Biochemical and Biophysical Research Communications **270,** 318–323 (2000)
doi:10.1006/bbrc.2000.2412, available online at http://www.idealibrary.com on IDEAL®

Possible Involvement of Orexin in the Stress Reaction in Rats

Takanori Ida,* Keiko Nakahara,* Takayuki Murakami,† Reiko Hanada,‡ Masamitsu Nakazato,§ and Noboru Murakami*,[1]

*Department of Veterinary Physiology and †Department of Veterinary Anatomy, Faculty of Agriculture, University of Miyazaki, Miyazaki 889-2155, Japan; ‡Department of Internal Medicine I, School of Medicine, Oita Medical University, Oita 879-5593, Japan; and §Third Department of Internal Medicine, Miyazaki Medical College, Kiyotake, Miyazaki 889-1692, Japan

Received February 25, 2000

We examined whether corticotropin-releasing factor (CRF) was involved in orexin-induced grooming and face-washing behaviors, and whether orexin was involved in the stress reaction. Administration of α-helical CRF, CRF antagonist, alone had no behavioral effect, but it blocked the orexin-induced grooming and face-washing behaviors in rats. The level of corticosterone increased in a dose-dependent manner 15 min after icv injection of orexin, and it remained high for at least 60 min. In 2-month-old rats, 1 h of immobilization stress increased orexin mRNA levels, but not the melanin-concentrating hormone (MCH) mRNA, in the lateral hypothalamic area (LHA). In 6-month-old rats, 30 min of cold stress increased the expression of orexin mRNA in the LHA. Unlike in the 2-month-old rats, immobilization stress did not change orexin mRNA expression in 6-month-old rats. These results suggest that CRF is involved in orexin-induced behaviors, and that orexin may play an important role in some stress reactions. © 2000 Academic Press

Orexins A and B, also known as hypocretins 1 and 2, are hypothalamic peptides isolated recently from the hypothalamus, and have been shown to stimulate food intake (1, 2). Cells expressing the pre-pro-orexin gene are distributed in the lateral hypothalamic area (LHA), the posterior hypothalamus and the perifornical nucleus of the adult rat brain. Immunoreactive axons emanating from these cells terminate at various hypothalamic sites, such as the locus coeruleus (LC), arcuate nucleus (ARC), paraventricular nucleus (PVN) and dorsal raphe (3, 4). Injection of orexin stimulates the expression of c-fos in the PVN and ARC, which are closely involved with feeding control through neuropeptide Y (NPY), melanin-concentrating hormone (MCH) and other neurotransmitters (3, 5). This wide distribution of orexin fibers suggests that orexin may play an important role in physiological functions other than feeding. Indeed, it has been recently demonstrated that orexin is involved in sleep and wakefulness or narcolepsy (6, 7).

We reported previously that injection of orexin induced various physiological behaviors, such as grooming, face washing, burrowing and searching (8). The predominant orexin-induced behavior was grooming. Although the physiological significance of grooming is not fully understood, it is known to be closely associated with the stress reaction (9). Environmental stress leads to grooming and face washing, and corticotropin-releasing factor (CRF) may be involved in this reaction (9, 10). CRF is synthesized mainly in the PVN, where orexin-containing neurons are distributed (11). Therefore, orexin-induced grooming may be associated with CRF. We also observed that high-dose of orexin (10 nmol) caused seizure activity (8). It may be important to note that high-dose intracerebroventricular (icv) injection of CRF has been reported to induce epileptogenic activity in the rat (12).

Immunohistochemical studies have also shown that long and descending axonal projections containing orexin innervate all levels of the spinal cord from cervical to sacral segments in the mouse, rat and human (13). Innervation of the intermediolateral column and lamina 10, as well as strong innervation of the caudal region of the sacral cord, suggest that orexin may participate in regulation of both the sympathetic and parasympathetic parts of the autonomic nervous system. In support of this suggestion, orexin dose-dependently increases heart rate, blood pressure and renal sympathetic activity in conscious, unrestrained rats (14, 15).

[1] To whom correspondence should be addressed. Fax: 81-985-58-7265. E-mail: a0d201u@cc.miyazaki-u.ac.jp.

0006-291X/00 $35.00

Copyright © 2000 by Academic Press
All rights of reproduction in any form reserved.

It also increases gastric acid secretion (16). In the light of these findings, we set out to determine whether orexin-induced behaviors were blocked by co-administration of a CRF antagonist. Next, we measured the expression of orexin mRNA in the LHA after exposure to cold stress or immobilization stress, in order to determine whether orexin was involved in the stress reaction, using different aged rats, since the tone of the autonomic nervous system shows age-related changes (17). The lateral hypothalamus has another distinct neuropeptide, melanin-concentrating hormone (MCH) which is a stimulator of food intake (18). MCH and orexin have similar neuronal fiber distributions (13, 19, 20). Therefore, we measured MCH mRNA levels in rats under stress in order to compare their levels with those of orexin mRNA.

MATERIALS AND METHODS

Experiment 1

Animals. We purchased adult male Wistar rats (300–350 g) from Charles River Japan Inc. (Yokohama, Japan) and housed them in Plexiglas cages in an animal room that was maintained under a constant light–dark cycle (lights on from 0700–1900 h) and temperature (22°C) for at least 2 weeks. Food and water were provided *ad libitum*. For at least 2 weeks before use, each animal was gently handled on a daily basis by the principal investigator.

ICV cannulation and behavior test. Both the cannulation method used for intracerebroventricular (icv) injection and the method used for behavior testing had been reported previously (8). Each rat received two 10-μl icv injections of either: (1) saline and orexin A (10 μg) (Peptide Inst., Osaka, Japan); or (2) saline and α-helical CRF (10, 50, 100 μg) (Peninsula Laboratories, Merseyside, England); or (3) α-helical CRF (10, 50, 100 μg) and orexin A (10 μg); or (4) saline and saline. We evaluated the following behaviors for 2 h after injection: (1) face washing: rubbing and stroking the face and head (behind the ear) with both forepaws; (2) grooming: face grooming with the hindpaws, cleaning the hind legs, body, tail and genitals; (3) resting time: sleeping and no movement. We also calculated the total time occupied by locomotor activity, including grooming, face washing, sniffing around, burrowing into the chip bedding, walking and looking around, as well as feeding. We analyzed the data statistically by analysis of variance for total reaction (8).

Blood collection. From each rat received an icv injection of 10 and 30 μg orexin A or saline, 50 μl of blood were taken at 0 min, 15 min, 30 min and 60 min after the initial injection by the tail-tip-incision method. Plasma corticosterone levels were measured with a radioimmunoassay (RIA) kit (SRS Co., Tokyo, Japan).

Experiment 2

Animals. Male Sprague-Dawley rats 2 and 6-months old were purchased from Charles River Japan Inc. (Yokohama, Japan) and housed in same conditions described in Experiment 1.

Stress. Each rat was assigned to one of the following groups: (1) rats left undisturbed (control); (2) rats exposed to 1 h of immobilization stress from 0900 h to 1000 h. Briefly, immobilization stress consisted of immobilizing the rats on wooden boards by taping all 4 limbs and the neck; (3) rats exposed to cold stress for 30 min from 0900 h to 0930 h and for 2 days from 0900 h to 0900 h. Rats were housed in Plexiglas boxes (4 animals per box) and placed in a room at 8°C. They were provided with sufficient food and water under a 12-h light–dark cycle. After the immobilization stress session, the animals were killed within 10 s of being removed from the apparatus. After the cold-stress session, the animals were killed in the cold room. After sacrifice, the brains were immediately collected and frozen on dry ice. The LHA was punched out of the frozen brain slices as reported previously (21). Total RNA was extracted with Trizol (Life Technologies Inc., Grand Island, NY). One μg of the total RNA was denatured with 16 ml of 1 M glyoxal and 50% dimethylsulfoxide, then electrophoresed on 0.8% agarose gel (FMC Bio Products, Rockland, ME) in 10 mM sodium phosphate buffer (pH 7.0). The sample was then transferred to a Zeta Probe membrane (Bio-Rad Laboratories, Richmond, CA) and fixed by ultraviolet irradiation. The probes used for Northern blot analyses were a 287 bp cDNA fragment of rat orexin (sense primer: 5′-TGTCGCCAGAAGACGTGTTCCTG-3′ and antisense primer: 5′-AAGACGGGTTCAGACTCTGGATC-3′), a 320 bp cDNA fragment of rat MCH (sense primer: 5′-AACGGGTCGGTAGACTCGT-3′ and antisense primer: 5′-ATCGGTTGTTGCTCCTTCTC-3′), and a 203 bp cDNA of G3PDH (sense primer: 5′-GTTTGTGATGGGTGTGAACC-3′ and antisense primer: 5′-TCACGCCACCAGCTTTC-3′). The membrane was first treated for 2 h at 37°C in 6 × SSPE (900 mM NaCl, 60 mM $NaH_2PO_4 \cdot H_2$), 7 mM EDTA, pH 7.4) containing 40% formamide, 5 × Denhardt's solution, 0.5% SDS and 0.1 mg/ml denatured salmon-sperm DNA, then hybridized for 18 h at 42°C in an identical solution that contained ^{32}P-labeled orexin, MCH and G3PDH cDNA probes. The RNA blot was washed with 2 × SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0)/0.1% SDS solution at 55°C and exposed to film. The membrane was boiled for 15 min at 70°C in 0.1 × SSC solution to strip it of the orexin and G3PDH probes, then used for sequential hybridization with the MCH and G3PDH probes. Hybridization signals were measured in a Fujix Bio-image analyzer, BAS 2000 (Fuji Photo Film Co., Tokyo, Japan). Levels of both the orexin and MCH mRNA were calculated as relative radioactivity of the G3PDH.

Statistics

The data were expressed as the mean ± SEM. The statistical analysis of mRNA amount was carried out by ANOVA followed by Fisher's PLSD test *post hoc*.

RESULTS

Experiment 1

Administration of orexin significantly increased face-washing and grooming behavior (Fig. 1A). Although administration of CRF antagonist alone had no significant effect on these activities at any of the doses used, it reduced orexin-induced face-washing and grooming activities in a manner that was almost dose dependent, except with 1 dose (face washing: antagonist 100 μg + orexin) (Fig. 1A). Locomotor activity and resting behavior during the 2 h following injection were significantly increased and decreased, respectively, by orexin administration. CRF antagonist alone had no direct effects, but it blocked the orexin-induced increase and decrease of locomotor activity and resting, respectively (Fig. 1B).

As the orexin-induced behavior was partly blocked by CRF antagonist, we considered that orexin might stimulate the CRF-ACTH-corticosterone secretion pathway. Therefore, we measured corticosterone levels after injection of orexin. The level of corticosterone increased 15 min after injection of orexin and stayed

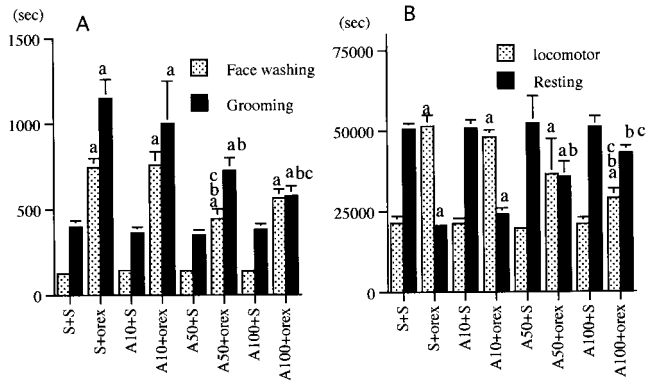

FIG. 1. Effect of α-herical CRF, a CRF receptor antagonist, on orexin-induced face-washing and grooming behavior (A) and on locomotor activities and resting (B) in rats. S, saline; A, α-herical-CRF; orex, orexin. The black and dotted bars and vertical lines represent the mean ± SEM (n = 8). Each activity was measured as total s over the 2 h after the injections. a, $P < 0.001$ versus S + S; b, $P < 0.005$ versus S + orex; c, $P < 0.005$ versus A10 + orex.

high for at least 60 min. This increase was dose dependent for orexin (Fig. 2).

Experiment 2

In the 2-month-old rats, 1 h of immobilization stress increased orexin mRNA expression in the LHA (Fig. 3). However, 30 min or 48 h of cold stress did not change the expression of orexin in the LHA. In 6-month-old rats, 30 min, but not 48 h, of cold stress increased the expression of orexin mRNA in the LHA (Fig. 4). In the 6-month-old rats, unlike the 2-month-old rats, immobilization stress did not change orexin mRNA expression. Expression of MCH mRNA in the LHA was not affected in rats of either age by any of the treatments (Figs. 3 and 4).

DISCUSSION

This study showed that pretreatment with α herical-CRF, a CRF receptor antagonist, significantly reduced orexin-induced grooming and face-washing behaviors. These results suggest that administration of orexin caused grooming and face washing behavior via, at least, CRF secretion. CRF is synthesized mainly by parvocellular neurons in the PVN (11), where orexin-containing neurons and orexin receptor 2 are distributed (3, 4, 22). Further, orexin induces expression of immunoreactive Fos in this area (3). Therefore, these data support the relationship between orexin and CRF. Of course, we cannot exclude the possibility that orexin causes grooming and face-washing behavior via vasopressin or other peptides, such as dopamine, ACTH or α-MSH (9, 14, 33, 40), since the blockade of behavior induced by the CRF antagonist was not complete. However, the fact that corticosterone secretion was stimulated by orexin also supports the concept that orexin-induced grooming is mainly triggered by CRF. Vasopressin-containing neurons are distributed in the parvocellular division of the PVN, and vasopressin also induces grooming (9, 23, 24, 25), but orexin does not induce the expression of Fos in this division (3). Dopamine-D1-agonist-induced grooming is blocked by a CCKA antagonist (25). ACTH-induced grooming is blocked by a non-selective melanocortin-3/4-receptor antagonist (SHU 9119) (27). α-MSH- and neuropeptide-E-1-induced grooming are blocked by MCH (11). Dopamine D-1 antagonist and naloxone block grooming induced by many peptides (28, 29, 30). However, our preliminary study showed that administration of naloxone, SHU 9119 and MCH did not reduce orexin-induced grooming activity (unpublished data).

In this study, administration of orexin stimulated locomotor activity and decreased resting behavior, as shown as our previous reports (8) and other studies (7). CRF also increases locomotor activity and time spent in the arousal state (31). These CRF-induced reactions are similar to stress reactions, and the stress reactions are nearly completely blocked by administration of CRF antagonist (31–34). One hour of immobilization in 2-month-old rats and 30 min of cold stress in 6-month-old rats increased orexin mRNA levels in the LHA. MCH mRNA levels in the LHA did not change in rats of either age under any of the conditions. Therefore, we considered that an increased level of orexin mRNA in the LHA was specific for stress. However, other research has shown that MCH mRNA expression decreases after chronic foot-shock stress (35). We do not know why stress did not change the MCH mRNA levels in our experiment. MCH influences feeding behavior but not grooming behavior, so it may have little direct involvement in the stress reaction, or it may be involved in very short-term stress, such as foot shock. These results suggest that orexin may be involved in the stress reaction, but that its involvement is limited by the kind of stress or the age of the rats.

Stress is considered to be a modulator of the autonomic nervous system. Central administration of CRF

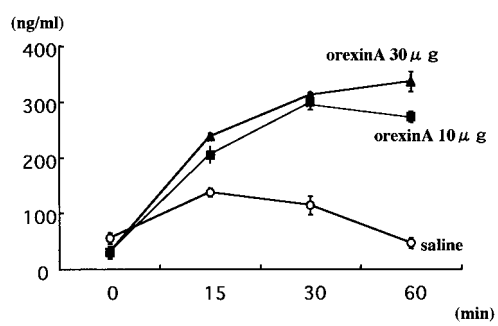

FIG. 2. Effect of icv injection of orexin A on plasma corticosterone levels in rats. Orexin was injected at 0900 h. The symbols and vertical lines represent the mean ± SEM (n = 8).

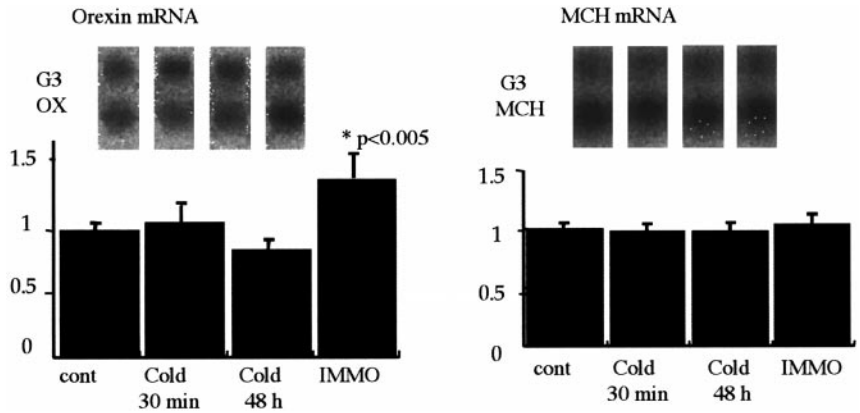

FIG. 3. Effect of immobilization and cold stress on the expression of orexin and MCH mRNA in 2-month-old rats. IMMO indicate 60 min immobilization. The lower figure shows the relative value of mRNA expression versus those of control rats. The bar and vertical lines represent the mean ± SEM (n = 8).

increases sympathetic and decreases central parasympathetic outflow (36). In rats, foot shock under restraint increases parasympathetic outflow, whereas foot shock without restraint increases sympathetic outflow (37, 38). Vagotomy antagonizes stress-induced hypocalcemia and stress-induced gastric ulceration (39, 40). These stresses activate parasympathetic outflow (39, 40). In our study, the rats were strongly immobilized. One hour after immobilization began they were very quiet, indicating an increased parasympathetic outflow. In contrast, cold stress is considered mainly to increase sympathetic outflow (41). Which autonomic nervous system predominates in the stress reaction depends on the age of the animal. The net autonomic tone of rats has been observed to be predominantly sympathetic at 2 week of age, but thereafter it becomes predominantly parasympathetic (17). There are significantly fewer primary dendritic branch points and a significantly smaller total dendritic length of the sympathetic preganglionic neurons in aged rats compared with adults (42). No significant differences have been found in any characteristics of the parasympathetic neurons among the different age groups. Among different age groups there are differences in the plasma levels of catecholamines in relation to stress (41, 43). Therefore, in immobilized young, orexin mRNA expression might increase to activate the parasympathetic outflow, but in adults that reaction might be weak, as parasympathetic tone predominates anyway. In contrast, cold stress provokes the opposite reaction. Orexin dose-dependently increases heart rate, blood pressure and renal sympathetic activity in conscious, unrestrained rats (14, 15). Orexin also increases gastric acid secretion (16). The innervation patterns of the intermediolateral column and lamina 10, as well as strong innervation of the caudal region of the sacral cord, nucleus of the solitary tract and dorsal motor nucleus of the vagus, suggest that orexin may participate in regulation of both the sympathetic and parasympathetic parts of the autonomic nervous system. Evidence from study of lesions in the LHA suggests that the LHA has a potential role in stress (44). Orexin may be a very important modulator for afferent and efferent nerves passing through the LHA.

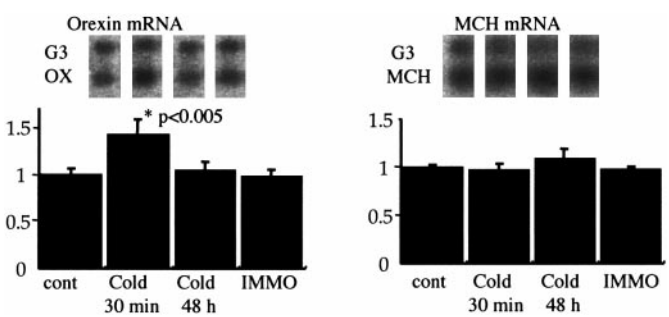

FIG. 4. Effect of immobilization and cold stress on the expression of orexin and MCH mRNA in 6-month-old rats. IMMO indicate 60 min immobilization. The lower figure shows the relative value of mRNA expression versus those of control rats. The bar and vertical lines represent the mean ± SEM (n = 8).

ACKNOWLEDGMENTS

We thank Akiko Kuroda for her technical assistance. This research was supported by a grant from the Ministry of Education, Science and Culture of Japan to N.M. (10460132), and a Sasakawa Scientific Research Grant to K.N. (11-239).

REFERENCES

1. Lecea, L. D., Kilduff, T. S., Peyron, C., Gao, X. B., Foye, P. E., Danielson, P. E., Fukuhara, C., Battenberg, E. L., Gautivik, V. T., Bartlett, F. S., Frankel, W. N., van den Pol, A. N., Bloom, F. E., Gautvik, K. M., and Sutcliffe, J. G. (1998) The hypocretins: Hypothalamus-specific peptides with neuroexcitatory activity. *Proc. Natl. Acad. Sci. USA* **95,** 322–327.
2. Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R. M., Tanaka, H., Williams, S. C., Richardson, J. A., Kozlowski,

G. P., Wilson, S., Arch, J. R., Buckingham, R. E., Haynes, A. C., Carr, S. A., Annan, R. S., McNulty, D. E., Liu, W. S., Terrett, J. A., Elshourbagy, N. A., Bergsma, D. J., and Yanagisawa, M. (1998) Orexin and orexin receptors: A family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* **92,** 573–585.

3. Date, Y., Ueta, Y., Yamashita, H., Yamaguchi, H., Matsukura, S., Kangawa, K., Sakurai, T., Yanagisawa, M., and Nakazato, M. (1999) Orexins, orexigenic hypothalamic peptides, interact with autonomic, neuroendocrine and neuroregulatory systems. *Proc. Natl. Acad. Sci. USA* **96,** 748–753.
4. Peyron, C., Tighe, D. K., van den Pol, A. N., Lecea, L. D., Heller, H. C., Sutcliffe, J. G., and Kilduff, T. S. (1998). Neurons containing hypocretin (orexin) project to multiple neuronal systems. *J. Neurosci.* **23,** 9996–10015.
5. Kalra, S. P., Dube, M. G., Pu, S., Xu, B., Horvath, T. L., and Kalra, P. S. (1999) Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. *Endocrine Reviews* **20**(1), 68–100.
6. Chemelli, R. M., Willie, J. T., Sinton, C. M., Elmquist, J. K., Scammell, T., Lee, C., Richardson, J. A., Williams, S. C., Xiong, Y., Kisanuki, Y., Fitch, T. E., Nakazato, M., Hammer, R. E., Saper, C. B., and Yanagisawa, M. (1999) Narcolepsy in orexin knockout mice: Molecular genetics of sleep regulation. *Cell* **98,** 437–451.
7. Hagan, J. J., Leslie, R. A., Patel, S., Evans, M. L., Wattam, T. A., Holmes, S., Benham, C. D., Taylor, S. G., Routledge, C., Hemmati, P., Munton, R. P., Ashmeade, T. E., Shah, A. S., Hatcher, J. P., Hatcher, P. D., Jones, D. N. C., Smith, M. I., Piper, D. C., Hunter, A. J., Porter, R. A., and Upton, N. (1999) Orexin A activates locus coeruleus cell firing and increases arousal in the rat. *Proc. Natl. Acad. Sci. USA* **96,** 10911–10916.
8. Ida, T., Nakahara, K., Katayama, T., Murakami, N., and Nakazato, M. (1999) Effect of lateral cerebroventricular injection of the appetite-stimulating neuropeptides, orexin and neuropeptide Y, on the various behavioral activities of rats. *Brain Res.* **821,** 526–529.
9. Gispen, W. H., and Isaacson, R. L. (1981) ACTH-induced excessive grooming in the rat. *Pharmac. Ther.* **12,** 209–246.
10. Dunn, A. J., Berridge, C. W., Lai, Y. I., and Yachabach, T. L. (1987) CRF induced excessive grooming behavior in rats and mice. *Peptides* **8,** 841–4.
11. Swanson, L. W., Sawchenko, P. E., and Lind, R. W. (1986) Regulation of multiple peptides in CRF parvocellular neurosecretory neurons: Implications for the stress response. *Prog. Brain Res.* **68,** 169–190.
12. Ehlers, C. L., Henriksen, S., Wang, J. M., Rivier, J., Vale, W., and Bloom, F. E. (1983) Corticotropin releasing factor produces increases in brain excitability and convulsive seizures in rats. *Brain Res.* **278,** 332–336.
13. Van den Pol, A. N. (1999) Hypothalamic hypocretin (orexin): Robust innervation of the spinal cord. *J. Neurosci.* **19**(8), 3171–3182.
14. Samson, W. K., Gosnell, B., Chang, J. K., Resch, Z. T., and Murphy, T. C. (1999) Cardiovascular regulatory actions of the hypocretins in brain. *Brain Res.* **831,** 248–253.
15. Shirasaka, T., Nakazato, M., Matsukura, S., Takasaki, M., and Kannan, H. (1999) Sympathetic and cardiovascular actions of orexins in conscious rats. *Am. J. Physiol.* **277,** R1780–R1785.
16. Takahashi, N., Okumura, T., Yamada, H., and Kohgo, Y. (1998) Stimulation of gastric acid secretion by centrally administered orexin-A in conscious rats. *Biochem. Biophys. Res. Commun.* **254,** 623–627, doi:10.1006/bbrc.1998.9994.
17. Kuwahara, M., Huh, M. D., Hirose, H., and Sugano, S. (1986) Alteration of the intrinsic heart rate and autonomic nervous tone during the growing process of rats and pig. *Jpn. J. Vet. Sci.* **48,** 703–709.
18. Qu, D., Ludwig, D. S., Gammeltoft, S., Piper, M., Pelleymounter, M. A., Cullen, M. J., Mathes, W. F., Przypek, J., Kanarek, R., and Flier, E. M. (1996) A role for melanin-concentrating hormone in the central regulation of feeding behaviour. *Nature* **380,** 243–247.
19. Bittencourt, J. C., and Elias, C. F. (1998) Melanin-concentrating hormone and neuropeptide EI projections from the lateral hypothalamic area and zona incerta to the medial septal nucleus and spinal cord: A study using multiple neuronal tracers. *Brain Res.* **805,** 1–19.
20. Broberger, C., Lecea, L. D., Sutcliffe, J. G., and Hoekfelt, T. (1998) Hypocretin/orexin and melanin-concentrating-hormone-expressing cells form distinct populations in the rodent lateral hypothalamus: Relationship to the neuropeptide Y and agouti gene-related protein systems. *J. Comp. Neurol.* **402,** 460–474.
21. Mondal, M. S., Nakazato, M., Date, Y., Murakami, N., Yanagiswa, M., and Matsukura, S. (1999) Widespread distribution of orexin in rat brain and its regulation upon fasting. *Biochem. Biophys. Res. Commun.* **256,** 495–499, doi:10.1006/bbrc.1999.0362.
22. Trivedi, P., Yu, H., MacNeil, D. J., Van der Ploeg, L. H. T., and Guan, X.-M. (1998) Distribution of orexin receptor mRNA in the rat brain. *FEBS Lett.* **438,** 71–75.
23. Drago, F., Stanciu, M., Salehi, S., and Scapagnini, U. (1997) The block of central vasopressin V1 but not V2 receptors suppresses grooming behavior and hypothermia induced by intracerebroventricular vasopressin in male rats. *Peptides* **18,** 1389–1392.
24. Sanchez, M., Baker, B. I., and Celis, M. (1997) Melanin-concentrating hormone (MCH) antagonizes the effects of α-MSH and neuropeptide E-I on grooming and locomotor activities in the rat. *Peptides* **18,** 393–396.
25. Van Kampen, J., and Stoessl, A. J. (1997) The effects of CCK-4 on dopamine D1 agonist-induced grooming are blocked by a CCKA receptor antagonist: Evidence for a novel CCK receptor subtype? *Neuropharmacology* **36,** 1679–1688.
26. Ma, X.-M., and Aguilera, G. (1999) Transcriptional responses of the vasopressin and corticotropin-releasing hormone genes to acute and repeated intraperitoneal hypertonic saline injection in rats. *Mol. Brain Res.* **68,** 129–140.
27. Von Frijitag, J. C., Croist, G., Gispen, W. H., Adan, R. A. H., and Wiegant, V. M. (1998) The role of central melanocortin receptors in the activation of the hypothalamus–pituitary–adrenal axis and the induction of excessive grooming. *Br. J. Pharmacol.* **123,** 1503–1508.
28. Stoessl, A. J. (1994) Dopamine D1 receptor agonist-induced grooming is blocked by the opioid receptor antagonist naloxone. *Eur. J. Pharmacol.* **259,** 301–303.
29. Van Wimersma greidanus, T. B., and Maigret, C. (1991) Neuromedin induced excessive grooming/scratching behavior is suppressed by naloxone, neurotensin and a dopamine D1 receptor antagonist. *Eur. J. Pharmacol.* **209,** 57–61.
30. Van Wimersma greidanus, T. B., Maigret, C., Torn, M., Ronner, E., van der Kracht, S., van der Wee, N. J. A., and Versteeg, H. G. (1989) Dopamine D-1 and D-2 receptor agonists and antagonists and neuropeptide-induced excessive grooming. *Eur. J. Pharmacol.* **173,** 227–231.
31. Dunn, A. J., and Berridge, C. W. (1990) Physiological and behavioral responses to corticotropin-releasing factor administration: Is CRF a mediator of anxiety or stress responses? *Brain Res. Rev.* **15,** 71–100.
32. Brown, M. R., Fisher, L. A., Webb, V., Vale, W. W., and Rivier, J. E. (1985) Corticotropin-releasing factor: A physiologic regulator of adrenal epinephrine secretion. *Brain Res.* **328,** 355–357.

33. Krahn, D. D., Gosnell, B. A., Grace, M., and Levine, A. S. (1986) CRF antagonist partially reverses CRF and stress-induced effects on feeding. *Brain Res. Bull.* **17,** 285–289.

34. Owens, M. J., and Nemeroff, C. B. (1991) Physiology and pharmacology of corticotropin-releasing factor. *Pharmacol. Rev.* **43,** 425–473.

35. Presse, F., Hervieu, G., Imaki, T., Sawchenko, P. E., Vale, W., and Nahon, J-L. (1992) Rat melanin-concentrating hormone messenger ribonucleic acid expression: Marked changes during development and after stress and glucocorticoid stimuli. *Endocrinology* **131,** 1241–1250.

36. Fisher, L. A. (1989) Corticotropin-releasing factor: Endocrine and autonomic interaction of responses to stress. *Trends Pharmacol. Sci.* **10,** 189–193.

37. Callahan, M. F., da Rocha, M. J., and Morris, M. (1992) Sinoaortic denervation does not increase cardiovascular/endocrine responses to stress. *Neuroendocrinology* **56,** 735–744.

38. Pascoe, J. P., and Kapp, B. S. (1985) Electrophysiological characteristics of amygdaloid central nucleus neurons during Pavlovian fear conditioning in the rabbit. *Behav. Brain Res.* **16,** 117–33.

39. Ma, J., Aou, S., Matsui, H., and Hori, T. (1993) Gastric vagus mediates immobilization-induced hypocalcemia in rats. *Am. J. Physiol.* **265,** R609–R614.

40. Zhang, J.-F., and Zheng, F. (1997) The role of the paraventricular nucleus of the hypothalamus in stress-ulcer formation in rats. *Brain Res.* **761,** 203–209.

41. McCarty, R. (1985) Sympathetic-adrenal medullary and cardiovascular responses to acute cold stress in adult and aged rats. *J. Auton. Nerv. Syst.* **12,** 115–122.

42. Dering, M. A., Santer, R. M., and Watson, A. H. D. (1996) Age-related changes in the morphology of preganglionic neurons projecting to the rat hypogastric ganglion. *J. Neurocytol.* **25,** 555–563.

43. Chiueh, C. C., Nespor, S. M., and Rapoport, S. I. (1980) Cardiovascular, sympathetic and adrenal cortical responsiveness of aged Fischer-344 rats to stress. *Neurobiol. Aging* **1,** 157–163.

44. Iwata, J., LeDoux, J. E., and Reis, D. J. (1986) Destruction of intrinsic neurons in the lateral hypothalamus disrupts the classical conditioning of autonomic but not behavioral emotional responses in the rat. *Brain Res.* **368,** 161–166.