCRT gene promoter (17). This transgene is expressed selectively in HCRT neurons, which, as a result, are selectively and progressively destroyed after birth (17). KO mice were homozygous for null mutation of the HCRT gene (5) and, therefore, they had a congenital deficiency of HCRT peptides with viable hypothalamic neurons (6). Mouse genotype was determined by PCR on DNA extracted from tail biopsies and lack of measurable brain levels of HCRT-1 peptide (6). Mouse colonies were maintained at the Department of Applied Biomedical Research, S. Orsola University Hospital, Bologna, Italy, as previously described in detail (1). The mice analyzed in the present study had been included in previously published studies with other purposes (1–3).

Experimental protocol. Mice were instrumented with a fronto-parietal differential electroencephalographic lead, a differential electromyographic lead from nuchal muscles, and a telemetric blood pressure transducer (TA11PAC10; Data Science International, Tilburg, The Netherlands), as described in detail elsewhere (31). Recordings were performed for 3 days on mice undisturbed and freely behaving in their own cages after a minimum of 11 days of postsurgical recovery and habituation. The electroencephalographic and electromyographic signals were transmitted via cable and synchronized to the telemetric blood pressure signal by means of simultaneous analogical recovery and habituation. The electroencephalographic and electromyographic signals were transmitted via cable and synchronized to the telemetric blood pressure signal by means of simultaneous analogical recovery and habituation.

Data analysis. Scoring of sleep-wake states (wakefulness, NREMS, and REMS) was performed visually on the basis of raw electroencephalographic and electromyographic recordings with a 4-s resolution as described in detail elsewhere (31). We took care to avoid including even brief arousals within sleep episodes. In particular, episodes of electroencephalographic desynchronization and/or sustained increases in nuchal muscle tone lasting more than 2 s (i.e., half a 4-s epoch) amidst sleep epochs were scored as wakfulness or as an indeterminate state (31). Episodes of REMS at sleep onset representing catalepsy-like states in rodents were scored following consensus criteria (25) and excluded from all analyses. Values of HP were computed as the standard deviation of the respective baseline values. Increase and averaged after subtraction of their respective baseline values.

The indexes SDSBP, SDHP, BRS, and BEI were computed on the basis of beat-to-beat values of HP and SBP. The other analyses were performed after resampling the time series of HP and SBP at 20 Hz with piecewise cubic Hermite interpolation. The CCF and the SBP surges were analyzed after low-pass filtering the time series of HP and SBP below 0.8 Hz (3-pole Butterworth filter) to focus the analysis on fluctuations slower than the breathing rate (30). The CCF analysis and the indexes SDSBP and SDHP were averaged over consecutive data subsets of 60-s duration overlapped for 45 s (30). Data analysis was performed in MatLab (Mathworks, Natick, MA).

Statistical analysis. Data were analyzed with 2-way mixed-model ANOVA (GLM procedure with Huynh-Feldt correction). In case of significant interaction between the mouse strain factor and the wake-sleep state factor, simple effects of the state were tested in each strain, and simple effects of the strain were tested in each state with preplanned comparisons using paired-sample (wakefulness vs. NREMS and REMS vs. NREMS) and independent-sample (TG vs. WT and KO vs. WT) t-tests with false-discovery rate procedure (8).

RESULTS

Duration of wake-sleep episodes and mean values of cardiovascular variables. The individual and total duration of the wake-sleep episodes analyzed and the mean values of SBP and HP in the different wake-sleep states are reported in Table 1.

Magnitude of SBP and HP variability. The values of SDSBP and SDHP estimating the magnitude of SBP and HP variability are reported in Fig. 1. Physiological sleep-dependent changes in SBP variability occurred in all experimental groups, with SDSBP decreasing in NREMS compared with wakefulness and increasing during REMS toward values in wakefulness. Conversely, both TG and KO lost the sleep-dependent modulation of heart rate variability, which consisted in WT of a significant increase in SDHP on passing from NREMS to REMS. This increase was not detected in HCRT-deficient mice. Accordingly, SDHP during REMS was significantly lower in either TG or KO than in WT.

Baroreflex analysis with the sequence technique. Fig. 2 shows the values of BRS and BEI estimated with the sequence technique. Both the gain (BRS) and the degree of engagement (BEI) of the arterial baroreflex differed significantly among wake-sleep states in all experimental groups. In particular, BRS was higher in NREMS than in wakefulness, whereas both
BRS and BEI decreased on passing from NREMS to REMS. Thus, these patterns of sleep-dependent changes in BRS and BEI were also fully preserved in TG and KO.

CCF analysis of central autonomic and baroreflex control of HP. The average CCFs between HP and SBP showed a positive peak at negative time shifts and a negative trough at positive time shifts in each wake-sleep state (Fig. 3). The first CCF pattern indicates a positive correlation between HP and previous SBP values, which is consistent with baroreflex buffering of the blood pressure changes elicited by vascular resistance fluctuations (33). The second CCF pattern indicates a negative correlation between HP and subsequent SBP values, which is consistent with central autonomic commands acting on the heart (33). Both CCF patterns differed significantly in magnitude among wake-sleep states, the first (i.e., baroreflex) pattern being more prominent in NREMS than either in wakefulness or REMS and the second (i.e., central autonomic)

Table 1. Duration of full-blown wake-sleep episodes >60 s and mean values of cardiovascular variables therein

<table>
<thead>
<tr>
<th>Group</th>
<th>State</th>
<th>Dm, s</th>
<th>Dt, h</th>
<th>SBP, mmHg</th>
<th>HP, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>W</td>
<td>196 ± 18</td>
<td>22.4 ± 2.0</td>
<td>137 ± 2</td>
<td>99 ± 1</td>
</tr>
<tr>
<td></td>
<td>NREMS</td>
<td>122 ± 5</td>
<td>20.2 ± 1.4</td>
<td>116 ± 2</td>
<td>119 ± 2</td>
</tr>
<tr>
<td></td>
<td>REMS</td>
<td>101 ± 1</td>
<td>2.4 ± 0.2</td>
<td>121 ± 2</td>
<td>110 ± 2</td>
</tr>
<tr>
<td>TG</td>
<td>W</td>
<td>199 ± 13</td>
<td>29.2 ± 1.8</td>
<td>137 ± 1</td>
<td>98 ± 1</td>
</tr>
<tr>
<td></td>
<td>NREMS</td>
<td>131 ± 5</td>
<td>23.3 ± 1.5</td>
<td>118 ± 1</td>
<td>122 ± 2</td>
</tr>
<tr>
<td></td>
<td>REMS</td>
<td>109 ± 2</td>
<td>2.7 ± 0.2</td>
<td>131 ± 1</td>
<td>103 ± 2</td>
</tr>
<tr>
<td>KO</td>
<td>W</td>
<td>186 ± 14</td>
<td>25.1 ± 1.3</td>
<td>138 ± 2</td>
<td>94 ± 1</td>
</tr>
<tr>
<td></td>
<td>NREMS</td>
<td>126 ± 8</td>
<td>24.2 ± 2.1</td>
<td>121 ± 2</td>
<td>111 ± 2</td>
</tr>
<tr>
<td></td>
<td>REMS</td>
<td>101 ± 2</td>
<td>2.5 ± 0.2</td>
<td>132 ± 2</td>
<td>100 ± 1</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE in congenic mice with ablation of hypocretin neurons (TG, n = 12), congenital deficiency of hypocretin peptides (KO, n = 8), and wild-type controls (WT, n = 15). W, wakefulness; NREMS, non-rapid eye movement sleep; REMS, rapid eye movement sleep. Dm and Dt, individual and total duration of the wake-sleep episodes analyzed, which were uninterrupted wake-sleep episodes of duration ≥60 s and free of cardiovascular artifacts; SBP, systolic blood pressure; HP, heart period.

Fig. 1. Standard deviation of beat-to-beat values in systolic blood pressure (SDSBP) (top) and heart period (SDHP) (bottom) during wakefulness (W), non-rapid eye movement sleep (NREMS), and rapid eye movement sleep (REMS). Values are expressed as means ± SE in congenic mice with ablation of hypocretin neurons (TG; n = 12), congenital deficiency of hypocretin peptides (KO; n = 8), and wild-type controls (WT; n = 15). ANOVA, SDSBP: state, P < 0.001; strain, P = 0.300; state × strain, P = 0.986. ANOVA, SDHP: state, P < 0.001; strain, P = 0.162; state × strain, P = 0.003. *P < 0.05 vs. NREMS; † and ‡, P < 0.05 vs. TG and KO, respectively. Brackets over bars refer to comparisons between marginal means of the three mouse strains within each state.

Fig. 2. Cardiac baroreflex sensitivity (BRS) and baroreflex effectiveness index (BEI) estimated with the sequence technique during W, NREMS, and REMS. Values are expressed as means ± SE in congenic mice with ablation of hypocretin neurons (TG, n = 12), congenital deficiency of hypocretin peptides (KO, n = 8), and wild-type controls (WT, n = 15). ANOVA, BRS: state, P < 0.001; strain, P = 0.427; state × strain, P = 0.149. ANOVA, BEI: state, P < 0.001; strain, P = 0.164; state × strain, P = 0.263. *P < 0.05 vs. NREMS. Brackets over bars refer to comparisons between marginal means of the three mouse strains within each state.

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pattern being less prominent in REMS than in NREMS. These sleep-dependent differences occurred in each mouse strain, irrespective of HCRT deficiency.

**Analysis of central autonomic and baroreflex cardiovascular control during SBP surges.** The frequency of occurrence of spontaneous SBP surges during each wake-sleep state (Fig. 4) mirrored the sleep-dependent differences in SDSBP (Fig. 1). The results of the coherent averaging of SBP surges are shown in Fig. 5. In each wake-sleep state, HP decreased before the SBP peak, consistently with the activity of central autonomic commands on the heart. HP then increased after the SBP peak, consistently with baroreflex control. In NREMS, the SBP peak was significantly lower, and the HP trough was significantly more pronounced than either in wakefulness or REMS, irrespective of the mouse strain. On the other hand, the HP peak was significantly blunted in TG and KO compared with WT during REMS.

**DISCUSSION**

This study demonstrates for the first time that HCRT signaling does not play a critical role in the control of BP and HP variability during undisturbed wake-sleep cycles. However, during REMS, HP control seems to be partially impaired in HCRT-deficient mice.

The effects of sleep on the magnitude and control of cardiovascular variability that we found in WT were similar to those in larger mammals, including humans, as far as SBP variability was concerned (28, 32), whereas results suggested that species differences may occur in HP variability. In particular, SDHP did not decrease on passing from wakefulness to NREMS in WT (Fig. 1), whereas it has been reported to do so in human subjects (32). In WT, BRS was the highest in NREMS (Fig. 2), whereas available evidence is contrasting in larger animals, including humans (28). In WT, the positive CCF peak, consistent with baroreflex control, was the highest during NREMS among wake-sleep states (Fig. 3), as it is in humans (32). However, BEI did not increase in WT on passing from wakefulness to NREMS (Fig. 2), whereas it does in humans (32). During NREMS, on the other hand, the CCF trough consistent with central autonomic control was as pronounced as in wakefulness and more pronounced than in REMS in WT (Fig. 3). These findings are similar to results obtained during stages 1 and 2 of NREMS in humans (32). Taken together, the results of the CCF analysis in the present study, thus, indicate that during NREMS, WT showed the greatest baroreflex contribution to HP control, as well as the greatest central autonomic contribution to HP control among wake-sleep states, i.e., the tightest overall link between HP and SBP variability among wake-sleep states. This conclusion agrees with results of co-
In the face of these heterogeneous mechanisms, it is striking that the coherent averaging procedure revealed the same temporal sequence of events during SBP surges in each wake-sleep state, with the maximum decrease in HP preceding the SBP peak and the maximum increase in HP following it (Fig. 5). This temporal sequence of events may be explained by baroreflex buffering of central autonomic commands that act on the heart and blood vessels (33).

We found that differences in cardiovascular variability between HCRT-deficient mice and controls were limited to REMS and involved total HP variability (SDHP index, Fig. 1) and the magnitude of baroreflex HP control during SBP surges (positive HP peak, Fig. 5). These differences occurred in both TG and KO and were thus because of the lack of HCRT peptides released by HCRT neurons, as opposed to the lack of other signaling molecules coreleased by these neurons. Results obtained on our mouse models of narcolepsy are not in agreement with data obtained on narcoleptic patients, which are quite discrepant themselves. In fact, no significant differences in HP variability have been detected during REMS between narcoleptic patients and controls (11), and increases in HP and SBP variability during wakefulness have been observed in one study on narcoleptic patients (12). We also did not find evidence of reductions in the magnitude of central autonomic and baroreflex changes in HP, which have been recently reported in narcoleptic patients (12). We also did not find evidence of reductions in the magnitude of central autonomic and baroreflex changes in HP, which have been recently reported in narcoleptic patients (12).
have been contrasting on animal models. In particular, a previous study on TG, which did not take the wake-sleep state into account, did not reveal any significant alteration in BRS and BEI (35). Conversely, a slight defect in BRS limited to decreasing SBP sequences was recently reported during REMS in a rat model of partial HCRT deficiency (26).

In a recent study on TG and KO mice, we have shown that lack of HCRT peptides released by HCRT neurons blunts the physiological differences in the mean value of blood pressure that occur between wakefulness and either NREMS or, particularly, REMS (1). The present study shifted the focus from the average values of cardiovascular variables in each wake-sleep state to the magnitude and control of the cardiovascular variability, which is superimposed on those average values in each state. Intriguingly, we found that the conclusions previously reached concerning the mean values of cardiovascular variables (1) did not hold for cardiovascular variability. In fact, the present study indicates that the effects of the loss of HCRT signaling on the physiological differences in cardiovascular variability between wake-sleep states are minor at best, particularly as far as blood pressure variability is concerned. The functional neuroanatomy underlying sleep-related central autonomic commands remains poorly understood (28). Our present results emphasize the complexity of these commands, suggesting that central neural mechanisms that determine the mean value of blood pressure during sleep differ, at least in part, from those that determine blood pressure variability during sleep. In particular, HCRT neurons appear involved in the former, but not in the latter, mechanisms.

In conclusion, we have investigated sleep-dependent changes in cardiovascular variability in HCRT-deficient mice with an array of techniques based on the analysis of spontaneous cardiovascular fluctuations and yielding complementary information. The results demonstrated that the chronic lack of HCRT peptides released by hypothalamic HCRT neurons may cause subtle alterations in HP control during spontaneous behavior. However, the integrity of HCRT signaling is not necessary for the occurrence of physiological sleep-dependent changes in SBP variability.

**Perspectives and Significance**

Evidence on defects in the magnitude and control of cardiovascular variability is contrasting in patients with narcolepsy-cataplexy (9, 11, 12, 16). Our data suggest that these defects are either linked to genetic or lifestyle factors in particular patient samples or are species-specific and cannot be translated to mouse models for mechanistic insight. In this respect, it should be kept in mind that physiological and/or behavioral adaptation may occur in knockout and transgenic models, potentially activating compensatory mechanisms that underpin the true effect of HCRT signaling in cardiovascular functioning. Thus, further studies will be needed to clarify whether HCRT signaling is redundant or, rather, is not involved at all in driving the sleep-dependent changes in blood pressure variability in mice. Finally, the stereotyped pattern of HP changes that we showed by coherent averaging of spontaneous SBP surges suggests that at least some of these events are driven by a common central autonomic generator in different wake-sleep states. This generator does not require hypothalamic HCRT neurons and, hence, may differ from neural pathways involved in classical defense response paradigms (19, 35, 36).

**GRANTS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

A.S. and G.Z. designed the study. S.B., C.B., and V.L.M. performed the experiments. A.S. performed data analysis and wrote the paper. G.Z. coordinated the group and reviewed the manuscript.

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