

**Depolarizing stimuli cause persistent and selective loss of orexin in rat
hypothalamic slice culture**

Hiroshi Katsuki^a, Shinsuke Kurosu^b, Shotaro Michinaga^a, Akinori Hisatsune^a, Yoichiro
Isohama^a, Yasuhiko Izumi^b, Toshiaki Kume^b, Akinori Akaike^b

^aDepartment of Chemico-Pharmacological Sciences, Graduate School of Pharmaceutical
Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan.

^bDepartment of Pharmacology, Graduate School of Pharmaceutical Sciences, Kyoto
University, 46-29 Yoshida-shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan.

Address correspondence to:

Hiroshi Katsuki, Ph.D.

Department of Chemico-Pharmacological Sciences

Graduate School of Pharmaceutical Sciences, Kumamoto University

5-1 Oe-honmachi, Kumamoto 862-0973, Japan

Phone: +81-96-371-4180 Fax: +81-96-362-7795

E-mail: hkatsuki@gpo.kumamoto-u.ac.jp

ABSTRACT

A hypothalamic neuropeptide orexin (hypocretin) is a critical regulator of physiological processes including sleep/wakefulness and feeding. Using organotypic slice culture of rat hypothalamus, we found that exposure to elevated extracellular concentration of K^+ (+10 - 30 mM) for 24 - 72 h led to a substantial decrease in the number of neurons immunoreactive for orexin and a co-existing neuropeptide dynorphin-A. In contrast, the same treatment affected neither the number of melanin-concentrating hormone-immunoreactive neurons nor the number of total neurons. A substantial decrease of orexin-immunoreactive neurons was also induced by 72 h treatment with 1 - 10 μ M veratridine, a Na^+ channel activator. The effect of elevated K^+ was only partially reversible, and that of veratridine was virtually irreversible, although the decrease in orexin immunoreactivity was not associated with signs of cell damage assessed by propidium iodide uptake and Hoechst 33342 nuclear staining. In addition, the level of preproorexin mRNA did not decrease during treatment with elevated K^+ or veratridine. After treatment with elevated K^+ and veratridine, c-Fos immunoreactivity appeared in orexin-immunoreactive neurons but not in melanin-concentrating hormone-immunoreactive neurons, suggesting selective excitation of orexin neurons. However, the amount of orexin released extracellularly was paradoxically decreased by treatment with elevated K^+ and veratridine. Overall, these characteristics of orexin neurons may be taken into consideration to understand the behaviors of these neurons under physiological and pathophysiological conditions.

Keywords: c-Fos; Depolarization; Dynorphin; Hypocretin; Hypothalamus;

Melanin-concentrating hormone

1. Introduction

The lateral hypothalamic area contains a distinct set of neuronal population possessing orexin (also called hypocretin) as a neuropeptide transmitter. Increasing lines of evidence indicate that orexins A and B, derived from a single preproorexin peptide, plays an important role in various biological functions [17]. Physiological functions of orexin include regulation of food intake, energy homeostasis and neuroendocrine functions, and other lines of evidence suggest involvement of orexin in drug addiction and dependence [12].

Regulation of sleep and wakefulness is among the most well-known functions of orexin, along with the relationship to narcolepsy, a neurological disorder characterized by excessive daytime sleepiness, sleep attacks and intrusions of rapid-eye-movement sleep into wakefulness [19]. Firm evidence linking narcolepsy with abnormalities in human orexin system comes from the facts that the concentration of orexin in the cerebrospinal fluid of the majority of narcolepsy patients is decreased to an undetectable level [15], and also that orexin neurons are selectively decreased in the hypothalamus of the patients [16, 21].

The mechanisms underlying selective loss of orexin neurons leading to narcolepsy remain unclear. We previously explored possible involvement of excitotoxicity, because excitotoxic events triggered by over-activation of glutamate receptors are involved in the pathogenesis of various neurological disorders associated with neuron loss [5]. We found that an endogenous *N*-methyl-D-aspartate receptor agonist, quinolinic acid, caused selective loss of orexin neurons as compared to melanin-concentrating hormone (MCH)-containing neurons in hypothalamic slice culture [9]. Although the precise mechanisms of the selective vulnerability to excitotoxic challenge are unclear, they might be related to the electrophysiological characteristics of orexin neurons. That is, orexin neurons have a

depolarized membrane potential as compared to MCH neurons and show little spike frequency adaptation [6], which may promote opening of *N*-methyl-D-aspartate receptor channels. We have also shown that GABA_A receptor stimulation, which is expected to suppress membrane depolarization, prevents quinolinic acid-induced death of orexin neurons [10].

Since these observations suggest that regulation of membrane excitation plays a critical role in the maintenance of viability of orexin neurons, here we examined effects of manipulations that influence neuronal membrane potential.

2. Materials and methods

2.1. Culture preparation and drug treatment

Organotypic hypothalamic slice cultures were prepared essentially according to the methods described previously [9, 13]. The procedures were approved by our institutional animal experimentation committee, and animals were treated in accordance with the Guidelines of the United States National Institutes of Health regarding the care and use of animals for experimental procedures. Seven to eight-day-old Wistar rats (Nihon SLC, Shizuoka, Japan) under deep anesthesia with diethyl ether inhalation were decapitated, and brains were removed from the skull. Brains were cut into two hemispheres and coronal slices of 300 μm thickness were prepared with a tissue chopper. Hypothalamic slices at rostrocaudal level containing the lateral hypothalamic area and the arcuate nucleus were selected for cultivation (consecutive five slices per each hemisphere). Slices from each hemisphere were embedded together onto a microporous membrane (Millicell-CM, Millipore,

Bedford, MA, USA) placed in a six-well culture plate. Culture medium, consisting of 50% minimal essential medium/Hepes (GIBCO, Invitrogen Japan, Tokyo, Japan), 25% Hanks' balanced salt solution (GIBCO) and 25% heat-inactivated horse serum (GIBCO) supplemented with 6.5 mg/ml glucose, 2 mM L-glutamine, 100 U/ml penicillin G potassium and 100 µg/ml streptomycin sulfate (GIBCO), was supplied at 0.9 ml/well. Culture medium was replaced with fresh medium on the next day of culture preparation, and thereafter, three times a week. Slices were maintained in a humidified atmosphere of 5% CO₂ and 95% air at 34 °C. At 18 - 21 days *in vitro*, slices were treated with culture medium with added KCl (10 or 30 mM) or culture medium containing veratridine (0.3 - 10 µM; Nacalai Tesque, Kyoto, Japan). Where indicated, colchicine (10 µM; Nacalai Tesque) or tetrodotoxin (1 µM; Nacalai Tesque) was concomitantly applied with KCl or veratridine.

2.2. Immunohistochemistry and cell counting

After drug treatment, cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 h at 4°C, and subjected to double immunofluorescence histochemistry for orexin and MCH, as described [9]. Goat polyclonal anti-orexin A (C-19) antibody (1:150, sc-8070, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and rabbit anti-MCH antiserum (1:800, H-070-47, Phoenix Pharmaceuticals Inc., Belmont, CA, USA) were used as primary antibodies, and Alexa Fluor 488-labeled donkey polyclonal anti-goat IgG(H+L) (1:200, A-11055, Molecular Probes, Eugene, OR, USA) and Alexa Fluor 594-labeled donkey polyclonal anti-rabbit IgG(H+L) (1:500, A-21207, Molecular Probes) were used as respective secondary antibodies. Fluorescence signals were observed with the use of an epifluorescence microscope (Olympus, Tokyo, Japan). The number of

immunoreactive neurons in an area of $420 \times 640 \mu\text{m}^2$ in individual slices was counted. As described previously [9], middle three slices within five consecutive coronal slices typically contained many orexin-immunoreactive neurons, whereas the number of immunoreactive neurons was considerably low in the remaining two (the most rostral and the most caudal) slices, because distribution of orexin neurons is confined to the area that encompasses about 1 mm rostrocaudally in rat brain [11]. Therefore, values obtained from three out of five slices per one culture insert were adopted for assessment of the number of orexin and MCH neurons.

In the case of dynorphin immunohistochemistry, rabbit anti-dynorphin A antibody (1:200, PC210, EMD Chemicals Inc., San Diego, CA, USA) was used as a primary antibody, and Tyramide Signal Amplification Kit with goat anti-rabbit IgG and Alexa Fluor 594 (T-20925, Molecular Probes) was used to detect specific labeling of dynorphin. Procedures for fluorescence observation and cell counting were the same as those for orexin and MCH.

In experiments of double staining for orexin and NeuN, mouse anti-NeuN monoclonal antibody (1:400; MAB377, Millipore) was used instead of anti-MCH antiserum, and immunoreactivity against NeuN was detected with Cy3-conjugated donkey anti-mouse IgG(H+L) (1:400; 610-704-124, Rockland Immunochemicals, Gilbertsville, PA, USA). Immunofluorescence was examined with a laser scanning confocal microscopy (MRC-1024, BioRad, Hercules, CA, USA), and the number of immunopositive neurons was counted in an area of $540 \times 540 \mu\text{m}^2$ in individual slices.

c-Fos immunoreactivity was detected with mouse anti-c-Fos monoclonal IgG (1:100, sc-8047, Santa Cruz) and Alexa Fluor 594 donkey anti-mouse IgG (1:200, A-21203, Molecular Probes) as a primary and a secondary antibody, respectively, in combination with

immunoreactivity for orexin or MCH. In this case, Alexa Fluor 488-labeled donkey polyclonal anti-rabbit IgG(H+L) (1:200, A-21206, Molecular Probes) was used as a secondary antibody to detect MCH immunoreactivity.

2.3. Nuclear staining with propidium iodide and Hoechst 33342

Propidium iodide (PI) fluorescence was used to detect occurrence of cell death [1]. PI (5 µg/ml) was applied to slice cultures during drug treatment. After the treatment, slices were fixed with 4% paraformaldehyde and were subjected to orexin immunohistochemistry as described above. For examination of nuclear morphology, fixed slices were treated with 0.1 mg/ml Hoechst 33342 for nuclear staining after orexin immunohistochemistry.

2.4. Reverse transcription - polymerase chain reaction (RT-PCR) for preproorexin mRNA

After treatment with KCl or veratridine for indicated periods, slices were collected in 400 µl of ISOGEN reagent (Nippon Gene, Tokyo, Japan), homogenized on ice, and total RNA was extracted according to the manufacturer's instructions. RT-PCR was performed with PrimeScript RT-PCR kit (RR014A, TaKaRa, Shiga, Japan): 42 °C for 30 min, 95 °C for 5 min, and 4 °C for 5 min for RT; and 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 1.5 min for PCR. PCR products were subjected to 1.5% agarose gel electrophoresis. The primer sequences were as follows: preproorexin forward 5'-CGGATTGCCTCTCCCTGAGC-3', preproorexin reverse 5'-CTAAAGCGGTGGCGGTTGC-3' (product: 397 bp); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5'-GCCAAGGTCATCCATGACAAC-3', GAPDH reverse 5'-AGTGTAGCCCAGGATGCCCTT-3' (product: 351 bp).

2.5. Radioimmunoassay for orexin

For quantification of orexin peptide released into culture medium, treatment of slice cultures with KCl or veratridine was performed in the presence of Halt™ protease inhibitor cocktail (5 µl/ml, Pierce Biotech., Rockford, IL, USA). After the treatment, culture medium was collected, and the concentration of orexin was determined with orexin A radioimmunoassay kit (RK-003-30, Phoenix Pharmaceuticals Inc.), according to the manufacturer's instructions.

2.6. Statistical analysis

Data are expressed as means ± S.E.M. Statistical significance was evaluated by one-way analysis of variance followed by Student-Newman-Keuls test. Probability values less than 0.05 were considered significant.

3. Results

3.1. Elevated extracellular K^+ decreases orexin neurons but not MCH neurons

Exposure to elevated concentrations of extracellular K^+ is a conventional method to cause membrane depolarization and neuronal excitation [8]. To test the effect of chronic membrane depolarization on orexin neurons, we maintained hypothalamic slice cultures in medium with added KCl. Double immunofluorescence histochemistry showed that 72 h treatment with 10 or 30 mM KCl caused a substantial decrease in the number of orexin-immunoreactive neurons, without significantly affecting the number of

MCH-immunoreactive neurons (Fig. 1A). The effect of KCl on orexin neurons was already apparent and significant at 24 h after initiation of the treatment, although at this time point the degree of the decrease of orexin-positive neurons was smaller than that after 72 h treatment (Fig. 1B). In a control experiment, we confirmed that 72 h treatment with 30 mM NaCl instead of KCl produced no effect on the number of orexin-immunoreactive neurons (data not shown). To examine whether the decrease of orexin-positive neurons was a reversible event, a group of slices was treated with added KCl for 72 h, then maintained in normal culture medium for further 72 h, and processed for immunohistochemical examination. In this case, the number of orexin-positive neurons was larger than that in cultures fixed immediately after 72 h treatment with KCl, suggesting partial recovery of orexin immunoreactivity (Fig. 1C). However, the decrease of orexin-positive neurons was still significant.

We also examined immunoreactivity for NeuN, a neuronal marker, to determine whether or not widespread loss of neurons occurred after KCl treatment. As shown in Fig. 2, 72 h treatment with 10 or 30 mM KCl did not significantly affect the number of NeuN-positive cells, whereas the number of orexin-positive neurons in the same area clearly decreased.

3.2. Veratridine selectively decreases orexin neurons

Elevated extracellular K^+ is expected to cause membrane depolarization by shifting K^+ equilibrium potential. We next examined if other procedures to cause membrane excitation could mimic the effect of KCl treatment. For this purpose, we used veratridine, a Na^+ channel activator that abolishes inactivation of voltage-dependent Na^+ channels, thereby maintains membrane depolarization [22, 23]. We found that 72 h treatment with veratridine at or over 1 μ M caused a significant decrease in the number of orexin-immunoreactive

neurons, without affecting the number of MCH neurons (Fig. 3A). The effect of veratridine was virtually irreversible, as the number of orexin-positive neurons did not show any recovery after 72 h of washout period (Fig. 3B).

3.3. Dynorphin-A is decreased concomitantly with orexin

Nearly all orexin neurons in the lateral hypothalamic area are shown to contain dynorphin-A as a co-transmitter [3], and a concomitant loss of dynorphin with orexin has been demonstrated in the brain of narcolepsy patients [4]. Accordingly, we examined if the treatment with elevated extracellular K^+ or with veratridine could result in loss of dynorphin. Double immunofluorescence histochemistry for orexin and dynorphin-A on slices cultured under control conditions revealed that the majority of orexin neurons exhibit immunoreactivity for dynorphin-A (Fig. 4A). Treatment of slice cultures with KCl or veratridine for 72 h caused a substantial loss of dynorphin-A immunoreactivity as well as orexin immunoreactivity (Fig. 4B, C).

3.4. Neither elevated extracellular K^+ nor veratridine induces death of orexin neurons

Theoretically, a decrease in orexin-immunoreactive neurons may result either from induction of cell death, or from depletion of orexin peptide stored within the cells. To determine whether induction of cell death was attributable to the observed reduction of orexin neurons, we used PI uptake for detection of dead cells in slice cultures [7]. Treatment of slice cultures with 30 mM KCl for 72 h in the presence of PI did not result in the appearance of cells exhibiting PI fluorescence. On the other hand, several cells exhibited PI fluorescence after treatment with 10 μ M veratridine for 72 h, but not for 48 h. However,

orexin immunoreactivity and PI fluorescence never merged on the same cells, suggesting that orexin neurons did not undergo cell death during this treatment (Fig. 5A). In addition, PI fluorescence was not observed in slices treated for 72 h with a lower concentration (1 μ M) of veratridine (data not shown), a treatment that effectively decreased orexin immunoreactivity (see Fig. 3A).

We also carried out nuclear staining with Hoechst 33342 to reveal if the loss of orexin neurons was accompanied by any changes in nuclear morphology indicative of cell degeneration. As shown in Fig. 5B, 72 h treatment with 30 mM KCl or 10 μ M veratridine did not induce alterations of nuclear morphology, such as shrinkage, fragmentation, or swelling, in orexin-immunoreactive neurons. As a control, we confirmed that 24-h treatment with 1 μ M staurosporine caused clear shrinkage of the nucleus of neurons (data not shown).

3.5. Preproorexin mRNA does not decrease, and orexin release does not increase, in response to elevated extracellular K^+ or veratridine

In the next set of experiments, we measured the levels of preproorexin mRNA to gain insight into the mechanisms of orexin depletion by high K^+ and veratridine. Results of semi-quantitative RT-PCR showed that treatment of slice cultures with 10 mM KCl for 6 - 72 h did not result in a decrease in the expression levels of preproorexin mRNA (Fig. 6A). The same was true for veratridine treatment: the levels of preproorexin mRNA did not decrease, but rather, showed a tendency to increase during treatment with 1 μ M veratridine for 6 - 72 h (Fig. 6B). Therefore, the decrease of orexin peptide cannot be explained by changes in transcriptional activity on the gene encoding orexin.

Because depolarization induced by high extracellular K^+ and veratridine is expected to

enhance neurotransmitter release, a plausible mechanism of orexin depletion is that the amount of neuropeptide released extracellularly overwhelms that of newly synthesized peptide. To obtain support for this view, we measured the concentration of orexin peptide released into culture medium. Unexpectedly, however, we found that 6 h treatment of slice cultures with 10 mM KCl or with 1 μ M veratridine significantly decreased the concentration of orexin in culture medium (Fig. 6C).

3.6. Redistribution of orexin within neurons may contribute to the disappearance of orexin in the cell body

During the course of investigations, we noted that disappearance of orexin immunoreactivity in cell bodies was frequently associated with an apparent increase in orexin immunoreactivity in axon-like processes (for example, see Fig. 1A). This increase in orexin immunoreactivity was observed as early as 3 h after initiation of KCl treatment (Fig. 7A). To examine whether axonal transport participated in depletion of orexin in the cell body, we used colchicine, a drug that inhibits tubulin polymerization and axonal transport. We found that concomitant application of 10 μ M colchicine for 72 h completely prevented the decrease in the number of orexin-immunoreactive cell bodies by 10 mM KCl or by 1 μ M veratridine (Fig. 7B).

3.7. c-Fos expression suggests preferential excitation of orexin neurons over MCH neurons by elevated extracellular K^+ and veratridine

The above results on orexin peptide release casted a concern about whether the treatment procedures employed in this study were indeed effective in causing neuronal excitation. To evaluate the extent of stimulus-induced neuronal excitation, we examined c-Fos expression in

orexin and MCH neurons. c-Fos is a product of an immediate early gene whose expression is initiated by electrical excitatory activities of neurons, and c-Fos expression has been used to examine activation of orexin neurons associated with behavioral states and physiological stimuli in vivo [14, 24].

c-Fos immunoreactivity was not observed in orexin neurons in slices maintained under control culture conditions, whereas a significant number of orexin-immunoreactive neurons showed c-Fos expression in response to KCl treatment (Fig. 8A). The number of c-Fos-positive orexin neurons reached a peak at 3 h after initiation of KCl treatment (Fig. 8B). During this short-term period, the number of orexin-immunoreactive neurons did not change (data not shown). Interestingly, no MCH neurons exhibited c-Fos immunoreactivity in response to KCl treatment (Fig. 8A). Similarly, treatment with 1 μ M veratridine for 3 - 6 h induced c-Fos expression in a significant population of orexin neurons (Fig. 8C), but not in MCH neurons. The increase in c-Fos expression by 3-h treatment with 10 mM KCl or 1 μ M veratridine was virtually abolished by concurrent application of 1 μ M tetrodotoxin, a Na⁺ channel blocker (data not shown).

4. Discussion

We demonstrated here that elevation of extracellular K⁺ concentration or application of veratridine caused a dramatic decrease in the number of orexin-containing neurons but not that of MCH-containing neurons in hypothalamic slice cultures. Manipulation of the K⁺ equilibrium potential by elevating extracellular levels of K⁺ is a conventional method to produce membrane depolarization. K⁺ equilibrium potential under control culture conditions

and conditions with added 10 and 30 mM KCl is estimated to be around -89 , -61 and -38 mV, respectively, with the assumption that intracellular concentration of K^+ is 150 mM. On the other hand, veratridine is known to sustain membrane depolarization by preventing inactivation of voltage-dependent Na^+ channels and keeping these channels open [22]. The different sensitivities of orexin neurons and MCH neurons to these stimuli are highlighted by the finding that c-Fos expression was induced after treatment with KCl or veratridine in orexin- but not MCH-containing neurons. Although the precise mechanisms remain unknown, distinct electrophysiological characteristics of these neurons, such as the levels of resting membrane potential and spike frequency adaptation [6], may be involved in the selectivity of the effects of depolarizing stimuli.

Although a substantial decrease in orexin-containing neurons was induced by high K^+ and veratridine, this decrease was unlikely to be mediated by induction of cell death in orexin neurons. Indeed, sustained excitation by elevated extracellular K^+ or by application of veratridine has been shown to produce a beneficial, rather than deleterious, effect on survival of several types of central neurons [8, 18]. We showed here that the number of NeuN-immunoreactive cells did not change after KCl treatment. Moreover, examinations with PI fluorescence and Hoechst 33342 staining revealed no signs of death or degeneration of orexin neurons during the treatment with KCl and veratridine. The observation that the levels of preproorexin mRNA expression did not decrease in response to KCl and veratridine is also consistent with the view that orexin neurons do survive these conditions. Therefore, we suggest that the neurons have lost the ability to retain orexin peptide within the cell bodies at sufficient amount detectable by immunohistochemical examinations. We should also note here that the loss of orexin-A immunoreactivity means depletion of preproorexin and

proorexin peptides as well as mature orexin-A peptide, as the precursor peptides should be immunoreactive for the antibody used in the present study.

An unexpected finding was that our procedures initially intended to increase neuron excitability did not lead to facilitation of orexin release into the extracellular compartment. That is, 6 h treatment with KCl and veratridine decreased orexin concentration in culture medium, although the same procedures were effective in inducing c-Fos expression indicative of activation of orexin neurons. Induction of c-Fos expression by KCl and veratridine was dependent on action potential generation, since it was blocked by tetrodotoxin. A plausible explanation for these apparently discrepant results is that conditions of excitation required to induce release of orexin are different from those sufficient for inducing c-Fos expression. Neuropeptide transmitters are generally stored in large dense-core vesicles positioned at a distance from the plasma membrane. In contrast to that of small molecule neurotransmitters such as monoamines and amino acids, exocytotic release of neuropeptides is initiated only by trains of action potential bursts allowing large amount of Ca^{2+} entry required to vesicle fusion [25]. On the other hand, much lower levels of activity may be able to induce c-Fos expression. For example, in mouse dorsal root ganglion neurons, one action potential every 10 s is sufficient to stimulate c-Fos expression [2, 20].

The precise mechanisms of depletion of orexin peptide (and its precursors) should be addressed in future investigations. Because the level of preproorexin mRNA expression did not decline in response to KCl and veratridine, the most straightforward interpretation is that translational processes for peptide synthesis may be compromised under continuous excitation. The observations that dynorphin A decreased concomitantly with orexin, and that orexin immunoreactivity did not easily recover after washout of KCl or veratridine, may also

provide a clue to elucidate detailed mechanisms of orexin depletion.

It is also unclear why orexin peptide already stored within the cells disappears in response to KCl and veratridine treatment without accompanying an increase in extracellular release of orexin. But one point deserving attention is that we identified orexin neurons by their immunoreactivity for orexin in the cell body. Therefore, redistribution of orexin peptide within the cells, particularly via enhancement of axonal transport of orexin-containing vesicles, can contribute to depletion of orexin at the cell body level. Indeed, we found that colchicine prevented disappearance of orexin immunoreactivity in the cell body, indicating that axonal transport may be crucial for orexin depletion we observed. We also do not formally exclude the possibility that some kinds of peptide degradation systems are recruited to eliminate stored neuropeptide during treatment with high K^+ and veratridine.

Whether or not neurodegeneration occurs in the hypothalamus of narcolepsy patients is still a matter of debate. One report demonstrating selective loss of orexin neurons in the patients has described glial cell activation suggestive of the involvement of degeneration processes [21], whereas another report found no evidence for inflammatory events [16]. A finding generally considered to be strong evidence for neurodegeneration in narcolepsy is that there is a concomitant decrease of neurons immunoreactive for dynorphin and orexin as well as of neuronal activity-regulated pentraxin, a subtype of pentraxin preferentially expressed in orexin neurons [4]. However, our findings indicate that similar changes in expression levels, at least for dynorphin and orexin, can be induced by altered neuronal excitatory activities without accompanying neuronal cell death. Apart from narcolepsy, depletion of orexin during continuous excitation might be related to development of irresistible sleepiness during long-term sleep deprivation, given that orexin-mediated neurotransmission is important for

maintaining wakefulness.

In conclusion, we demonstrated here that continuous excitation could cause depletion of orexin peptide from orexin neurons. Notably, we have recently shown that inhibition of neural activity results in reversible depletion of orexin [13]. These findings, indicating that proper levels of neural activity are crucial for maintaining normal functions of orexin neurons, may provide valuable clues to understand behaviors of orexin neurons under physiological and pathophysiological conditions.

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Figure Legends

Fig. 1. Elevated extracellular K^+ induces selective loss of orexin-containing neurons. (A) Representative photomicrographs showing orexin and MCH immunoreactivity in hypothalamic slice cultures. Slices were cultured under control conditions or treated with added KCl (10 mM) for 72 h, then processed for immunohistochemical examinations. Scale bar, 50 μ m. (B) Effect of KCl treatment on the number of orexin- and MCH-immunoreactive neurons. Slice cultures were maintained in medium with added KCl (10 and 30 mM) for 24 - 72 h. The number of immunoreactive neurons was normalized with reference to the mean number in control cultures as 100%. $n = 9 - 12$ for each condition. ** $P < 0.01$, *** $P < 0.001$ vs. control. (C) Effect of KCl treatment was partially reversible. Immunohistochemistry was performed on slices promptly after 72 h treatment with KCl (72 h), or after cultivation in normal medium for 72 h following 72-h KCl treatment (72 h + post.). $n = 15-21$ for each condition. *** $P < 0.001$ vs. control; # $P < 0.05$.

Fig. 2. Elevated extracellular K^+ does not affect the number of total neurons. (A) Representative confocal fluorescence images showing orexin and NeuN immunoreactivity in the same field of a hypothalamic slice cultured under control conditions (left panels) or a slice treated with 30 mM KCl for 72 h (right panels). Scale bar, 50 μ m. (B) Summary of the effect of KCl treatment on the number of orexin- and NeuN-immunoreactive cells. Immunohistochemistry was performed after 72 h treatment of slice cultures with KCl (10 and 30 mM). $n = 18$ for each condition. *** $P < 0.001$ vs. control.

Fig. 3. Veratridine causes selective and virtually irreversible loss of orexin-containing

neurons. (A) The number of orexin- and MCH-immunoreactive neurons was determined after 72 h treatment with indicated concentrations of veratridine. $n = 9 - 12$ for each condition. $*** P < 0.001$ vs. control. (B) Same as in (A) for veratridine treatment, but slices were maintained in drug-free normal culture medium for 72 h before immunohistochemical examination. $n = 12 - 15$ for each condition. $*** P < 0.001$ vs. control.

Fig. 4. Dynorphin-A immunoreactivity is lost in response to KCl and veratridine. (A) Shown are representative confocal fluorescence images of orexin and dynorphin-A immunoreactivities, observed in the same field of a slice cultured under control conditions (left panels) and a slice treated with 10 mM KCl for 72 h (right panels). Scale bar, 50 μ m. (B) Effect of KCl treatment on the number of dynorphin-A-immunoreactive neurons. Slices were exposed to KCl at indicated concentrations for 72 h. $n = 12 - 15$ for each condition. $*** P < 0.001$ vs. control. (C) Effect of veratridine on the number of dynorphin-A-immunoreactive neurons. Veratridine at indicated concentrations was applied to slice cultures for 72 h. $n = 18 - 21$ for each condition. $** P < 0.01$, $*** P < 0.001$ vs. control.

Fig. 5. Orexin neurons do not show signs of cell damage in response to KCl and veratridine. (A) Propidium iodide (PI) staining was performed during 72 h treatment of slice cultures with 30 mM KCl and 10 μ M veratridine, then slices were processed for orexin immunohistochemistry. Orexin-immunoreactive neurons (green) are pointed by arrows. PI fluorescence (red) was observed only after veratridine treatment in several cells other than

orexin-containing neurons (right panel). Scale bar, 20 μm . (B) Orexin immunoreactivity (green, upper panels), Hoechst 33342 nuclear staining (blue, middle panels), and their merged images (lower panels) of slices cultured under control conditions, or treated with 30 mM KCl or 10 μM veratridine for 72 h. Arrows indicate nuclei of orexin-immunoreactive neurons. Scale bar, 20 μm .

Fig. 6. KCl and veratridine do not decrease preproorexin (PPO) mRNA expression, and do not increase orexin release. Slice cultures were treated with 10 mM KCl (A, n = 6) or 1 μM veratridine (B, n = 7) for indicated periods, and the level of PPO mRNA was determined by semi-quantitative RT-PCR with reference to the level of GAPDH mRNA. Typical results of RT-PCR for PPO and GAPDH mRNAs are shown above the graphs. (C) After 6 h treatment with 10 mM KCl (n = 8) or 1 μM veratridine (n = 8) in the presence of protease inhibitor cocktail, culture medium was harvested and orexin concentration was measured with enzyme immunoassay. * $P < 0.05$, ** $P < 0.01$ vs. control (n = 12).

Fig. 7. Redistribution of orexin may be involved in the disappearance of orexin within cell bodies. (A) Representative confocal images showing orexin immunoreactivity in axon-like processes in slices cultured under control conditions (left) or treated for 3 h with 10 mM KCl (right). Scale bar, 50 μm . (B) Effect of colchicine (col.) on KCl- or veratridine-induced decrease in the number of orexin-immunoreactive neurons. Colchicine (10 μM) was applied concomitantly with 10 mM KCl or 1 μM veratridine for 72 h. n = 9 for each condition. *** $P < 0.001$ vs. control (without drug treatment); ### $P < 0.001$ vs. group without colchicine.

Fig. 8. c-Fos expression is induced in orexin neurons by KCl and veratridine. (A) Double immunofluorescence histochemistry was performed for c-Fos (red) and orexin (green, upper panels), or for c-Fos (red) and MCH (green, lower panels). Before immunohistochemical processing, slice cultures were exposed to 10 mM KCl for 3 h. Arrows indicate several (but not all) c-Fos-immunoreactive orexin neurons. Scale bar, 50 μ m. (B) Effect of KCl on the number of neurons immunopositive for both orexin and c-Fos. KCl (10 mM) was applied for indicated periods. n = 15 for each condition. (C) Effect of veratridine (1 μ M) on the number of neurons immunopositive for both orexin and c-Fos. n = 15 for each condition.

Figure 1

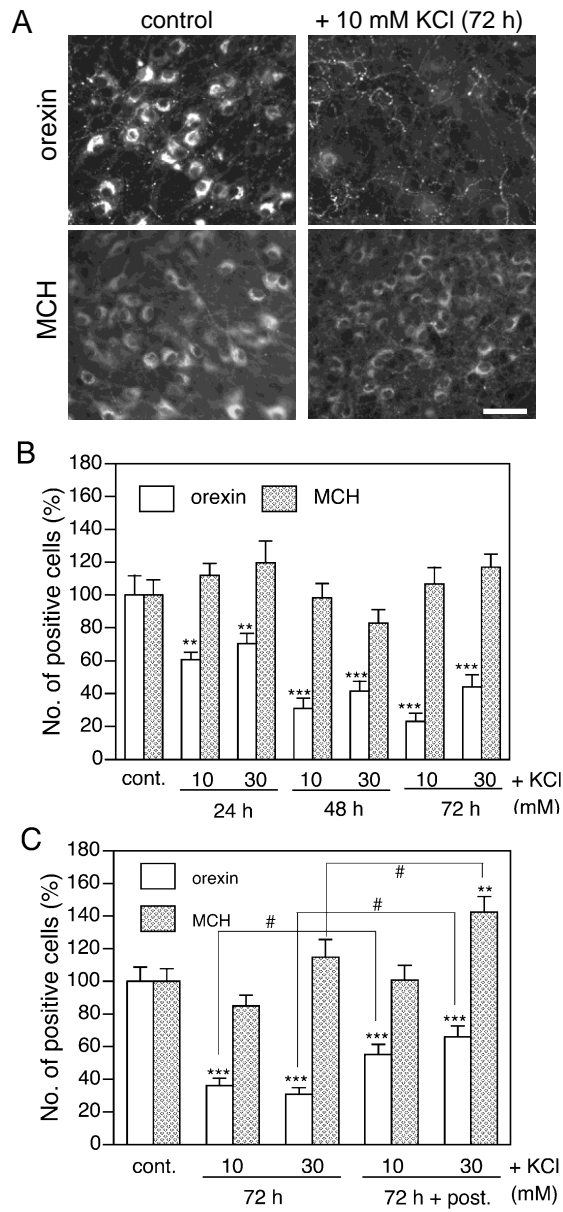


Figure 2

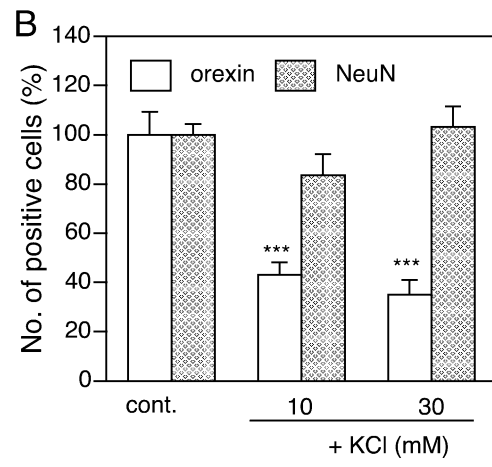
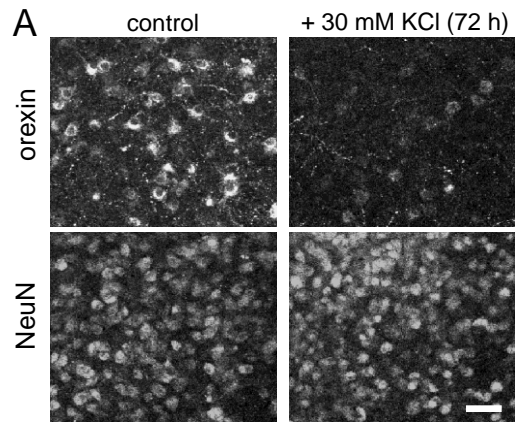


Figure 3

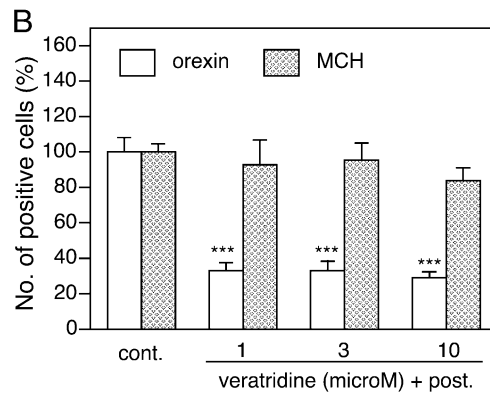
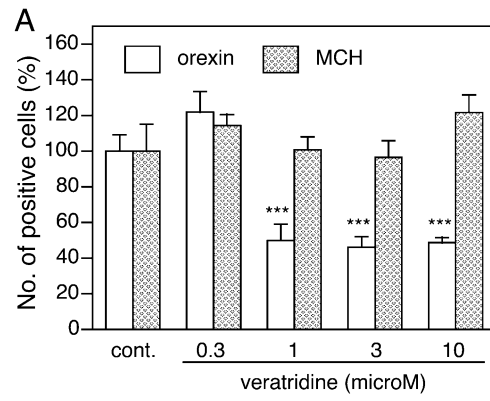


Figure 4

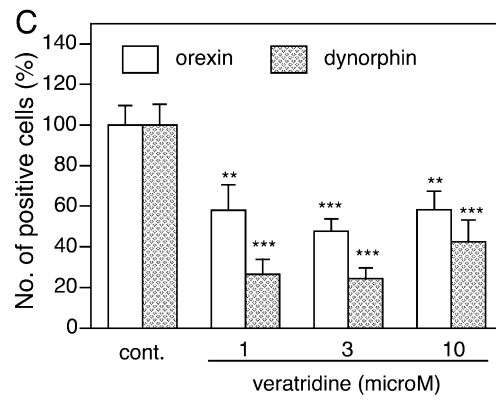
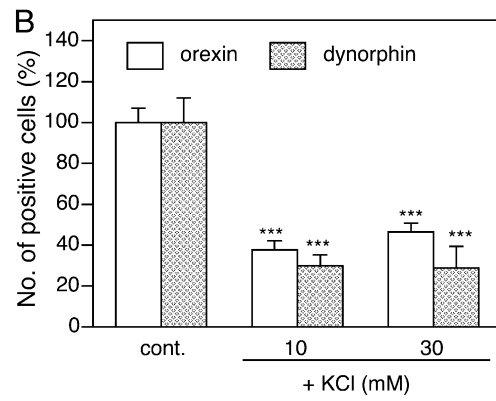
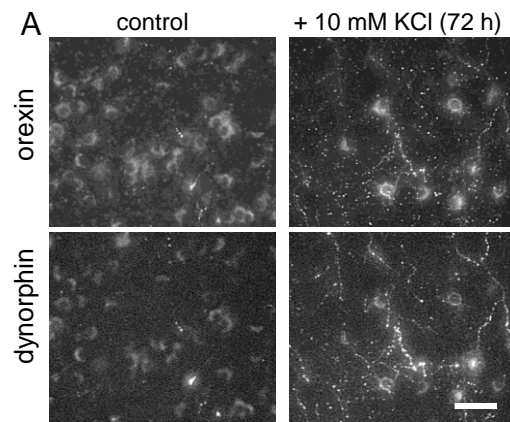


Figure 5

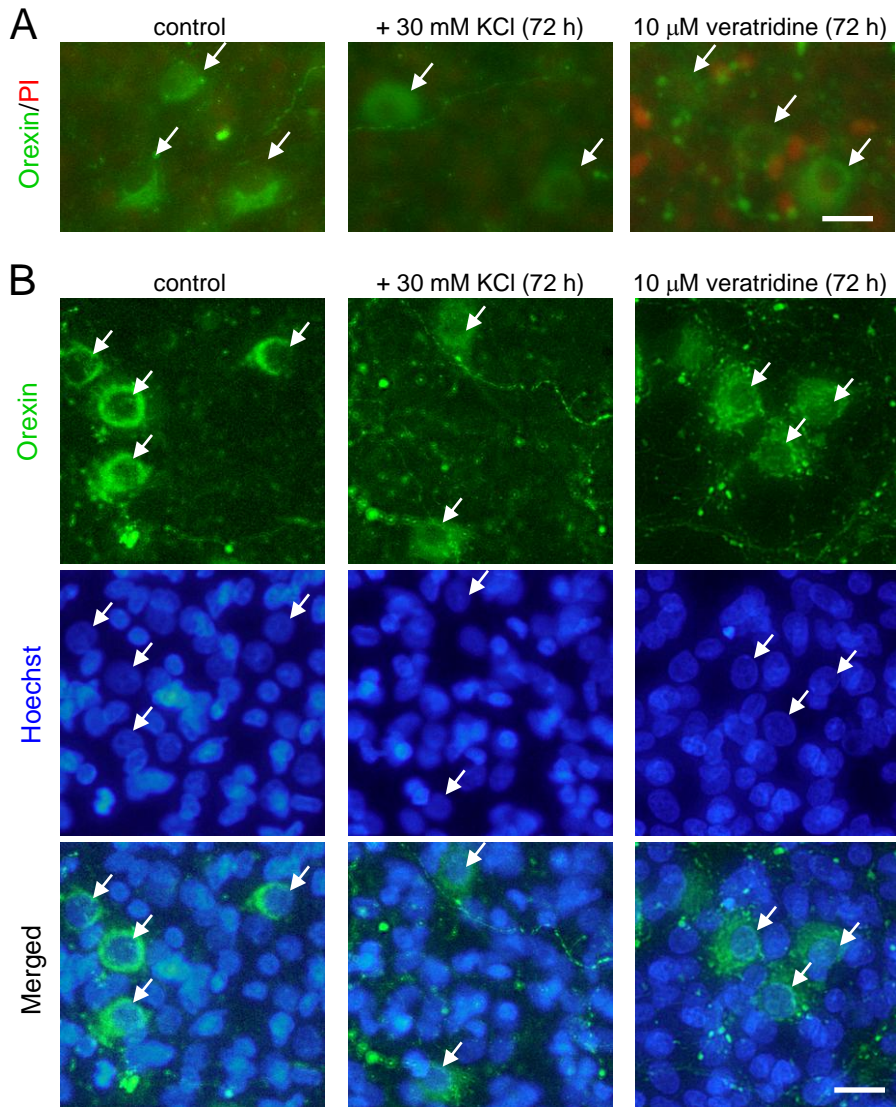


Figure 6

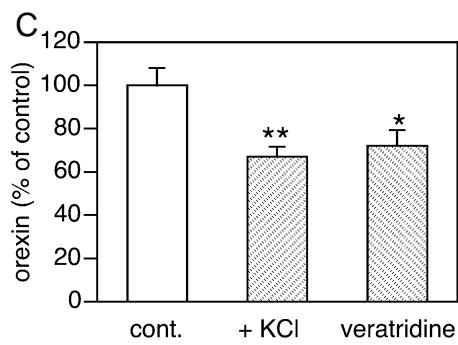
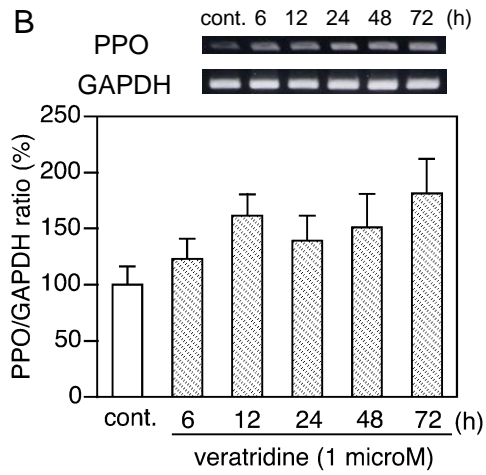
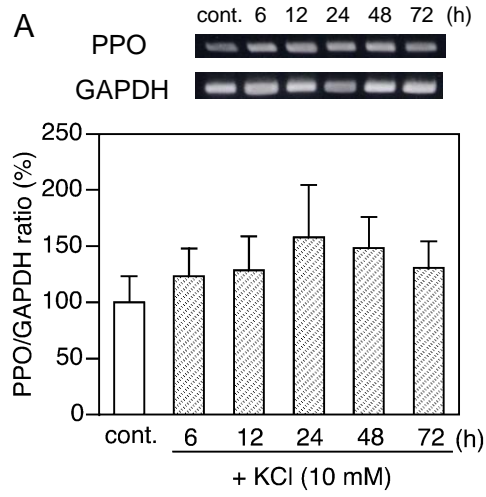


Figure 7

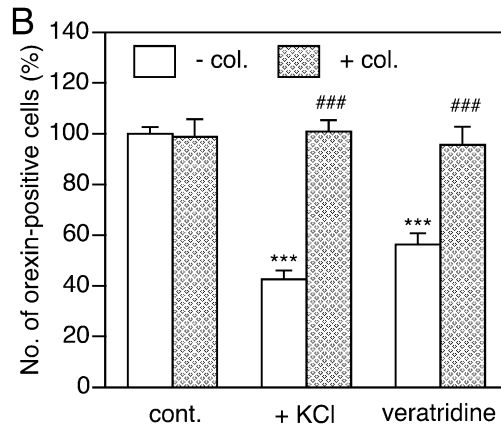
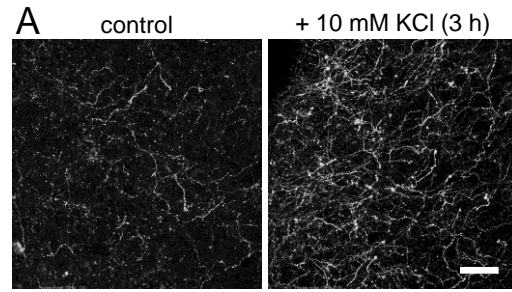


Figure 8

