Central Deficiency of Corticotropin-Releasing Hormone Receptor Type 1 (CRH-R1) Abolishes Effects of CRH on NREM But Not on REM Sleep in Mice

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Study Objectives: Corticotropin-releasing hormone (CRH) is the major activator of the hypothalamic-pituitary-adrenocortical (HPA) system and orchestrates the neuroendocrine, autonomous as well as behavioral responses to stress. Many studies suggest an influence of CRH on sleep-wake regulation even in the absence of stressors. However, none of these studies yet clearly distinguished between central and peripheral effects of CRH. Therefore, we investigated in CNS-specific CRH receptor type 1 deficient mice whether centrally administered CRH could induce its sleep-wake modulatory effects without peripheral induction of HPA activity.

Design: Male mice (C57BL/6J, CNS-specific CRH-R1 knockout [CKO] mice and their control littermates [CL]) were intracerebroventricularily (i.c.v.) injected with vehicle or 3 different doses of CRH shortly before the beginning of the light period. Electroencephalogram (EEG) and electromyogram (EMG) were monitored to compare the effects of CRH on vigilance states with or without presence of central CRH-R1. To quantify HPA-axis reactivity to CRH injections in CKO and CL animals, blood samples were analyzed to determine plasma corticosterone concentrations.

Results: I.c.v. injections of CRH promoted wakefulness while decreasing NREMS in C57BL/6J and CRH-R1 CL animals, whereas such changes were not exerted in CKO mice. However, REMS suppression after CRH application persisted in all animals. I.c.v. injected CRH increased plasma corticosterone levels in both CL and CKO mice.

Conclusions: The results demonstrated that CRH has a major impact on wake and NREMS regulation which is predominantly mediated through central CRH-R1. Peripheral actions of CRH, i.e., elevated HPA activity, may interfere with its central effects on REMS but not on NREMS suppression.

Keywords: CRH, stress, wake, NREM, REM, sleep regulation, conditional CRH-R1 knockout mice

Citation: Romanowski CPN; Fenzl T; Flachskamm C; Wurst W; Holsboer F; Deussing JM; Kimura M. Central deficiency of corticotropin-releasing hormone receptor type 1 (CRH-R1) abolishes effects of CRH on NREM but not on REM sleep in mice. *SLEEP* 2010;33(4):427-436.

SINCE ITS DISCOVERY IN 1981, NUMEROUS INSIGHTS INTO THE ROLE AND FUNCTION OF CORTICOTROPIN-RELEASING HORMONE (CRH) HAVE BEEN accumulated.[1] It is commonly regarded as one of the major factors controlling the activity of the hypothalamic-pituitary-adrenocortical (HPA) axis and autonomic components in responses to stress.[2,3] Secretion of CRH from the paraventricular nucleus (PVN) of the hypothalamus constitutes the initial step in these processes which finally peak in arousal and wakefulness.

Additionally, extrahypothalamic actions of CRH indicate that this neuropeptide acts as a neuromodulator in the CNS and thus might be involved in the regulation of spontaneous behaviors (e.g., food intake, locomotor activity, anxiety) even in the absence of stressors.[4] Up to now it remains unclear whether the arousal effects of CRH are exerted in consequence of its action in the periphery, through other stress hormones, or centrally, by a direct action of this neuropeptide within the brain. Many studies, however, hypothesize a regulatory influence of CRH on sleep and waking as one of its central effects. Previously, Opp reported that Lewis rats, in which CRH synthesis and its secretion from the hypothalamus are decreased, have reduced amounts of waking and increased non-rapid eye movement sleep (NREMS) in comparison to Fischer 344 and Sprague-Dawley (Sp-D) rats in which the level of the neuropeptide in the brain is normal.[5] In another experiment, Chang and Opp demonstrated that intracerebroventricular (i.c.v.) application of an antisense oligonucleotide probe directed against CRH mRNA reduces spontaneous waking in Sp-D rats during their activity phase.[6] Further corroborating this hypothesis, a possible involvement of central CRH on sleep reflects the receptor distribution, as found consistently in many brain regions, such as the basal forebrain, the thalamus, hypothalamus, midbrain, pons, and the medulla, which are closely related to waking.[7,8]

Concerning the degree of involvement and depending on the kind of behavior regulated by the interplay of CRH and its receptor subtypes (CRH receptor type 1 and 2; CRH-R1 and CRH-R2) literature sometimes presents conflicting results. For example, 3 independent laboratories studied 3 differently generated CRH-R2 knockout (CRH-R2 KO) mouse lines with regard to behavioral and endocrine activity. Only 2 groups could show significant increases of anxiety-related behavior[9,10]; only one of these 2 observed augmentation of adrenocorticotropic hormone (ACTH) and corticosterone (CORT) release in response to stress,[9] whereas the third group showed opposite endocrine changes with absence of anxiety-related behavior.[11] While the role of CRH-R1 in anxiety seems to be more established, sleep-wake rhythmicity results also diverge. Chang and Opp could show that central application of a CRH-R1 antagonist (α-helical CRH) prior to the onset of the dark period reduced spontaneous waking in rats,[12] whereas Gonzalez and colleagues did not find such effects.[13] To elucidate more closely whether this neuro-

Submitted for publication June, 2009
Submitted in final revised form August, 2009
Accepted for publication September, 2009
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peptide and its receptors are involved in the central mechanism of sleep-wake regulation, the central effects of CRH need to be considered separately from those on the HPA system in an experimental approach, where its central (spontaneous) and peripheral (stress-induced) regulatory influences are distinguishable. Along these lines, we have recently demonstrated that central CRH is driving REM sleep in a genetic mouse model of CRH excess. This conditional model overexpresses CRH in either the entire central nervous system, or only in the anterior forebrain without affecting basal HPA-axis activity[14]; both genetic mutant lines exhibit similarly altered REM sleep.[15] Complementing the previously used gain-of-function model, the present study made use of conditional (CNS-specific) CRH-R1 knockout (CKO) mice and their control littermates (CL), in which the impact of central CRH mediated via CRH-R1 on sleep-wake behavior can be separately analyzed with the presence of a functional HPA axis.

MATERIALS & METHODS

Animals

Fourteen male C57BL/6J mice (Harlan Winkelmann GmbH, Borchen, Germany) were used in this study. CNS-restricted deletion of CRH-R1 was achieved by breeding mice with a homozygous floxed *Crhr1* gene, where exons 9-13 are flanked by *loxP* sites,[16] to *nestin-cre* mice.[17] In this transgenic mouse line cre expression is controlled by the nestin promoter and neural enhancer, which drives cre expression in neuronal and glial precursors as early as embryonic day 10.5. Resulting heterozygous $Crhr1^{+/loxP}$ *nestin-cre* and $Crhr1^{+/loxP}$ F_1 animals were intercrossed to obtain the F_2 generation animals of the desired genotypes: $Crhr1^{loxP/loxP}$ *nestin-cre* (conditional knockout; CKO) and $Crhr1^{loxP/loxP}$ (control littermate; CL). Subsequently, the conditional CNS-specific CRH-R1 knockout mouse line was maintained by breeding $Crhr1^{loxP/loxP}$ *nestin-cre* and $Crhr1^{loxP/loxP}$ animals. In $Crhr1^{loxP/loxP}$ *nestin-cre* animals CRH-R1 expression is disrupted throughout the brain but not in the pituitary (Supplementary Figure 1 available online at www.journalsleep.org). Our CRH-R1 CKO and CL mice were kept on a C57BL/6J x 129S2/SvPas background and have been bred to transgenic *nestin-cre* mice that were generated using (C57BL/6J x SJ)F_2 oocytes.[18] A total of 21 male, CNS-specific CRH-R1 knockout (n = 12) mice (CKO) and their control (n = 9) littermates (CL) were used in the sleep study. Animals were housed in custom-made Lucite cages (transparent Lucite walls, 35 cm height; replaceable, grey Lucite floor, 26 cm × 26 cm) under constant environmental conditions (12 h:12 h light/dark cycle, 100 lx during the light period, 22°C ± 1°C, 50% ± 10% humidity). A feeding rack and a water bottle were attached to the front panel of the cage which allowed animals access to food and water *ad libitum*. All animal studies were conducted according to the guidance of the European Community Council Directive. Experimental protocols were approved by the local commission for the Care and Use of Laboratory Animals of the State Government of Bavaria, Germany.

Surgery

During surgery, animals were anesthetized using inhalation narcosis (custom-made vaporizing device) with an isoflurane/oxygen mixture (Isofluran, DeltaSelect GmbH, Dreieich, Germany). To maintain a constant position of the head, mice were fixed with a stereotaxic apparatus and before and after operation received subcutaneously atropine sulfate (0.05 mg/kg, Atropin, Braun Melsungen AG, Melsungen, Germany) to stabilize circulation and meloxicam (0.5 mg/kg, Metacam, Braun Melsungen AG, Melsungen, Germany) as a postoperative analgesic, respectively. An i.c.v. guide cannula was implanted stereotaxically in the left lateral ventricle and cemented to the skull with dental acrylic resin (Paladur, Heraeus Kulzer, Hanau, Germany). Four electrodes made of gold wires with ball-shaped ends were placed epidurally on the cortex (one in the frontal and one in the parietal field of each hemisphere) for electroencephalogram (EEG) recordings, and 2 more of these electrodes were inserted in the neck muscle to record electromyogram (EMG) signals. All electrodes were soldered to a connector (BCP socket connector) which was affixed to the skull with Paladur. Mice were allowed to recover from surgery for 2 weeks in their experimental cages before the initiation of baseline recordings. At the end of the experiments, animals were euthanized with an overdose of sodium pentobarbital. Brains were removed and processed for Nissl staining (cresyl violet acetate, Sigma-Aldrich, St. Louis, MO) to examine the positions of the i.c.v. cannulae (tissue lesions). Only animals with correctly placed cannulae were accounted for further analysis.

EEG/EMG Recordings

After the recovery period a recording cable was plugged into the connector and attached to an electric swivel system which allowed EEG and EMG recordings from the freely behaving animal. All signals were preamplified (1000 x, custom made) and sent to a main amplifier (10 x, custom made) before transformation by an analog/digital card (64 Hz sampling rate; National Instruments, Austin, TX) and storage on a computer. The EEG signals were analogue band pass-filtered (0.5–29 Hz, filter frequency roll off 48 dB/octave) and root mean square was applied to the non-filtered EMG signals before its digital conversion (sample rate: 64 Hz). Continuous EEG/EMG recordings were made for 23 h per experimental day to allow animal care and maintenance of the recording setup during the remaining hour, if necessary. Data obtained in this manner were analyzed by a LabView-based acquisition program (EGEraVigilanz, SEA, Köln, Germany), which enabled semiautomatic classification of vigilance states applying a FFT algorithm adapted from a report by Louis et al.[19] Vigilance states were defined as wake, NREMS, or rapid eye movement sleep (REMS) in 4-sec epochs. Semiautomatically scored data were confirmed and corrected if necessary by visual rescoring.

CRH Administration

For central application of CRH, a bolus injection was achieved i.c.v. by using a Hamilton syringe (Type 700 Hamilton Microliter, Banaduz, Switzerland) with an injection cannula (ID 0.11 mm). Each experimental animal either received 3 µL of a vehicle control (sterile water, Braun Melsungen AG, Melsungen, Germany) or 3 different doses of human/rat CRH (0.3, 1.0, 3.0 µg; h/r CRF, Bachem AG, Bubendorf, Switzerland) dissolved into an equal volume of the vehicle. Based on previous findings that at 1 µg/mouse i.c.v. administered CRH elevated

levels of plasma CORT,[4] we chose the other doses lower and higher than that to detect differences in their responses. Upon injection the animals were gently restrained, and the injection cannula was inserted into the guide cannula. Each i.c.v. application was finished within 30 sec after insertion of the injection cannula, and animals were released into their respective recording cages. All injections were completed within the last 30 min of the dark period under red light conditions. To include manipulatory effects of injections, EEG and EMG recordings started one hour before the onset of the light period. The order of CRH application at several doses was randomized in each animal, including 2 baseline days between treatment days to prevent accumulation of drug effects.

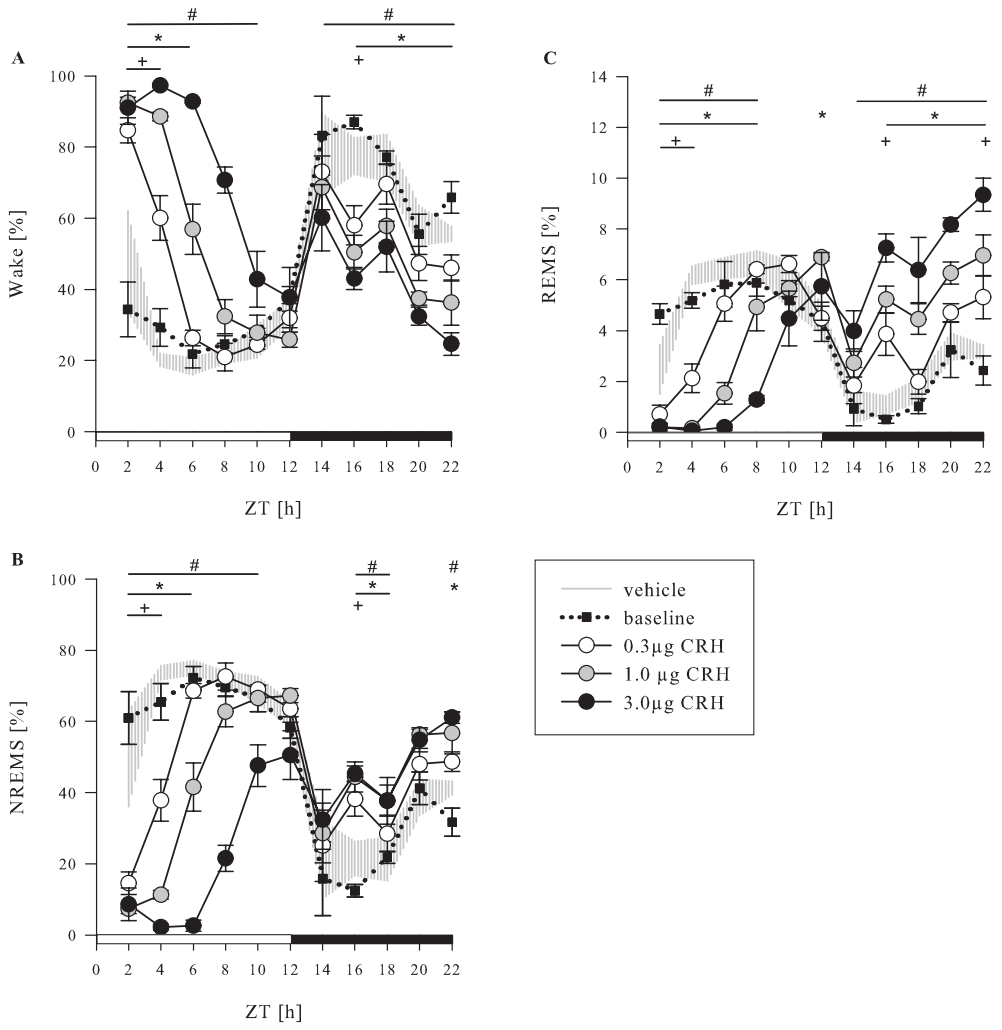

Figure 1—Effects of i.c.v. CRH injections on vigilance states in C57BL/6J mice. Corticotropin-releasing hormone (CRH) dose-dependently increased wake levels during the light period for up to 10 h after application and decreased those levels for almost the entire course of the dark period. Contrariwise, NREMS was dose dependently decreased during the light period and showed increases during the successive dark period. REMS was similarly reduced immediately after CRH application and almost totally blocked for up to 4 and 6 h after injection of 1.0 µg and 3.0 µg CRH, respectively. Shortly after transition to the dark period a dose-dependent increase in REMS was observed. Depicted are percentages of the given vigilance states (indicated on the y-axis) of 2-h means ± SEM for baseline (■) and each CRH treatment (○: 0.3 µg, ●: 1.0 µg, ●: 3.0 µg). The hatched area indicates mean values ± SEM after vehicle treatment. The symbols +,*, and # denote statistically significant differences by comparison of vehicle versus treatment with 0.3, 1.0, or 3.0 µg of CRH, respectively (tests with contrasts in ANOVA, $P < 0.05$). Solid lines under those symbols connect consecutive time points showing statistical differences. The open and filled bar on the x-axis indicates the light and dark period, respectively.

Analysis of Plasma Corticosterone

To quantify HPA-axis reactivity in our CRH-R1 CKO animals and their CL in response to CRH administration, plasma CORT levels were measured by radioimmunoassay (Corticosterone^{125}I RIA Kit, MP Biomedicals, Orangeburg, USA). Hence control blood (15 µL) was collected one week before and an equal volume of sample blood after vehicle or 1.0 µg of CRH treatment was taken from the tail vein at 2 h after injection (Zeitgeber Time [ZT] 2). Blood samples were centrifuged immediately (3500 rpm, 15 min, 4°C), and separated plasma supernatant was collected and stored at −80°C until later analysis. Test plasma samples were diluted 1:150. The sensitivity of the CORT RIA kit is 25 ng/mL. The intra-assay and inter-assay coefficients of variation were < 12 % for CORT.

Statistical Analysis

Time spent in wake, NREMS and REMS was calculated in consecutive 2-h intervals, and effects of different doses and time intervals on these vigilance states were statistically evaluated for significance in each time period (light or dark) and each mouse group by two-factorial analyses of variances (ANOVAs) with a repeated measures design. Thereby the 2 influential factors were dose and time, both within-subjects factors with 5 levels (baseline, vehicle, 0.3, 1.0, and 3.0 µg CRH) and 6 (light period; 2-h intervals) or 5 levels (dark period; 2-h intervals), respectively. Furthermore, the mean duration of the wake, NREMS, and REMS episodes within the light and dark period were determined, and the effects of treatment, time period and mouse groups on given means were statistically analyzed by 3-factorial ANOVA. As mentioned previously, in addition to dose and time, group was an additional influential factor, with 3 levels: C57BL/6J, CRH-R1 CKO, and CL. Data for plasma CORT levels were compared between mouse groups and different treatments by 2-factorial analyses of variance with a

repeated measures design. In the event of significant main or interaction effects in ANOVA, post hoc tests for simple effects (contrasts tests or Student-Neumann-Keuls tests) were applied in order to locate significant differences among the levels of the within- or between-subjects factors, respectively. As nominal level of significance $\alpha = 0.05$ was accepted. All post hoc tests (tests with contrasts and/or Student-Neumann-Keuls tests) were performed at a reduced level of significance (Bonferroni adjusted), in order to keep the type 1 error ≤ 0.05. Values presented in tables and graphs indicate mean values ± SEM.

RESULTS

Sleep and Wake Responses to CRH Injections

In C57BL/6J mice, significant interaction effects of treatment and time on wake were found during the light period ($F_{treatment\ x\ time}$ (20/220) = 18.74, $P < 0.001$) but in the dark period only significant treatment effects could be detected ($F_{treatment}$ (4/44) = 33.78, $P < 0.001$). Compared to the baseline recording, injection itself even with vehicle elicited a significant increase in wakefulness during the first 2 h (contrasts tests, $P = 0.039$), which afterwards returned to and remained at baseline levels (Figure 1A). CRH application, however, caused significantly higher rises of wake by postinjection hour 2 in comparison to vehicle and thus baseline (contrasts tests, $P < 0.05$). Arousal levels remained significantly elevated in a dose-dependent manner during most of the entire light period. The highest dose of CRH caused significantly increased wakefulness for up to 10 h after injection (contrasts tests: ZT 2-8: $P < 0.001$; ZT 10: $P \leq 0.014$), whereas the wake-promoting effects of CRH disappeared roughly 6 and 8 h after application of 0.3 µg or 1.0 µg (Figure 1A; contrasts tests, $P < 0.001$), respectively.

At the beginning of the dark period, however, a dose-dependent reduction in time spent awake was observed in all animals injected with CRH in comparison to vehicle treatment. This reduction in wake persisted to be significant at the 2 higher doses (contrasts tests: 1.0 µg: $P \leq 0.012$; 3.0 µg: $P < 0.001$) during most of the dark period (Figure 1A; ZT16-22).

Regarding NREMS, interaction effects of treatment and time were significant during the light period ($F_{treatment\ x\ time}$ (20/220) = 18,521, $P < 0.001$), whereas during the dark period only significant treatment effects arose ($F_{treatment}$ (4/44) = 65.694, $P < 0.001$). Vehicle injections showed as compared with baseline levels, only a slight and non-significant reduction of NREMS during the first 2 h (Figure 1B). Application of CRH, however, induced clear, dose-dependent decreases of this vigilance state in comparison to control and therefore baseline levels. The higher the dose of applied CRH, the larger were the changes and effect durations observed in NREMS. While 0.3 µg of CRH significantly reduced time spent in NREMS for up to 4 h, 1.0 µg and 3.0 µg of CRH affected significant reductions for 6 and 10 h, respectively (contrasts tests in ANOVA, $P < 0.001$; Figure 1B). After transition to the dark period, all CRH-treated animals showed increases in NREMS compared with vehicle treated animals, although the dose dependency was not as clear as seen for wake. Whereas NREMS after treatment with 0.3 µg of CRH was only slightly elevated and reached significantly higher levels only at one time point (ZT 16; contrasts tests, $P \leq 0.022$), injections of 1.0 µg and 3.0 µg of CRH similarly led to more distinct rises in NREMS levels. Both doses entailed significant increases of NREMS at several timepoints of the dark period (ZT 16, 18, 22; contrasts tests, $P \leq 0.035$).

In the case of REMS, significant interaction effects were found in the light period ($F_{treatment\ x\ time}$ (20/220) = 9.675, $P < 0.001$), whereas significant treatment effects could be observed during the dark period ($F_{treatment}$ (4/44) = 24.356, $P < 0.001$). Vehicle injections significantly suppressed REMS appearance for up to 2 hours compared with baseline (contrasts tests in ANOVA, $P = 0.008$; Figure 1C). However, no further differences in REMS amounts could be detected in comparison to baseline levels. As for other vigilance states, effects induced by CRH treatment were dose-dependent. The lowest dose of CRH (0.3 µg) significantly reduced REMS levels for 4 post-injection h compared to vehicle treatment and baseline (contrasts tests, $P \leq 0.025$). Two other doses of CRH, 1.0 µg and 3.0 µg, almost completely blocked REMS for the first 4 or 6 h, respectively, and significant reduction of REMS lasted for 8 h after application (contrasts tests, $P \leq 0.017$). On the other hand, CRH promoted REMS during the dark period. At 0.3 µg of CRH, REMS levels were slightly increased, reaching significant levels at 2 time points (ZT 16, 22; contrasts tests, $P \leq 0.025$). The higher doses exhibited distinct effects on REMS enhancement. Elevation of REMS remained significant almost throughout the entire dark period after treatment with 1.0 µg (contrasts tests, $P \leq 0.013$) and 3.0 µg of CRH (contrasts tests, $P < 0.001$).

In CRH-R1 CKO and CL mice, significant interaction effects of treatment and time on wake were detected during the light period only (CRH-R1 CKO: $F_{treatment\ x\ time}$ (20/220) = 4.552, $P < 0.001$; CL: $F_{treatment\ x\ time}$ (20/160) = 13.353, $P < 0.001$). As similarly seen in C57BL/6J mice, vehicle treatment led to a slight but significant, short lasting increase of wake levels in both CRH-R1 CKO and CL animals during the first 2 h after injection in comparison to baseline (contrasts tests, $P \leq 0.008$). No further effects of vehicle treatment on wakefulness could be observed for the rest of the recording session if compared to baseline (Figure 2A and D). However, CRH application in CL mice elicited a significant, dose-dependent increase in wakefulness in comparison to baseline and vehicle conditions, as similarly seen in C57BL/6J mice. These effects were significant for up to 8 h after injection of 3.0 µg of CRH and up to 6 and 2 h after injection of 1.0 µg or 0.3 µg of CRH respectively (contrasts tests, $P \leq 0.002$; Figure 2A). In contrast, such a wake-inducing effect of CRH did not appear in CRH-R1 CKO animals. Although all CRH treatments led to slight elevations in wake levels at the beginning of the experiment (Figure 2D), no significant differences from vehicle and baseline levels could be detected with the exception of wake levels at ZT 2 after application of 0.3 µg CRH, ZT 4 after application of 1.0 µg CRH, and ZT 6 after application of 3.0 µg CRH (contrasts tests, $P \leq 0.047$). During the dark period, contrary to the effects in C57BL/6J animals, no reduction in wakefulness could be induced after any dose, neither in CL nor in CRH-R1 CKO animals.

Concerning NREMS, treatment and time effects showed a significant interaction during the light period in CL animals only ($F_{treatment\ x\ time}$ (20/160) = 11.794, $P < 0.001$). Vehicle treatment induced a slight but significant decrease within the first 2 h after injection (contrasts tests, $P \leq 0.035$) in comparison

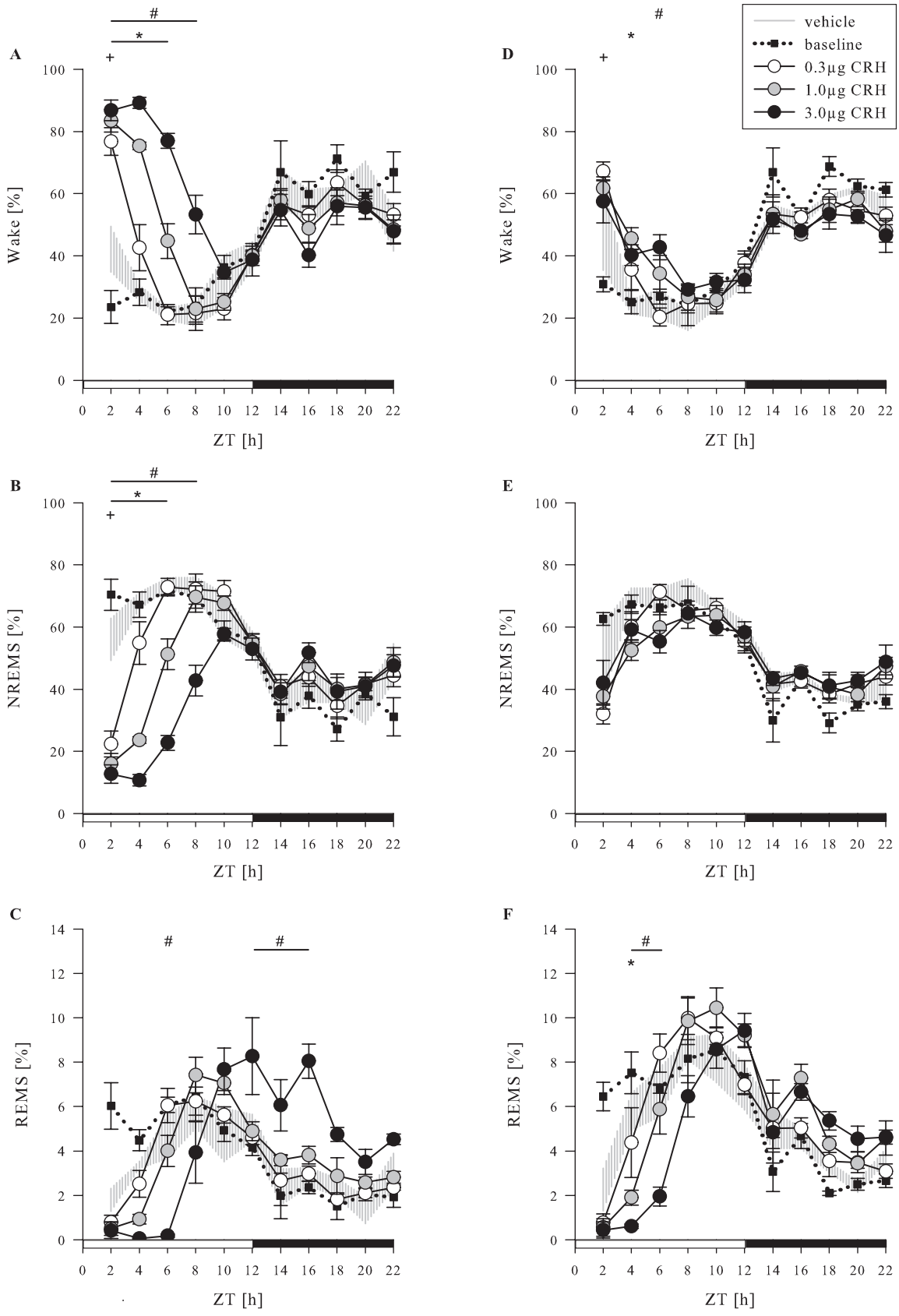

Figure 2—Effects of i.c.v. CRH injections on vigilance states in CL (A, B, C) and CRH-R1 CKO mice (D, E, F). CRH increased wake levels in CL mice in a dose-dependent fashion during the light period and decreased NREMS amounts at the same time. REMS reduction tended to occur also in a dose-dependent fashion, although only the highest dose of CRH induced significant reductions in comparison to vehicle treatment. In the conditional knockout animals, in contrast, wake promoting and NREMS reducing effects of CRH were almost totally blunted. However, dose-dependent REMS reduction could still be induced by CRH injections during the light period. For graph denotations please refer to Figure 1.

Table 1—Effects of i.c.v.-injected CRH on mean duration (min) of episodes of each vigilance state in C57BL/6J, CRH-R1 CL, and CRH-R1 CKO mice.

	WAKE			NREMS			REMS		
	C57	R1 CL	R1 CKO	C57	R1 CL	R1 CKO	C57	R1 CL	R1 CKO
vehicle	1.52 ± 0.13‡	1.20 ± 0.09	1.18 ± 0.09‡	2.53#+ ± 0.09‡	1.89*+ ± 0.09‡	1.57*# ± 0.10‡	0.61#+ ± 0.06‡	0.31* ± 0.03‡	0.31* ± 0.02‡
	12.53#+ ± 3.93‡	2.08* ± 0.20	2.12* ± 0.33‡	1.57#+ ± 0.10‡	1.18*+ ± 0.20‡	0.93*# ± 0.08‡	0.28 ± 0.02‡	0.20 ± 0.02‡	0.21 ± 0.08‡
0.3µg CRH	3.39 ± 1.21	2.34 ± 0.66	1.58 ± 0.16	2.04+ ± 0.10‡	1.79+ ± 0.18‡	1.40*# ± 0.12‡	0.52#+ ± 0.05‡	0.29* ± 0.04‡	0.31* ± 0.03‡
	4.58#+ ± 0.79	2.29* ± 0.43	1.76* ± 0.19	1.54+ ± 0.06‡	1.24 ± 0.11‡	0.93* ± 0.07‡	0.28 ± 0.02‡	0.20 ± 0.02‡	0.24 ± 0.02‡
1.0µg CRH	9.87#+ ± 2.49	3.92* ± 0.98	1.48* ± 0.17	1.82#+ ± 0.09	1.47* ± 0.17	1.41* ± 0.16‡	0.38#+ ± 0.03	0.25* ± 0.02	0.30* ± 0.04
	4.15 ± 0.51	1.99 ± 0.23	1.58 ± 0.11	1.61#+ ± 0.08	1.22*+ ± 0.10	0.88*# ± 0.08‡	0.35#+ ± 0.03	0.22* ± 0.02	0.24* ± 0.02
3.0µg CRH	17.42#+ ± 4.90‡	7.44* ± 2.66‡	1.72* ± 0.34	1.46 ± 0.20	1.13 ± 0.13	1.55 ± 0.19‡	0.26 ± 0.04	0.22 ± 0.03	0.26 ± 0.03
	3.19 ± 0.54‡	1.95 ± 0.23‡	1.78 ± 0.28	1.49+ ± 0.09	1.20 ± 0.09	0.95* ± 0.09‡	0.31 ± 0.02	0.25 ± 0.02	0.24 ± 0.02
P value	≤ 0.001	≤ 0.006		≤ 0.025	≤ 0.001		≤ 0.006	≤ 0.014	≤ 0.035

The table shows 12-h means (light and dark boxes indicate the light or dark period) of average bout lengths in minutes (± SEM) of wake, NREMS, and REMS for all treatments and all mouse lines. Significant effects of treatment are denoted by P-values (least significance level given) in the bottom line of the table. The symbol ‡ as appendix to the mean episode duration of a group points to significant differences between the light and dark period for that group (tests with contrasts, $P < 0.05$), whereas symbols *, #, and + denote significant differences between the actual group and C57BL/6J, or CRH-R1 CL, or CRH-R1 CKO, respectively (Student-Neumann-Keuls tests, $P < 0.05$).

to baseline levels in both CL littermates and CRH-R1 CKO animals (Figure 2B and E). No further vehicle effect could be observed for the rest of the recording session in either of the 2 animal lines. As similarly seen in C57BL/6J, CRH in CL elicited profound and significant dose-dependent reductions in NREMS (Figure 2B). Whereas reduced levels returned to baseline by 2 hours after injecting 0.3 µg of CRH (contrasts tests, $P < 0.001$), 1.0 µg, and 3.0 µg of CRH prolonged this time effect to 6 (contrasts tests, $P \leq 0.005$) and 8 h (contrasts tests, $P < 0.001$). In CRH-R1 CKO animals, all CRH treatments failed to induce significant decreases in NREMS in comparison to vehicle control (Figure 2E). During the dark period, as seen in wake responses, no treatment effects on NREMS levels could be detected in both genotypes.

Similar to wake, significant interaction effects between treatment and time factors on REMS were shown during the light period of both CRH-R1 CKO and CL mice (CRH-R1: $F_{treatment \times time}(20/220) = 10.255$, $P < 0.001$; CL: $F_{treatment \times time}(20/160) = 7.562$, $P < 0.001$). Vehicle injection itself decreased REMS significantly only at ZT 2 in both genotypes (contrasts tests, $P = 0.002$) but induced no further changes (Figure 2C and F). The clear responses of REMS to CRH injections were blurred in both CRH-R1 CL and CKO mice compared with those in C57BL/6J. Although REMS levels decreased in a dose-dependent fashion and were almost totally blunted after 3.0 µg of CRH (ZT 4 and 6; Figure 2C) in CRH CL, these decreases reached significant levels in comparison to vehicle levels at the highest dose and ZT 6 only (contrasts tests, $P \leq 0.002$). Reduced REMS started returning to baseline values after 2, 4, and 6 h respectively according to the applied dose of CRH, and remained comparable to vehicle and baseline levels except for the case after treatment with 3.0 µg of CRH. After this highest dose of CRH, REMS levels significantly increased from the end of the light period (ZT 12) to the beginning of the dark period (ZT 14 and 16) in comparison to the control conditions (contrasts tests, $P \leq 0.018$; Figure 2C). In CRH R1 CKO animals, the dose-dependent effects of CRH on REMS were also evident (Figure 2F). In a dose-dependent fashion, REMS levels were significantly reduced at ZT 4 and returned to vehicle and baseline levels by ZT 6 (1.0 µg CRH) and ZT 8 (3.0 µg CRH) respectively (contrasts tests, $P \leq 0.011$). Contrary to their CL, CRH-R1 CKO animals displayed no further significant CRH effects on REMS levels during the dark period.

CRH-Induced Changes in Sleep Architecture

We further analyzed the effects of CRH on sleep to investigate whether CRH affected more episode frequency or duration in sleep architecture. As shown in Figure 1 and Table 1, C57BL/6J mice displayed sleep-wake distributions typical of nocturnal animals. ANOVA with treatment, phases and group as influential factors showed that during the resting phase (light period) the mean duration of wake episodes was significantly shorter than during the active phase ($P < 0.001$; Table 1). However, the higher the dose of CRH injected, the longer a wake bout became during the inactive phase. After the lowest dose of CRH (0.3 µg), bout lengths during the light and dark period equalized. With 1.0 µg of CRH, mean wake bout length during the light period already exceeded bout lengths of the dark period. Only at the highest dose of CRH, the increase in the mean time spent awake per bout significantly differed from those during the dark period ($P < 0.001$). After this dosage, the nocturnal rhythmicity appeared to be inverted, opposing total wake levels seen after vehicle treatment, and the mean wake bout length during the light period was greater than that after the treatment of either vehicle or lower doses of CRH (0.3 µg, $P \leq 0.001$; 1.0 µg, $P = 0.052$). Therefore, arousal effects of CRH contributed to prolonging each episode length but not the frequency of entry to wake episodes. Within the dark period, the magnitude of decrease in wake bout length depended on the applied dose of CRH and developed into a trend.

As with wake, vehicle treatment had no effect on the length of NREMS or REMS episodes if compared to baseline. For both

NREMS and REMS, episode length was significantly greater in the resting than in the active period of the animals (contrasts tests, $P < 0.001$). However, application of CRH caused a dose-dependent decrease in NREMS and REMS episodes during the light period. At the lowest dose of CRH the mean NREMS or REMS bout during the resting period remained significantly longer than that in the active period of the animals (contrasts tests, $P \leq 0.001$). However, this difference disappeared after the 2 higher doses of CRH. Within the light period the decrease in length of NREMS and REMS bouts was dependent on the applied dose of CRH (contrasts tests, $P \leq 0.025$). The effects of CRH on sleep were due to shortened bout durations and reduced episode numbers (data not shown). During the course of the dark period, CRH seemed to affect neither NREMS nor REMS bout duration.

In CRH-R1 CKO and CL, some differences in sleep architecture were observed in comparison with C57BL/6J mice under baseline and vehicle conditions (Table 1). During the light period, NREMS and REMS bouts were significantly longer in C57BL/6J mice than in CRH-R1 CKO or CL animals (Student-Neumann-Keuls test, $P < 0.001$). During the dark period, however, wake and NREMS bouts displayed significantly greater durations in C57BL/6J as compared to those of CRH-R1 CKO or CL animals (Student-Neumann-Keuls test, $P \leq 0.005$). CRH-R1 CL as well as CKO mice consistently displayed longer wake bouts and shorter NREMS and REMS bouts during their active phase than during their resting phase under baseline and vehicle conditions, which reached significant levels except for wake in CRH-R1 CL animals (contrasts tests, $P \leq 0.016$; Table 1). Furthermore, compared with CRH-R1 CL, CRH-R1 CKO animals presented significantly shorter NREMS bout length during the light and dark period under both baseline and control conditions (Student-Neumann-Keuls test, $P \leq 0.033$).

In CRH-R1 CL animals, vehicle treatment did not affect wake and NREMS bout length, but slightly reduced REMS bout length during the inactive phase of the animals (contrasts tests, $P < 0.001$; data not shown) if compared to baseline levels, whereas, no differences in each bout length between baseline and vehicle treatment were detected in CRH-R1 CKO animals. Following CRH treatment, wake bout length in CRH-R1 CL mice was increased dose-dependently from 1.20 ± 0.09 to a maximum of 7.44 ± 2.66 min (as similarly seen in C57BL/6J mice), albeit only the highest dose entailed significant changes compared to all other treatments (contrasts tests, $P \leq 0.006$). Already at the lowest dose of CRH, wake bout lengths were equalized over the day. Toward the dark period, CRH treatment did not have any significant impact on wake bout lengths. However, in CRH-R1 CKO animals no differences in wake bout lengths were to be found during the light period after any dose of CRH, although mean wake episode length during the light period became closer to those during the dark period. As for NREMS and REMS, CRH injections also influenced their bout duration in CRH-R1 CL animals. With increasing amounts of injected CRH, NREMS and REMS episodes became shorter during the light period. After 1.0 µg and 3.0 µg of CRH, NREMS and REMS durations were significantly decreased (contrasts tests, $P \leq 0.004$), resulting in disappearance of circadian differences in NREMS and REMS bouts between the light and dark period. During the dark period CRH treatments did not induce significant changes in bout length. In CRH-R1 CKO animals, CRH treatment had no influence on NREMS bout length at all. Meanwhile, REMS episodes were exiguously shortened with increasing doses of CRH, but only after the highest dose this reduction reached a significant level (contrasts tests, $P = 0.035$). Effects of i.c.v. injected CRH on sleep architecture appeared to a lesser extent in CRH-R1 CKO than in CL mice.

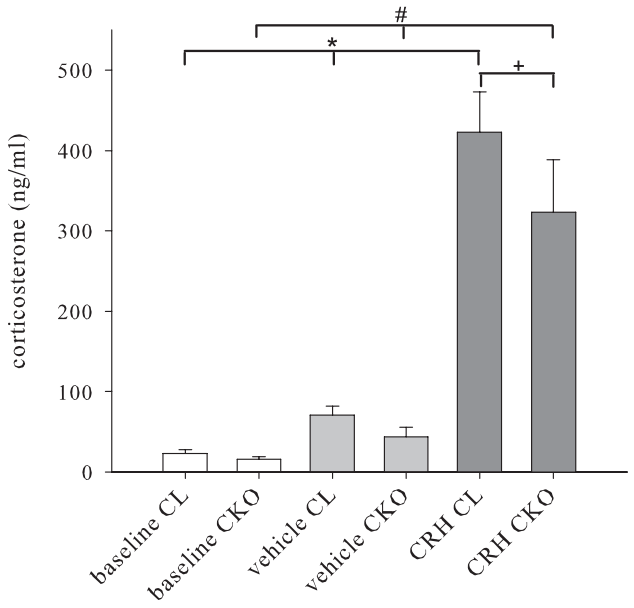

Figure 3—Effects of i.c.v. CRH injections on CORT levels in CL and CRH-R1 CKO mice. Compared to baseline and vehicle conditions a significant increase in plasma CORT was induced by 1.0 µg of CRH by 2 h after application in both CL (n = 8) and CRH-R1 CKO (n = 8) mice, although CL animals displayed significantly higher CORT levels after neuropeptide treatment compared to CKO animals. Symbols indicate significant differences in obtained CORT values with respect to different treatment conditions and their according genotypes (tests with contrasts in ANOVA, $P < 0.05$). For graph denotations please refer to Figure 1.

Changes in Plasma CORT Levels after CRH Injection in CRH-R1 CKO and CL Mice

Under baseline conditions at ZT 2 (Figure 3) both genotypes showed similar levels of plasma corticosterone (CORT; CL: 22.7 ± 5.2 ng/mL; CRH-R1 CKO: 16.2 ± 3.0 ng/mL). Two h after vehicle treatment (ZT 2), an increase in plasma CORT levels was detected in both mouse lines (CL: 70.5 ± 11.8 ng/mL; CRH-R1 CKO: 43.7 ± 11.8 ng/mL). However, differences in these levels were insignificant, neither within nor between the 2 genotypes. On the other hand, treatment with 1.0 µg of CRH distinctly increased plasma CORT in CL (423.2 ± 49.5 ng/mL) after two hours in comparison to their vehicle and baseline values ($F_{treatment} = 44.57$, $P < 0.001$; $F_{group} = 4.768$, $P = 0.034$; contrasts tests, $P < 0.001$). A similar effect could be seen in CRH-R1 CKO mice, where CRH treatment significantly increased plasma CORT levels (323.2 ± 65.8 ng/ml) 2 h after injection as well (contrasts tests, $P < 0.001$). Although increased levels of plasma CORT were lower in CRH-R1 CKO mice than in CL mice (Student-Neumann-Keuls test, $P = 0.007$), i.c.v. injected CRH was able to stimulate the HPA axis even with a deficiency of central CRH-R1.

DISCUSSION

The results demonstrated that i.c.v. injected CRH immediately promotes wakefulness and in turn suppresses sleep, namely both NREMS and REMS, in C57BL/6J mice. The effects of CRH on their sleep-wake patterns were clearly dose-dependent and also evident when CRH was injected into CRH-R1 CL mice. In both control mouse strains, wake-promoting effects of CRH were characterized by prolonging the episode duration but not increasing the episode occurrence. However, responses to CRH in CRH-R1 CKO mice were quite different from the other mouse lines. Acute effects of i.c.v. injected CRH that were supposed to increase waking and decrease NREMS almost disappeared, suggesting that wake-promoting/NREMS-suppressing effects of CRH are mediated by central CRH-R1. Since CRH effects on REMS still partly remained in CRH-R1 CKO mice, CRH-R1 is not the sole mediator of REMS suppression. Despite acute effects of CRH seen in the light period, only C57BL/6J mice responded to CRH for a longer period, resulting in increases in NREMS/REMS and suppression of wakefulness during the dark period. However, changes observed in the dark period would not be elicited by a direct action of CRH injected 12 h before. Those changes were shown also in a dose-dependent fashion, therefore loss of sleep produced in the light period might have caused the rebound of sleep debt. Such rebound responses were not significant in CRH-R1 CL mice. The mechanism accounting for different CRH effects between these 2 control mouse strains remains unclear. Slight differences seen in their basal levels of sleep and wakefulness could be one reason and may be caused by their different genetic backgrounds.

Genetic background and its influence on the homeostatic regulation of sleep and the sleep EEG itself have been the topic of many studies extensively discussed for the last decade.[20,21] Different inbred mouse strains are reported to display marked variations in sleep-wake distribution, mean duration of vigilance episodes, slow wave activity (SWA), and EEG power density under baseline, as well as under certain experimental conditions.[22-24] In our studies, dissimilarities in sleep-wake behavior of the 2 investigated mouse lines were also evaluated. C57BL/6J mice showed greater mean durations for wake, NREMS and REMS in comparison to those in CRH-R1 CL and CKO mice, and consequently sleep in our knockout mouse model was more fragmented. During the dark period, C57BL/6J mice displayed higher wake and lower NREMS levels than the knockout model; whereas REMS levels were comparable between C57BL/6J and CRH-R1 CL mice, although slightly elevated in CRH-R1 CKOs. Light-dark differences in time spent in wake and NREMS were most prominent in C57BL/6J mice. On the other hand, the baseline sleep profile of the CRH-R1 CKO/CL mouse line resembles more the one of 129/Ola mice described in literature than that of C57BL/6J mice as reported previously by Huber et al.[25] Despite the close inheritance to homozygous C57BL/6J mice, mixed genetic lineage with 129/Ola mice could contribute to differences in sleep architecture of CRH-R1 CKO/CL mice from that of C57BL/6J mice, and possibly influence the appearance of CRH effects in general.

However, differences in sleep responses to exogenous CRH between CRH-R1 CL and CKO animals are attributed to the lack of central CRH-R1 in the knockout mice. During baseline recordings and under vehicle conditions, slight but significant differences were found in the mean duration of NREMS and the total amount of REMS. Compared with their CL, CRH-R1 CKO mice demonstrated shorter NREMS episodes, but increased REMS due to more frequent episode entry. Although earlier studies reported a possible role of CRH in spontaneous wake control, neither the amount of wakefulness, nor its architecture significantly changed in the model of central CRH-R1 deficiency. Presently, there is no apparent explanation for sleep phenotypes of our CRH-R1 CKO mice; however, slight changes in their sleep architecture could be indirectly acquired as a result in order to postnatally compensate a lack of CRH signaling via CRH-R1.

In our study, C57BL/6J mice were employed to determine a dose-dependent impact of CRH on sleep-wake regulation, and as such, should serve as a comparison whether a similar dose-response relationship would appear in the case of central CRH-R1 deficiency. Therefore, CRH doses for this study were carefully selected to elicit clear behavioral and neuroendocrine effects.[4] CRH exerts its regulatory mechanism through a central neurotransmitter-like pathway either alone, or in combination with its activating effect upon the HPA system. To clarify which pathway(s) would participate in sleep-wake modulation, we tested relatively high CRH doses and mimicked a situation how stress-provoked excess secretion of CRH affects sleep in central CRH-R1 knockout mice. A recent study conducted by Sanford et al. compared effects of i.c.v. injected CRH on sleep-wake behavior in several mouse strains, including C57BL/6J, endowed with differential responsiveness to stress.[26] Although the dose range used in that study was presumably too low as to stimulate HPA-axis activity, the authors demonstrated that CRH caused only at the highest dose (0.4 μg) significant increases in wake and decreases in NREMS and REMS. As indicated above, we chose a low (non stress-inducing) dose of CRH (0.3 μg), and 2 higher doses (1.0 and 3.0 μg) that were expected to reliably evoke HPA-axis activity. One would speculate that the latter doses we used are too high, as they may increase HPA axis activity up to a non-physiological level. However, it was necessary to examine how CRH-R1 CKO animals would respond to the higher doses, while in intact animals the HPA axis hormone release could be fully stimulated. In CRH-R1 CKO animals, we failed to observe effects of CRH on wake and NREMS, although plasma CORT levels were increased. Thus, we propose that CRH effects on NREMS suppression were mediated centrally through CRH-R1, which is not counteracted by CRH-elicited peripheral effects that via CORT may act back on sleep regulation by activating central corticosteroid receptors. Our findings suggest that the wake-promoting action of CRH is at least to a certain extent dissociated from its action on the HPA system.

The main purpose of this study was to assess whether CRH directly modulates sleep-wake behavior in mice through the CRH receptor type 1, or if its impact on sleep is rather mediated as a secondary side effect of HPA axis components and arousal. CRH has long been implicated to be a mediator of arousal, or spontaneous wakefulness as mentioned above. Furthermore, it could be shown in rats that i.c.v. injections of low amounts of CRH dose-dependently affect EEG activity (e.g., EEG spectral power), as they increase brain excitability and alertness.[27] It is known that many parts of the brain contrib-

ute to sleep-wake regulation such as the basal forebrain, the hypothalamus, the thalamus and the brainstem.[28,29] The arousal systems are implicated also in different behavioral and emotional states and regulate them in secreting different excitatory and inhibitory neurotransmitters. Whereas the brainstem reticular formation (glutamatergic neurons) seems to be essential for the maintenance of arousal and cortical activation in general,[30] other brainstem arousal systems like the locus coeruleus (LC; noradrenergic neurons) have been shown to discharge maximally during aroused conditions such as stress by local release of CRH.[31,32] The raphe nuclei (RN; serotonergic neurons) are linked to arousal processes associated with positive emotions and rhythmic movements such as grooming,[29,33] and the pontomesencephalic areas, such as the laterodorsal tegmental and the pedunculopontine tegmental nuclei (LDT and PPT; cholinergic neurons) are active during behaviorally quite wake periods. In all of the above mentioned areas, CRH receptor-like immunoreactivity has been detected (predominantly CRH-R1) to a more or less strong extent,[8,34] and many of the wake-promoting or arousal-stimulating systems contain neurons expressing CRH themselves, notably the lateral hypothalamus (LH), the LDT and the LC.[35,36] Thus it seems likely that CRH is in a position to participate in sleep-wake control not only during stress, but also within its diurnal rhythm under non-stressful conditions, although our current data cannot directly support this possibility. Particular brain regions expressing CRH-R1 may be more crucial for affecting arousal than other regions. Such spatial specificities regarding CRH effects and CRH-R1 function should be further considered in future studies.

In contrast to wake and NREMS, a dose-dependent suppression of REMS was still observed in all animals including CRH-R1 CKO, pointing to a potential role of CRH-R2 in these animals, and its involvement in suppression of REMS. However, it is noteworthy that the expression of CRH-R2 was not altered in the brain of conditional CRH-R1 knockout animals compared to control littermates (data not shown) similar to conventional CRH-R1 knockout animals.[37] Regarding a mechanism of REMS regulation, Lu et al. proposed a putative flip-flop switch model consisting of REM-off and REM-on neurons, which enables the brain to perform sharp switches from other vigilance states to REMS and vice versa.[38] These neurons presumably inhibit each other's activity reciprocally via GABAergic projections, which could be influenced by several other brain areas or nuclei. The orexin (hypocretin) system, the serotonergic dorsal raphe nucleus (DRN), and the noradrenergic LC probably excite REM-off cells, whereas inhibitory input to REM-off cells derives from the GABAergic extended part of the ventrolateral preoptic nucleus, the GABAergic sublaterodorsal nucleus and the cholinergic PPT and LDT. In most of these areas either CRH-R1 (PPT, LDT, LC, and orexinergic neurons) or CRH-R2 (DRN and orexinergic neurons) is expressed.[8,34,39] Therefore an influence of CRH on REMS regulation could be mediated by both receptor types. Apart from that, REMS regulation could be affected by another pathway rather than central CRH receptors. Since i.c.v. injected CRH increased circulating CORT concentrations even with central CRH-R1 deficiency, the activation of the HPA axis through peripheral CRH-R1 might have also contributed to REMS alteration in this experimental condition. Elevated plasma CORT possibly affects CNS function including sleep via glucocorticoids and/or mineralcorticoid receptors along the neuroendocrine negative feedback loop.[40]

It is noteworthy that in CRH-R1 CKO mice centrally administered CRH can enhance HPA activity. Thus, it is likely that elevated peripheral hormones were responsible for the perpetual (or "sustained") REMS suppression. Therefore, the role of central CRH-R1 regarding REMS regulation still remains partially obscure. As reported previously CRH-R1 receptors in the pituitary and in the periphery are still intact in CRH-R1 CKO animals, thus centrally injected CRH most probably triggered this HPA-axis response. So far, it has been shown that i.c.v. injected CRH is actively transported out of the brain across the blood-brain barrier and capable of reaching peripheral sites of action.[41] Recently, we demonstrated that conditional CRH-overexpressing (CRH-COE) mice that present an undisturbed HPA system characteristically show elevated REM sleep, which can be normalized with CRH-R1 antagonism suggesting that regional-specific overexpression of CRH as reported previously accounts for REM sleep enhancement.[15] In contrast, extensive studies by Sanford's group have shown first REMS suppression by CRH, but recently bi-directional REMS responses to CRH that are mediated through CRH-R1 located in the central nucleus of the amygdala.[42-44] Indeed, REMS is a very fragile vigilance state, therefore depending on the experimental design the CRH system could contribute to either case triggering increases or reductions in REMS. Nevertheless, in the CRH-R1 CKO animals even an elicited HPA activity did not accompany pronounced increases in wakefulness or decreases in NREMS. Consequently a functional HPA axis does not seem to be essential for a CRH-mediated wake and NREMS regulation, but might have resulted in REMS suppression.

In summary, our study using CNS-specific CRH-R1 knockout mice demonstrates that CRH is able to centrally modulate sleep-wake regulation via CRH-R1. Although a possible role of central CRH-R2 in sleep-wake control has to be considered, our results suggest that central CRH-R1 plays a dominant role regarding effects on arousal. Enhanced HPA-axis activity is not a prerequisite for CRH exerting NREMS suppression, whereas REMS suppression might be influenced by increased stress hormone release.

ABBREVIATIONS

CRH, corticotropin-releasing hormone
ACTH, adrenocorticotropic hormone
HPA, hypothalamic-pituitary-adrenocortical
CNS, central nervous system
CRH-R1, CRH receptor type 1
CRH-R2, CRH receptor type 2
CKO, conditional knockout
CL, control littermate
EEG, electroencephalogram
EMG, electromyogram
i.c.v., intracerebroventricular
NREMS, non-rapid eye movement sleep
REMS, rapid eye movement sleep
CORT, corticosterone
RIA, radioimmunoassay
ZT, zeitgeber time
SWA, slow wave activity

ACKNOWLEDGMENTS

We would like to thank Sabrina Meyr and Stefanie Unkmeir for excellent technical assistance and Dr. Alexander Yassouridis for statistical expertise. This work was partially supported by the Bundesministerium für Bildung und Forschung within the framework of NGFN-Plus (FKZ: 01GS08155).

DISCLOSURE STATEMENT

This was not an industry supported study. The authors have indicated no financial conflict of interest.

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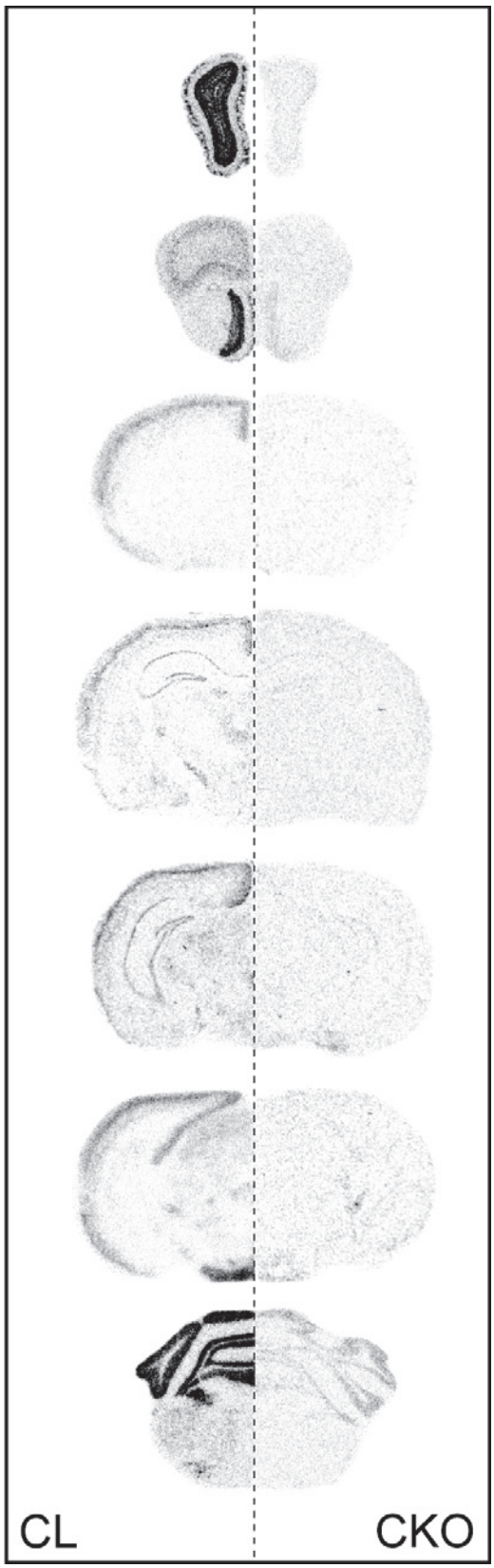

Supplementary Figure 1—CNS-restricted deletion of CRH-R1 expression in *Crhr1*$^{loxP/loxP}$ *nestin-cre* animals. Autoradiographs of a CRH-R1-specific in situ hybridization of coronal brain sections from rostral (top) to caudal (bottom) demonstrating the spatial expression pattern of CRH-R1 throughout the brain of *Crhr1*$^{loxP/loxP}$ control animals (CL, left). In the brain of *Crhr1*$^{loxP/loxP}$ *nestin-cre* animals (CKO, right), no CRH-R1 expression is detectable.

SUPPLEMENTARY METHODS

In situ hybridization

10-week-old mice were sacrificed in the morning (10:00) by an overdose of isoflurane. Brains were carefully removed and immediately shock frozen on dry ice. Frozen brains were cut on a cryostat in 20 µm-thick sections. For in situ hybridization cryostat sections of CL and CKO brains were mounted side by side on SuperFrost Plus slides (Menzel GmbH, Braunschweig, Germany). This procedure allowed for parallel *in situ* hybridization of sections under identical conditions assuring meaningful quantification and comparison of hybridization signals. All sections were processed for *in situ* hybridization as previously described (Deussing et al., 2007). For CRH-R1 a riboprobe covering nucleotides 969-1363 of GenBank accession no. NM_007762 was used. The riboprobes were generated by PCR, labeled, and hybridized as previously described (Deussing et al., 2007).

REFERENCE

1. Deussing JM, Kuhne C, Putz B, et al. Expression profiling identifies the RH/CRH-R1 system as a modulator of neurovascular gene activity. J Cereb Blood Flow Metab. 2007;8:1476-95