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**Hypothalamic prepro-orexin mRNA level is inversely correlated to the non-rapid
eye movement sleep level in high-fat diet-induced obese mice**

Running head: Hypothalamic prepro-orexin mRNA level and sleep

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Abstract

Orexins are hypothalamic neuropeptides, which play important roles in the regulation and maintenance of sleep/wakefulness states and energy homeostasis. To evaluate whether alterations in orexin system is associated with the sleep/wakefulness abnormalities observed in obesity, we examined the mRNA expression of *prepro-orexin*, *orexin receptor type 1*, (*orexin 1r*), and *orexin receptor type 2* (*orexin 2r*) in the hypothalamus in mice fed with a normal diet (ND) and high-fat diet (HFD)-induced obese mice. We also compared their relationships with sleep/wakefulness. Twenty-four, 4-week-old, male C57BL/6J mice were divided randomly into 3 groups, which received the following: (1) ND for 17 weeks; (2) HFD for 17 weeks; and (3) ND for 7 weeks and HFD for a further 10 weeks. The body weights of mice fed the HFD for 10–17 weeks were 112–150% of the average body weight of the ND group. The daily amount of non-rapid eye movement (NREM) sleep increased significantly in HFD-fed mice. These changes were accompanied by increases in the number but decreases in the duration of each NREM sleep episode. In addition, brief awakenings (<20 s epoch) during NREM sleep was nearly 2-fold more frequent. The mRNA level of *prepro-orexin* in the hypothalamus was significantly reduced in HFD-induced obese

mice, whereas the levels of *orexin 1r* and *orexin 2r* were unaffected. The daily amount of NREM sleep was negatively correlated with the hypothalamic *prepro-orexin* mRNA level, so these results suggest that the increased NREM sleep levels in HFD-induced obese mice are attributable to impaired orexin activity.

Key words: obesity, high-fat diet, orexin, non-rapid eye movement sleep

Introduction

Orexins (hypocretins) are a pair of neuropeptides derived from the precursor prepro-orexin and they are expressed by neurons in the perifornical nucleus, as well as in the adjacent lateral hypothalamic areas [1, 2]. Orexin fibers project into nuclei involved in the regulation of many important physiological functions including body temperature regulation, feeding, and the sleep/wakefulness cycle [3]. There are 2 types of orexin receptors, i.e., orexin receptor type 1 (orexin 1r) and orexin receptor type 2 (orexin 2r), which have segregated mRNA expression in the brain [4]. Lack of orexin signaling is associated with narcolepsy, which is characterized by excessive daytime sleepiness, sleep fragmentation, and increased body mass index in humans [5]. Narcoleptic symptoms are also evident in mice if orexins are ablated genetically [6]. Similarly, a decreased level of orexin is found in genetically obese mice [7, 8]. These mice are known to have sleep/wakefulness disorders such as increased non-rapid eye movement (NREM) sleep and sleep fragmentation [9-11]. These findings suggest that lower orexin levels may be related to the abnormal sleep/wakefulness patterns observed in genetically obese mice.

Exposure to a high-fat diet (HFD) may induce hyperphagia and a positive energy

balance, which leads to obesity. HFD is also known to affect postprandial sleepiness in humans [12]. Similarly, obese mice fed a HFD for 6 weeks experience increased NREM sleep and sleep fragmentation [13, 14], and their sleep/wakefulness phenotype is quite similar to that of genetically obese *ob/ob* [7] and *db/db* [8] mice. The hypothalamic orexin level is also affected in HFD-induced obesity model animals, although reports differ. For example, the orexin level show no change [15, 16] or a slight increase [17] after a HFD for 2–3 weeks, whereas the orexin level decrease after a HFD for 50 days [18]. In addition, the *orexin 1r* and *2r* mRNA levels are found to be elevated in obesity-resistant rats [19]. Overall, these results suggest that changes in the orexin levels and their receptor expression may be related to the sleep/wakefulness abnormalities observed in HFD-induced obese mice.

Therefore, to elucidate the role of the orexin system in sleep/wakefulness regulation in diet-induced obese animals, we examined the sleep/wakefulness parameters as well as the mRNA expression of *prepro-orexin*, *orexin 1r*, and *orexin 2r* in the hypothalamus in obesity-susceptible C57BL/6 mice that received a HFD for 10–17 weeks.

Materials and methods

Animals, diets, and HFD-induced obesity procedures

Twenty-four, 3-week-old, adult male C57BL/6J mice were purchased from CREA Japan (Hamamatsu, Japan). After a week of acclimation with a normal diet (ND), the mice were randomly divided into 3 groups (n = 8 per group) and provided with the following diets: (1) ND for 17 weeks (ND group); (2) HFD for 17 weeks (HFD 17 week group); and (3) ND for 7 weeks followed by HFD for 10 weeks (HFD 10 week group). The ND (CE-2; fat = 4.8 wt%, protein = 25.1 wt%, and carbohydrate = 4.2 wt%) and HFD (HFD-32; fat = 31.9 wt%, protein = 24.5 wt%, and carbohydrate = 7.1 wt%) were purchased from CREA Japan. The mice were housed in groups of 4 per cage until 18 weeks of age, and then subjected to surgery to allow sleep recording. The mice were housed individually for another 2 weeks before the sleep recordings. The mice were maintained in an AAALAC-approved animal facility at an ambient temperature of $22 \pm 2^{\circ}\text{C}$ with a 12 h light/dark cycle (lights on at 7:00 am) and they were provided with food and distilled water *ad libitum*. All animal experiments were carried out in accordance with the guidelines of the Animal Care and Use Committees of Hokkaido

University. All efforts were made to minimize the number of animals used and any pain or discomfort experienced by the subjects.

Surgery and polygraphic recording

Mice were anesthetized by intraperitoneal (i.p.) injection of a drug cocktail containing ketamine (75 mg/kg) and xylazine (10 mg/kg). The mice were chronically implanted with electroencephalograph (EEG) and electromyograph (EMG) electrodes for polysomnographic recording of their sleep/wakefulness states. In brief, for EEG monitoring, two stainless steel screws were implanted in the skull above the left cerebral hemisphere using the atlas of Franklin and Paxinos [20]. The EMG activity was monitored using stainless steel Teflon-coated wires, which were inserted bilaterally into the neck muscle. Mice were allowed to recover for 2 weeks before their transfer to the sleep recording chambers.

After recovering, the mice were housed in a plastic cage, which was placed in an insulated and soundproofed recording chamber. Mice were allowed 1 week to acclimatize to the recording environment. The EEG, EMG, and spontaneous locomotory activity were recorded for 24 h, beginning when the lights were switched on. The EEG and EMG signals

were amplified and filtered (EEG = 0.5–30 Hz; EMG = 16–128 Hz), digitized at a sampling rate of 128 Hz, and recorded using the SleepSign ver. 3 program (Kissei Comtec, Nagano, Japan). Spontaneous locomotory activity was monitored using an infrared sensor (Biotex, Kyoto, Japan), which was mounted on top of the cage.

Vigilance state determination

Vigilance states were classified automatically off-line using the SleepSign ver. 3 program based on the EEG, EMG, and spontaneous locomotory activity in every 10 seconds (1 epoch). The defined vigilance stages were examined visually and corrected if necessary. Brief awakenings were short wakefulness episodes that occurred during sustained NREM sleep [21]. In this study, we defined a brief awakening as a period of wakefulness lasting less than 2 epochs (<20 s). Using this scoring system, we determined the amount of NREM sleep, REM sleep, wakefulness, and the number of brief awakenings during a 24-h period. The average number and duration of the episodes for each vigilance state were calculated every hour during the 24 h period, and these data were averaged.

Gene expression measurements

After the sleep recordings, all animals were weighed and sacrificed by cervical dislocation. Their brains were removed immediately, and the hypothalamus was dissected. Tissue samples were stored at -30°C until RNA extraction. Total RNA was isolated from the hypothalamus using the guanidine–isothiocyanate method with RNAiso reagent (Takara Bio, Shiga, Japan). Final RNA concentrations were determined spectrophotometrically. The *prepro-orexin*, *orexin 1r*, and *orexin 2r* mRNA levels were measured quantitatively by real-time RT-PCR using the appropriate cDNA fragment as a standard and their levels were expressed relative to the *β -actin* mRNA level. In brief, 2 μg of total RNA was reverse transcribed using an oligo (dT) 15-adaptor primer and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). Real-time quantitative PCR was performed in a fluorescence thermal cycler (LightCycler system; Roche Diagnostics, Mannheim, Germany) using SYBR Green I (Roche Diagnostics). The primers used were as follows: 5'-GGC ACC ATG AAC TTT CCT TC-3' and 5'-CGT GCA ACA GTT CGT AGA GA-3' for mouse *prepro-orexin*, 5'-CAC TGG CTA GTG TAC GCC AA-3' and 5'-GAC TTG TGT CTG GCA GAG GA-3' for mouse *orexin 1r*, 5'-GCT CAT GGT TGT ACT TCT GG-3' and 5'-GTC TTC

CGT GTG TGT GAA CA-3' for mouse *orexin 2r*, 5'-TCG TAC CAC AGG CAT TGT GAT-3'

and 5'-TGC TCG AAG TCT AGA GCA AC-3' for mouse *β -actin*.

Statistical analysis

All data were expressed as the mean $\pm$ SE. One-way analysis of variance (ANOVA) followed by a post hoc Scheffe's F-test was used to compare the sleep/wakefulness parameters among groups. p values <0.05 were considered statistically significant. Pearson's correlation coefficient was used to evaluate the correlations between 3 pairs of the following variables: the sleep/wakefulness parameters, body weight, and *prepro-orexin* mRNA level. The Pearson's correlation coefficients (r) and p values were calculated using data from 24 mice. The significance level was $p < 0.05/3 = 0.017$. The result of a single test was only considered significant if its p value was less than the Bonferroni-adjusted level.

Results

Effects of HFD on body weight and sleep/wakefulness parameters

The body weight of 16 mice fed the HFD were 112–150% of the average body weights of the ND group. As shown in Table 1, the average body weight increased significantly in the HFD 10 week and 17 week groups, i.e., they were 127% and 136% relative to the ND-fed group, respectively.

The effects of the HFD on sleep/wakefulness parameters are also summarized in Table 1. The average daily amount of NREM sleep increased significantly in the HFD 10 week and 17 week groups, i.e., they were 109% and 114% relative to the ND-fed group, respectively. By contrast, the HFD 10 week and 17 week groups had significantly lower average daily wakefulness levels, i.e., they were 93% and 91% relative to the ND-fed group, respectively. These changes in NREM sleep and wakefulness were accompanied by an increased number of episodes, but each episode had a decreased duration. HFD 10 week and 17 week groups had significantly higher average daily brief awakening counts, i.e., they were 188% and 160% relative to the ND-fed group, respectively. The REM sleep parameters were not significantly altered.

Effects of HFD on prepro-orexin, orexin 1r, and orexin 2r mRNA levels

As shown in Fig. 1A, the average hypothalamic *prepro-orexin* mRNA level in the mice groups fed with the HFD for 10 weeks was significantly reduced, i.e., to 55% of that of the ND-fed group. The mice groups fed with HFD for 17 weeks also showed a reducing trend ($p = 0.09$). By contrast, the mRNA levels of *orexin 1r* (Fig. 1B) and *orexin 2r* (Fig. 1C) in the hypothalamus were not affected by the HFD.

Correlations among sleep/wakefulness parameters, body weight, and the prepro-orexin mRNA level

As shown in Fig. 2A, the regression analysis detected a negative correlation between body weight and the hypothalamic *prepro-orexin* mRNA level. As shown in Fig. 2B, there was a positive correlation between body weight and the daily NREM sleep amount. A positive correlation was also found between body weight and the daily brief awakening counts ($p < 0.006$, $r = 0.545$). Body weight was negatively correlated with the daily wakefulness amount ($p < 0.001$, $r = -0.665$) and the average wakefulness duration ($p < 0.001$, $r = -0.654$). As shown in Fig. 2C, there was a negative correlation between the hypothalamic

prepro-orexin mRNA level and the daily NREM sleep amount. A positive correlation was found between the hypothalamic *prepro-orexin* mRNA level and the daily wakefulness amount ($p < 0.001$, $r = 0.709$).

Discussion

In this study, we found that hypothalamic *prepro-orexin* mRNA level, but not that of *orexin 1r* and *2r*, were significantly decreased in mice fed with HFD. Regression analysis revealed that the mRNA level of *prepro-orexin* was negatively correlated with body weight. In good agreement with our results, the hypothalamic *prepro-orexin* mRNA was reported to decrease in mice fed a HFD for 50 days [18]. These results suggest that decrease in mRNA of *prepro-orexin* occurs according to weight gain but this change is independent from that of *orexin receptors* in the hypothalamus. At present, we cannot be certain whether this decrease was due to a reduction in the mRNA copy number per neuron or the number of neurons that produced *prepro-orexin* mRNA. The increased amount of sleep and sleep fragmentation induced by weight gain can be reversed by weight loss [13], which suggests that the effects of HFD on sleep involve neural plasticity. Thus, it is likely that the decrease in hypothalamic

prepro-orexin mRNA levels observed in HFD-induced obese mice may reflect the impairment of orexin neuronal activities rather than a reduced number of orexin neurons.

Our group and another group [18] have shown that the orexin levels are lower in mice fed a HFD for 7–10 weeks, but it was interesting to note that the orexin level was not lower during the early phase of HFD-induced obesity in mice fed a HFD for 2–3 weeks. Indeed, the *prepro-orexin* mRNA level is shown to be increased in the perifornical region and not the lateral region of the hypothalamus of mice given HFD for 3 weeks [17]. Moreover, there are reports showing no change in *prepro-orexin* mRNA [15] and orexin A peptide [16] in the whole hypothalami of mice fed a HFD for 2–3 weeks. Taken together, the hypothalamic orexin response may have depended on the HFD-induced obesity phase. The factors responsible for altering orexin levels in an obese state are not fully understood. Wortley *et al.* have shown that parallel increase in the *prepro-orexin* mRNA expression and blood triglycerides levels after 3 weeks of a HFD. They also have found that i.p. injection with intralipid increased the *prepro-orexin* mRNA level in the perifornical region of the hypothalamus along with increased circulating triglyceride level, which suggested that the blood triglycerides may be an important component of enhanced *prepro-orexin* mRNA expression [17]. However, by feeding HFD for 9 weeks, blood glucose level, but not

triglycerides level is shown to be increased [22]. It has been reported that most orexin neurons are glucose sensitive, while the activity of isolated orexin neurons is hyperpolarized and their firing frequency is decreased by a high glucose level [23]. Thus, it is suggested that the hyperglycemic state induced by HFD cause the decrease of *prepro-orexin* mRNA level directly or indirectly in the hypothalamus in our feeding conditions.

We have also found increased amount of sleep, especially NREM sleep, with reduced NREM sleep duration and increased NREM sleep episode number. Regression analysis revealed that the daily NREM sleep amount positively correlated with the body weight. Thus, it is suggested that these obese mice had an increased number of transitions between sleep/wakefulness states. The HFD-induced obese mice were also characterized by an increased incidence of brief awakening episode during NREM sleep. Brief awakenings may be used as an indicator of sleep quality because they are negatively correlated with the amount of slow wave activity, which is a behavioral measure of the sleep intensity [21, 24]. Overall, it is demonstrated that HFD-induced obese mice are behaviorally less stable and have lower sleep quality compared with normal mice. In general, our results agreed with earlier studies that evaluated the sleep/wakefulness profiles of HFD-induced obese C57BL/6 mice with a similar degree of weight gain [13, 14].

We found that the sleep/wakefulness profiles and hypothalamic *prepro-orexin* mRNA levels differed significantly between mice fed a HFD and ND. Regression analysis revealed that the daily amount of NREM sleep was negatively correlated with the hypothalamic *prepro-orexin* mRNA level. Deficits in the orexin system lead to narcolepsy, which is characterized by excessive daytime sleepiness and sleep fragmentation [5]. Orexin administration reduces sleep [25] whereas sleep is induced by a reversible antagonist of orexins 1r and 2r that is currently in clinical development for the treatment of insomnia [26]. Acute inhibition of orexin neurons via optogenetic silencing induces NREM sleep and this is accompanied by reduced serotonergic neuron firing rates, which are known to be involved with arousal state regulation [27]. Fluctuations in the extracellular orexin A levels in the lateral hypothalamic area are related to the light/dark cycle and sleep/wakefulness activities, and their release is increased after 6 h of sleep deprivation [28]. Given that orexin has an important role in the regulation of physiological sleep/wakefulness, our results suggest that the impairment of orexin neuronal activities may contribute to an increased amount of NREM sleep in HFD-induced obese mice.

In summary, HFD-induced obese mice had an increased amount of daily NREM sleep concomitant with a decrease in the *prepro-orexin* mRNA level but not *orexin 1r* and *2r*

mRNA levels, in the hypothalamus. In addition, regression analysis revealed that the daily amount of NREM sleep was negatively correlated with hypothalamic *prepro-orexin* mRNA level. These results suggest that impaired orexin activities may contribute to an increased amount of NREM sleep, which could explain the molecular basis of sleep alterations in high-fat diet-induced obesity mice.

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References

- [1] de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, et al. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci U S A* 1998;95(1):322-7.
- [2] Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, et al. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 1998;92(4):573-85.
- [3] Peyron C, Tighe DK, van den Pol AN, de Lecea L, Heller HC, Sutcliffe JG, et al. Neurons containing hypocretin (orexin) project to multiple neuronal systems. *J Neurosci* 1998;18(23):9996-10015.
- [4] Marcus JN, Aschkenasi CJ, Lee CE, Chemelli RM, Saper CB, Yanagisawa M, et al. Differential expression of orexin receptors 1 and 2 in the rat brain. *J Comp Neurol* 2001;435(1):6-25.
- [5] Nishino S. The hypocretin/orexin system in health and disease. *Biol Psychiatry* 2003;54(2):87-95.
- [6] Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, et al. Narcolepsy

- in orexin knockout mice: molecular genetics of sleep regulation. *Cell* 1999;98(4):437-51.
- [7] Stricker-Krongrad A, Richy S, and Beck B. Orexins/hypocretins in the ob/ob mouse: hypothalamic gene expression, peptide content and metabolic effects. *Regul Pept* 2002;104(1-3):11-20.
- [8] Yamamoto Y, Ueta Y, Date Y, Nakazato M, Hara Y, Serino R, et al. Down regulation of the prepro-orexin gene expression in genetically obese mice. *Brain Res Mol Brain Res* 1999;65(1):14-22.
- [9] Danguir J. Sleep patterns in the genetically obese Zucker rat: effect of acarbose treatment. *Am J Physiol Regul Integr Comp Physiol* 1989;256:R281-3.
- [10] Laposky AD, Bradley MA, Williams DL, Bass J, and Turek FW. Sleep-wake regulation is altered in leptin-resistant (db/db) genetically obese and diabetic mice. *Am J Physiol Regul Integr Comp Physiol* 2008;295(6):R2059-66.
- [11] Laposky AD, Shelton J, Bass J, Dugovic C, Perrino N, and Turek FW. Altered sleep regulation in leptin-deficient mice. *Am J Physiol Regul Integr Comp Physiol* 2006;290(4):R894-903.
- [12] Wells AS, Read NW, Uvnas-Moberg K, and Alster P. Influences of fat and

- carbohydrate on postprandial sleepiness, mood, and hormones. *Physiol Behav* 1997;61(5):679-86.
- [13] Guan Z, Vgontzas AN, Bixler EO, and Fang J. Sleep is increased by weight gain and decreased by weight loss in mice. *Sleep* 2008;31(5):627-33.
- [14] Jenkins JB, Omori T, Guan Z, Vgontzas AN, Bixler EO, and Fang J. Sleep is increased in mice with obesity induced by high-fat food. *Physiol Behav* 2006;87(2):255-62.
- [15] Taheri S, Mahmoodi M, Opacka-Juffry J, Ghatei MA, and Bloom SR. Distribution and quantification of immunoreactive orexin A in rat tissues. *FEBS Lett* 1999;457(1):157-61.
- [16] Ziotopoulou M, Mantzoros CS, Hileman SM, and Flier JS. Differential expression of hypothalamic neuropeptides in the early phase of diet-induced obesity in mice. *Am J Physiol Endocrinol Metab* 2000;279(4):E838-45.
- [17] Wortley KE, Chang GQ, Davydova Z, and Leibowitz SF. Peptides that regulate food intake: orexin gene expression is increased during states of hypertriglyceridemia. *Am J Physiol Regul Integr Comp Physiol* 2003;284(6):R1454-65.
- [18] Ferretti S, Fornari A, Pedrazzi P, Pellegrini M, and Zoli M. Developmental overfeeding alters hypothalamic neuropeptide mRNA levels and response to a high-fat diet in adult

- mice. *Peptides* 2011;32(7):1371-83.
- [19] Teske JA, Levine AS, Kuskowski M, Levine JA, and Kotz CM. Elevated hypothalamic orexin signaling, sensitivity to orexin A, and spontaneous physical activity in obesity-resistant rats. *Am J Physiol Regul Integr Comp Physiol* 2006;291(4):R889-99.
- [20] Franklin KGB and Paxinos G. *The Mouse Brain in Stereotaxic Coordinates*. Academic Press: San Diego, CA 1997
- [21] Tobler I, Deboer T, and Fischer M. Sleep and sleep regulation in normal and prion protein-deficient mice. *J Neurosci* 1997;17(5):1869-79.
- [22] Nishikawa S, Yasoshima A, Doi K, Nakayama H, and Uetsuka K. Involvement of sex, strain and age factors in high fat diet-induced obesity in C57BL/6J and BALB/cA mice. *Exp Anim* 2007;56(4):263-72.
- [23] Yamanaka A, Beuckmann CT, Willie JT, Hara J, Tsujino N, Mieda M, et al. Hypothalamic orexin neurons regulate arousal according to energy balance in mice. *Neuron* 2003;38(5):701-13.
- [24] Franken P, Dijk DJ, Tobler I, and Borbely AA. Sleep deprivation in rats: effects on EEG power spectra, vigilance states, and cortical temperature. *Am J Physiol Regul Integr Comp Physiol* 1991;261(1 Pt 2):R198-208.

- [25] Piper DC, Upton N, Smith MI, and Hunter AJ. The novel brain neuropeptide, orexin-A, modulates the sleep-wake cycle of rats. *Eur J Neurosci* 2000;12(2):726-30.
- [26] Winrow CJ, Gotter AL, Cox CD, Tannenbaum PL, Garson SL, Doran SM, et al. Pharmacological characterization of MK-6096 - a dual orexin receptor antagonist for insomnia. *Neuropharmacology* 2012;62(2):978-87.
- [27] Tsunematsu T, Kilduff TS, Boyden ES, Takahashi S, Tominaga M, and Yamanaka A. Acute optogenetic silencing of orexin/hypocretin neurons induces slow-wave sleep in mice. *J Neurosci* 2011;31(29):10529-39.
- [28] Yoshida Y, Fujiki N, Nakajima T, Ripley B, Matsumura H, Yoneda H, et al. Fluctuation of extracellular hypocretin-1 (orexin A) levels in the rat in relation to the light-dark cycle and sleep-wake activities. *Eur J Neurosci* 2001;14(7):1075-81.

Figure Legends

Figure 1. Effects of HFD on the mRNA levels of *prepro-orexin* (A), *orexin 1r* (B), and *orexin 2r* (C) in the hypothalamus. Values are the means $\pm$ SE for 8 mice. Differences among groups were tested using a one-way ANOVA followed by Scheffe's post hoc test. * $p < 0.05$ compared with the ND group.

Figure 2. Correlations between the following pairs of variables: (A) *prepro-orexin* mRNA level vs. body weight; (B) daily amount of NREM sleep vs. body weight; (C) daily amount of NREM sleep vs. the *prepro-orexin* mRNA level. Open circles represent ND group (n=8). Closed circles represent HFD 10 week group (n=8). Closed squares represent HFD 17 week group (n=8). Pearson's correlation coefficients (r) and p values were calculated based on 24 mice. The significance level was $p < 0.05/3 = 0.017$. The result of a single test was only considered significant if its p value was less than the Bonferroni-adjusted level.

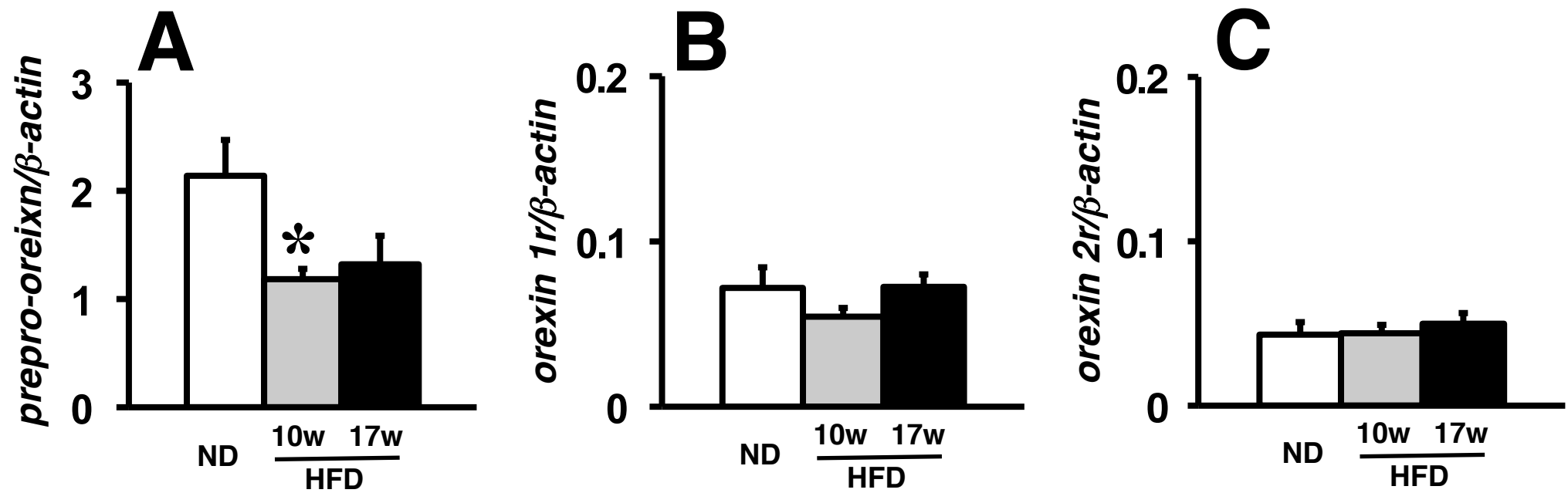


Figure 1

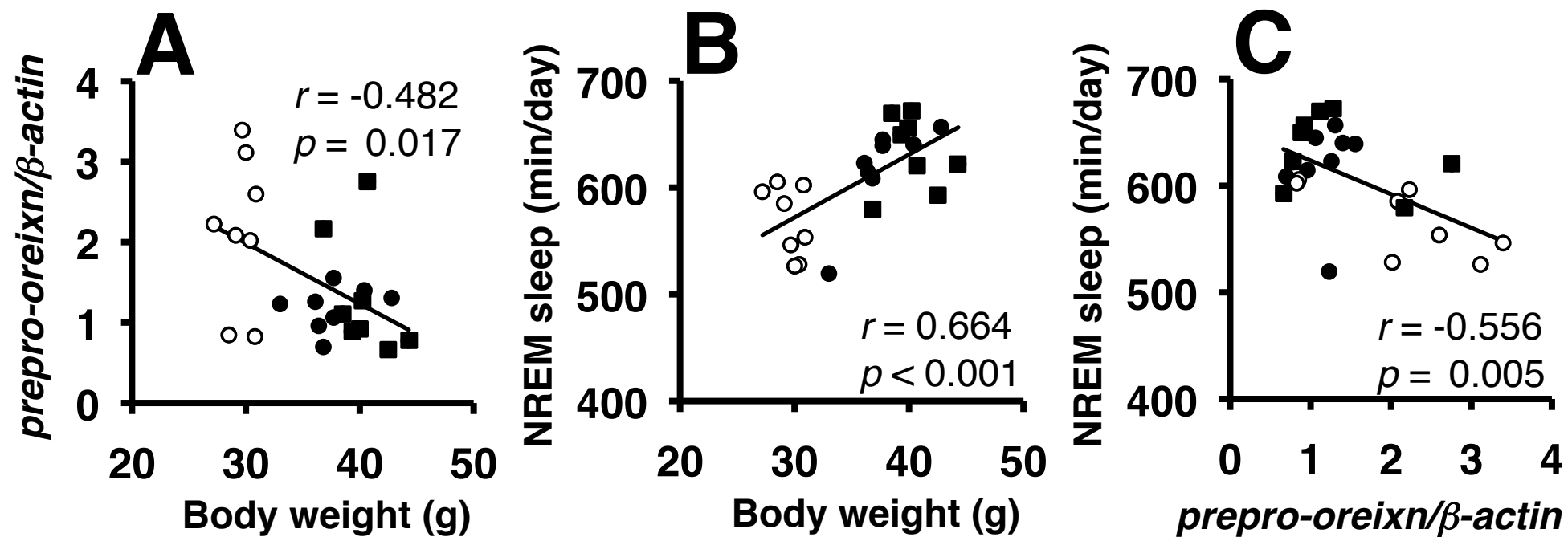


Figure 2

Table 1. Effects of high-fat diet on sleep/wakefulness parameters and body weight

	ND	HFD 10 week	HFD 17 week
Body weight (g)	29.6 ± 0.4	37.6 ± 1.0 **	40.3 ± 0.8 **
NREM sleep			
daily amount (min)	568.0 ± 13.0	618.0 ± 16.0 *	633.0 ± 13.0 **
episode duration (min)	1.8 ± 0.1	1.5 ± 0.1 *	1.6 ± 0.1
episode number (counts)	311.6 ± 12.2	417.9 ± 16.6 **	390.1 ± 18.8 **
REM sleep			
daily amount (min)	88.0 ± 6.1	95.0 ± 5.4	93.2 ± 6.8
episode duration (min)	0.9 ± 0.0	0.8 ± 0.1	0.8 ± 0.0
episode number (counts)	99.3 ± 8.7	115.9 ± 8.5	107.3 ± 11.4
Wakefulness			
daily amount (min)	784.1 ± 14.9	726.6 ± 14.4 *	713.9 ± 14.3 **
episode duration (min)	2.6 ± 0.1	1.8 ± 0.1 **	1.9 ± 0.1 **
episode number (counts)	313.5 ± 11.1	418.9 ± 17.1 **	392.0 ± 13.0 **
Brief awakening (counts)	140.3 ± 14.5	263.8 ± 17.1 **	224.0 ± 12.3 **

Values are the means ± SE for 8 mice. Twenty-four mice were divided randomly into 3 groups and subjected to different feeding conditions, as follows: (1) Normal diet (ND) for 17 weeks (ND group); (2) ND for 7 weeks followed by HFD for 10 weeks (HFD 10 week group); and (3) High-fat diet (HFD) for 17 weeks (HFD 17 week group). Differences among groups were analyzed using one-way ANOVA followed by Scheffe's post hoc test. * $p < 0.05$, ** $p < 0.01$ compared with the ND group.