Studies on Improvement of Sleep Environment Based on Function of Natural Product and Orexin Neuron

January 2017

Yo MURAKI

Studies on Improvement of Sleep Environment Based on Function of Natural Product and Orexin Neuron

A Dissertation Submitted to
the Graduate school of Life and Environmental Sciences,
the University of Tsukuba,
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Environmental Studies
(Doctoral program in Sustainable Environmental Studies)

Yo MURAKI

Abstract

Sleep/wake homeostasis is important for human health. Due to recent changes in the environment, the number of patients suffering from sleep disorders such as insomnia is increasing by rapid change of human environment. The author conducted physiological study for sleep/wake homeostasis to understand relationship between environment factors and sleep disorder and analyzed possibility for treatment of sleep disorder by natural products.

The number of patients with sleep disorder is increasing by change of environments, such as shift work, jet lug by travel abroad, increase of stress and aging. Especially aging and increase of stress can change level of neurotransmitters in the brain including serotonin and noradrenaline. For treatment of insomnia, drug-therapy and non-drug therapy are used. As drug therapy, benzodiazepine drug, melatonin receptor agonist and orexin receptor antagonist was approved based on understanding for sleep/wake homeostasis. As non-drug therapy, natural products have been used for treatment method for insomnia. The natural products can ameliorate sleep disorder by modulation of the effect of neurotransmitters. To understand physiological effects of neurotransmitters to important system for sleep/wake homeostasis can provide useful information to develop treatment method for insomnia using natural products.

The author analyzed the effect of neurotransmitters and input pathway for orexin neuron because of their impotence in sleep/wake homeostasis. The role of orexin neuron, which produce orexin peptides, in sleep/wake homeostasis have been clarified by animal and clinical analysis. Orexin neuron deficient mice showed impairment of sleep/wake homeostasis and orexin receptor antagonist ameliorate sleep disorder. In this study, the effect of neurotransmitters for orexin neurons was analyzed using slice patch clamp

methods on transgenic mice expressing green fluorescent protein specifically in orexin neuron and fluorescent microscope.

Serotonin and noradrenaline hyperpolarized membrane potential of orexin neuron. Serotonin-induced hyperpolarization was mediated by serotonin 1A receptor. On the other hands, noradrenaline-induced hyperpolarization was mediated by alpha 2 receptor and activation of gamma aminobutyric acid (GABA)-ergic neuron. Moreover, immunohistochemical study showed that serotonergic and adrenergic neural terminal was located near orexin neurons. Intracerebral administration of serotonin 1A antagonist for third ventricle increased locomotor activity in the night. The results indicating change of sleep time. The modulation of orexin neurons can be affect animal behavior.

The target molecule of sleep promoting natural products was analyzed based on database analysis and the possibility for modulation of orexin neuron was discussed. Valerenic acid, which is activator of GABA receptor, geissoschizine methyl ether, which is activator of serotonin 1A receptor, and rosmarinic acid, which can increase production of noradrenaline, is reported as sleep promoting natural products. These natural products can be considered to suppress orexin neuron activity based on the effect of neurotransmitters on orexin neuron. The results of this research can provide useful information for understanding the mechanism of action of natural products and development of new treatment method for sleep disorder using drug and natural products.

Contents

Abstract i
Contents iii
List of Tables vi
List of figures vi
Abbreviation and Acronyms viii
Publications xi
Chapter 1 Introduction 1
1.1 Sleep disorders and the environment 2
1.2 Epidemiology of insomnia 4
1.3 The use of natural products for insomnia 4
1.4 Current treatment methods for insomnia 8
1.5 The orexin system for sleep/wake homeostasis 8
1.6 Analysis of the regulation of the orexin system to understand the mechanism of natural products with sleep promoting effects 11
Orexin neurons have a key role in sleep/wake homeostasis in spite of their limited numbers. Compounds that can modulate the neuronal activity of orexin neurons can be an effective method for treatment of sleep disorders. 11
Chapter 2 Electrophysiological properties of orexin neurons 13
2.1 Introduction 14
2.2 Materials and methods 15
2.2.1 Animal usage 15
2.2.2 Slice preparation 15

2.2.3 Identification of EGFP-expressing orexin neurons by fluorescent microscopy 16
2.2.4 Electrophysiological recordings 16
2.2.5 Drugs 17
2.3 Results 19
2.3.1 Detection of orexin neurons in slice conditions 19
2.3.2 The effects of glutamatergic and GABAergic neurotransmitters 24
2.3.3 The effect of monoaminergic, cholinergic, and adrenergic neurotransmitters 26
2.3.4 The mechanism of suppression by serotonin 28
2.3.5 The mechanism of suppression of orexin neurons by NA 35
2.3.6. The mechanism of activation of orexin neurons by NA 40
2.3.7. The effect of NA on calcium currents 43
2.4 Discussion 45
Chapter 3 The importance of the regulation of orexin neurons by sleep/wake homeostasis-related pathways 49
3.1 Introduction 50
3.2 Materials and methods 51
3.2.1 Recording of postsynaptic current 51
3.2.2. Immunohistochemistry 52
3.2.3. Intracerebroventricular administration 53
3.2.4 Statistical analysis 54
3.3 Results 55
3.3.1. The excitatory and inhibitory input to orexin neurons 55
3.3.2. TH-ir neurons are in apposition to orexin-ir neurons 57

3.3.3. The influence of NA on excitatory and inhibitory synaptic inputs 59
3.3.4. Orexin-ir neurons are in apposition to serotonin transporter-ir nerve endings 64
3.3.5. Modulation of locomotor activity by serotonin 1A receptor antagonists in wild type mice and orexin-neuron-deficient mice 66
3.4. Discussion 68
Chapter 4 The possible treatment of sleep disorders via regulation of input pathways for the orexin system 72
4.1 Introduction 73
4.2. Materials and methods 74
4.3 Results 74
4.3.1 The natural products used for treatment of insomnia 74
4.3.2 The effects of natural products for insomnia 77
4.3.3 The relationship between the mechanism of insomnia-modulating natural products and orexin neuron modulation 80
4.4 Discussion 82
Chapter 5 General conclusions 85
Summary 89
Acknowledgements 90
References 91

List of Tables

Table 2.1 Comparison of electrical properties of orexin neurons and non-orexin neurons 21

Table 4.1 Natural products modulating sleep/wake homeostasis 76

Table 4.2 Clinical trials of natural products for insomnia 78

Table 4.3 Preclinical studies of the effects of herbal medicine on insomnia..... 79

List of figures

Fig. 1.1 Schematic of sleep disorders affected by environmental changes 3

Fig. 1.2 Natural products with sleep-promoting effects 6

Fig. 1.3 The active components of natural products..... 7

Fig. 1.4 The orexin system 10

Fig. 1.5 The design of the study 12

Fig. 2.1 Detection of orexin neurons under the fluorescent microscope. 20

Fig. 2.2 Active membrane properties of orexin neurons 22

Fig. 2.3 The presence of hyperpolarization-activated current in orexin neurons 23

Fig. 2.4 The effect of glutamate receptor and GABA receptor agonists on orexin neurons 25

Fig. 2.5 The effects of monoaminergic and cholinergic neurotransmitters on orexin neurons 27

Fig. 2.6 The concentration dependency of serotonin-induced hyperpolarization 30

Fig. 2.7 Serotonin increases potassium conductance 31

Fig. 2.8 Serotonin 1A receptor expression in orexin neurons 32

Fig. 2.9 Single channel characteristics of the channels activated by serotonin..... 33

Fig. 2.10 The effect of inhibition of inward rectifier potassium channel 34

Fig. 2.11 Hyperpolarization of orexin neurons by catecolamine..... 37
Fig. 2.12 The effect of adrenergic receptor antagonists on NA induced hyperpolarization 38
Fig. 2.13 Inward rectifier potassium channels activated by NA 39
Fig. 2.14 NA-induced inward current in orexin neurons via α1ARs 41
Fig. 2.15 Activation of non-selective cation channels by NA 42
Fig. 2.16 inhibition of Ca^{2+} channel current by NA 44
Fig. 2.17 Summary of receptors and ion channels identified in orexin neurons 48
Fig. 3.1 Spontaneous PSC in orexin neurons 56
Fig. 3.2 TH-ir nerve endings in the lateral hypothalamic area 58
Fig. 3.3 Reduction of sEPSC and induction of sIPSC by NA 61
Fig. 3.4 Reduction of mEPSCs and mIPSCs by NA 62
Fig. 3.5 The effect of NA on eEPSC and eIPSC 63
Fig. 3.6 Serotonin transporter-ir nerve ending in the lateral hypothalamic area 65
Fig. 3.7 The effect of the serotonin receptor antagonist on locomotor activty..... 67
Fig. 3.8 Summary of results in chapter 3..... 70
Fig. 3.9 Feedback loop between orexin and serotonin/NA neurons..... 71
Fig. 4.1 Possible modulation of orexin neurons via herbal medicines that ameliorate sleep/wake disorders..... 81
Fig. 4.2 The possibility of combination therapy using approved drugs and natural products as complementary medicine..... 84

Abbreviation and Acronyms

α1AR: alpha 1 adrenergic receptor

α2AR: alpha 2 adrenergic receptor

AMPA: α-Amino-2,3-dihydro-5-methyl-3-oxo-4-isoxazolepropanoic acid

ANOVA: analysis of variance

AP-5: (2R)-amino-5-phosphonopentanoate

BZD: benzodiazepine

CCD: charge-coupled device

CCh: carbachol

CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione

DA: dopamine

DR: dorsal raphe nucleus

EC_{50}: 50% effective concentration

eEPSC: evoked excitatory postsynaptic current

EGFP: enhanced green fluorescent protein

eIPSC: evoked inhibitory postsynaptic current

EPSC: excitatory postsynaptic current

FDA: food and drug administration

FITC: fluoresceinisothiocyanate

GABA: gamma aminobutyric acid

GIRK: G protein-coupled inwardly-rectifying potassium channel

GPCR: G protein coupled receptor

IC_{50}: 50% inhibitory concentration

IPSC: inhibitory postsynaptic current

ir: immunoreactive

LC: locus coeruleus

LDT: laterodorsal tegmental nucleus

LHA: lateral hypothalamic area

LPO: lateral preoptic area

MCH: melanin-concentrating hormone

mEPSC: miniature excitatory postsynaptic current

mIPSC: miniature inhibitory postsynaptic current

MnR: median raphe nucleus

MPO: medial preoptic nucleus

NA: noradrenaline

NBQX: 2,3-Dioxo-6-nitro-1,2,3,4- tetrahydrobenzo[f]quinoxaline-7-sulfonamide

NMDA: N-methyl-D-aspartate

NSCC: non selective cation channel

OX1R: orexin receptor 1

OX2R: orexin receptor 2

POA: preoptic area

PPN: pedunculopontine nucleus

PSC: postsynaptic current

S.E.: standard error

sEPSC: spontaneous excitatory postsynaptic current

sIPSC: spontaneous inhibitory postsynaptic currents

TH: tyrosine hydroxylase

TMN: tuberomamillary nucleus

TTC: tetanus toxin C-fragment

TTX: tetrodotoxin

VLPO: ventrolateral preoptic nucleus

5HT: 5-hydroxytryptamine

8-OH-DPAT: (S)-5,6,7,8-Tetrahydro-7α-dipropylamino-1-naphthol

Publications

Muraki Y, Yamanaka A, Tsujino N, Kilduff TS, Goto K, Sakurai T. Serotonergic regulation of the orexin/hypocretin neurons through the 5-HT1A receptor. J Neurosci. 2004; 24(32): 7159-66.

Yamanaka A, Muraki Y, Ichiki K, Tsujino N, Kilduff TS, Goto K, Sakurai T. Orexin neurons are directly and indirectly regulated by catecholamines in a complex manner. J Neurophysiol. 2006; 96(1): 284-98.

Yamanaka A, Muraki Y, Tsujino N, Goto K, Sakurai T. Regulation of orexin neurons by the monoaminergic and cholinergic systems. Biochem Biophys Res Commun. 2003; 303(1): 120-9.

Tsujino N, Yamanaka A, Ichiki K, Muraki Y, Kilduff TS, Yagami K, Takahashi S, Goto K, Sakurai T. Cholecystokinin activates orexin/hypocretin neurons through the cholecystokinin A receptor. J Neurosci. 2005; 25(32): 7459-69.

Sakurai T, Nagata R, Yamanaka A, Kawamura H, Tsujino N, Muraki Y, Kageyama H, Kunita S, Takahashi S, Goto K, Koyama Y, Shioda S, Yanagisawa M. Input of orexin/hypocretin neurons revealed by a genetically encoded tracer in mice.Neuron. 2005; 46(2): 297-308.1.

Presentation in international conference

Muraki Y, Yamanaka A, Hirashima N, Tsujino N, Goto K, Sakurai T.

Regulation of orexin neurons by the monoaminergic and cholinergic systems

Neuroscience 2003, Presentation Number: 889.6

Muraki Y, Yamanaka A, Isoda H

Serotonergic and noradrenergic regulation of orexin neuron and possibility for modulation by natural product

The 29th Annual and International Meeting of Japanese Association for Animal Cell Technology, 2016, Submission number: JAACT16-P0134

Chapter 1 Introduction

1.1 Sleep disorders and the environment

Sleep/wake homeostasis is important for human health. Due to recent changes in the environment, the number of patients suffering from sleep disorders such as insomnia is increasing. In the United States, about half of patients have problems with sleep. Sleep disorders are caused by several environmental factors such as the increase of shift work; jetlag induced by increased travel abroad; aging [1]; and long working hours, which can cause stress, [2].

The number of patients with insomnia has increased over the past ten years. Sleep disorders can be a cause of other diseases such as diabetes because of disturbance to the endocrine system [3]. Insomnia is also associated with the risk of dementia and myocardial infarction [4, 5]. Non-alcoholic fatty liver disease can also be caused by disturbances in sleep/wake homeostasis [6].

In the development of sleep disease triggered by the environment, stress and aging are important factors. Recent rapid changes in one's environment cause stress and aging and affect various brain systems. For example, stress decreases serotonin [7] and noradrenaline (NA) [8] production. Aging also decreases serotonin and noradrenaline. To understand the physiological changes induced by rapid environmental changes, studies focused on brain neurotransmitters are important.

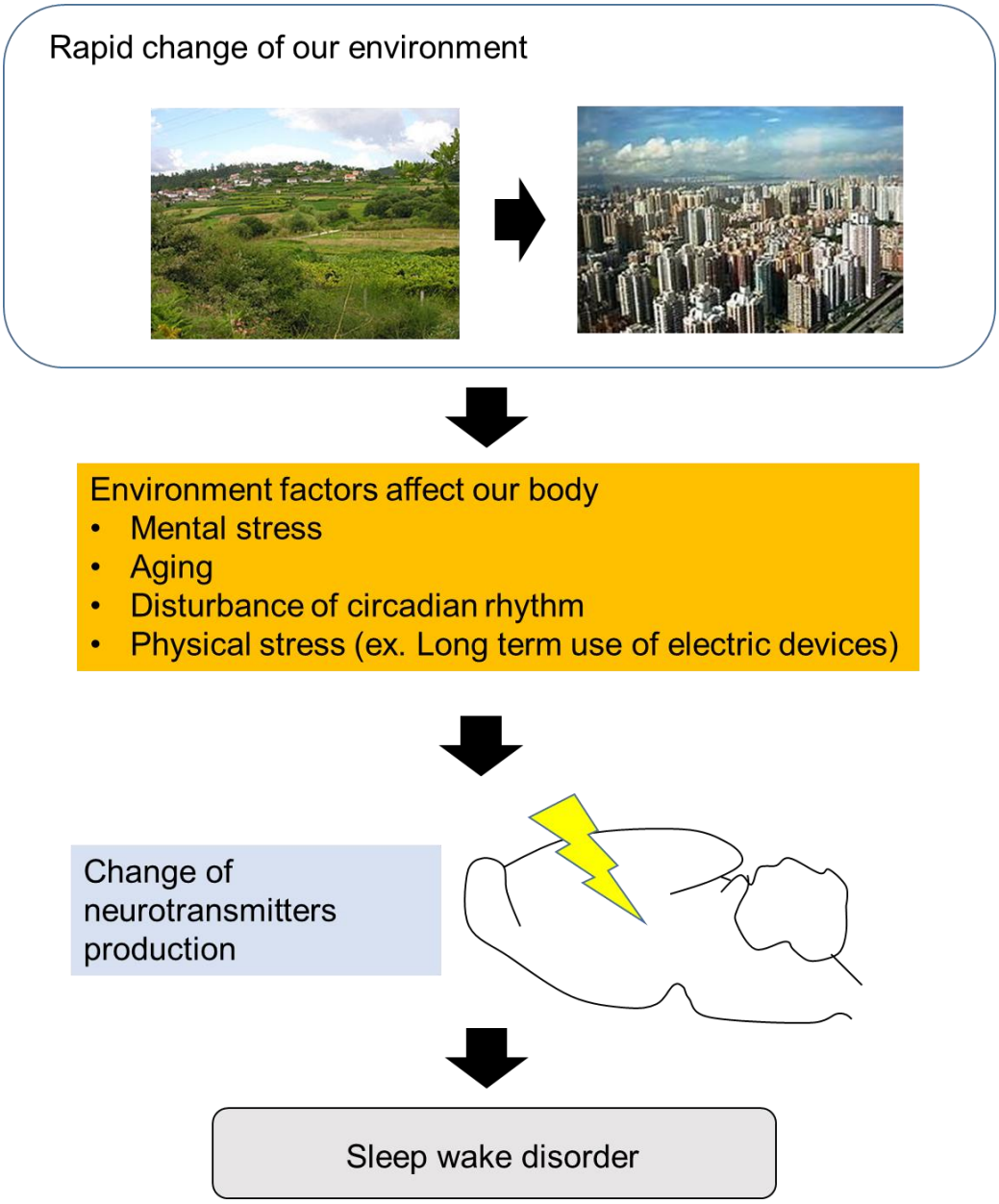

Fig. 1.1 Schematic of sleep disorders affected by environmental changes

(Pictures from https://ja.wikipedia.org)

1.2 Epidemiology of insomnia

Insomnia is characterized by long sleep latency, frequent nocturnal awaking. Sleep disorder have following characteristics: (i) difficulty in falling asleep despite adequate opportunity and circumstance to sleep: (ii) impairment of sleep is associated with daytime distress: (iii) sleep difficulty occurs at least 3 times per week and has been a problem for at least 1 month [9].

Large number of patients were suffered in all over the world. Based on Diagnostic and Statistical Manual of Mental Disorder-IV classification, which is representative criteria for diagnosis of sleep disorder in the world, the preference is estimated from 4.4% to 6.4 %[10]. The number of patient with insomnia is increased twofold in Japan [11]. Sleep disorder is big problem all over the world.

1.3 The use of natural products for insomnia

Complementary medicine is widely used to treat insomnia as it is a common disease with symptoms recognizable to patients. In Japan, several kinds of supplements are used for the induction of sleep. For example, the amino acid glycine has been used to induce sleep [12].

Throughout history, several kinds of natural products have been used for the treatment of insomnia (Fig. 1.2). In Europe, herbal medicines such as extract of valerian and St John's wort were used for the treatment of sleep disorders [13] [14]. In Japan, herbal medicine has long been utilized. For example, yoku-kan-san and Suan Zao Ren Tang can be used for insomnia [15] [16]. Kava, a Polynesian plant, can induce sleep in humans and has been used for insomnia [13]. Several compounds are considered the active components (Fig. 1.3).

According to recent research, the active components of herbal medicines and the target molecules of these herbal medicines have been identified. The possible modulation of orexin neurons by these molecules is discussed in Chapter 4, based on experimental data about receptor subtype expression in orexin neurons and reported information for the target molecules of herbal medicine.

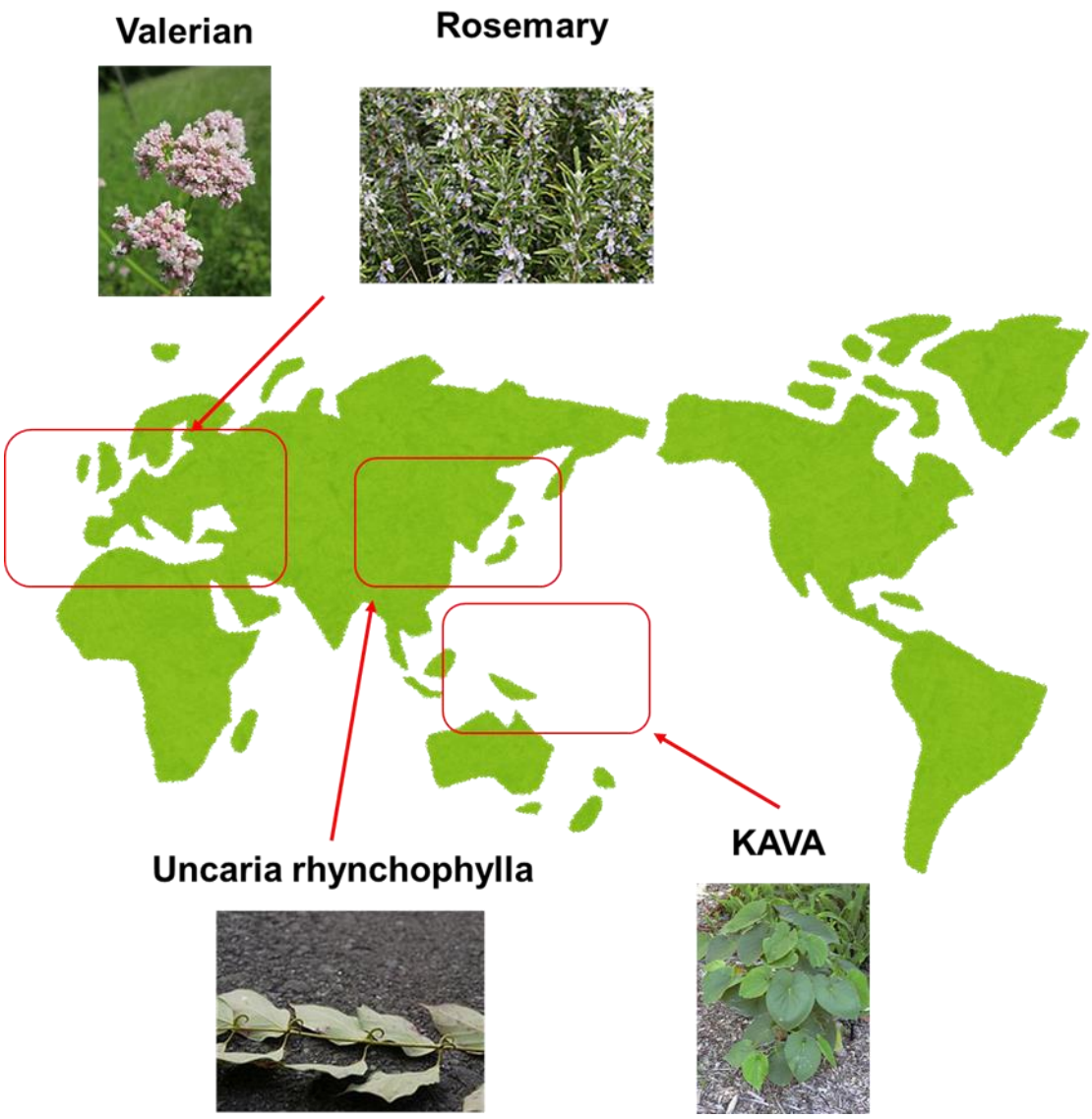

Fig. 1.2 Natural products with sleep-promoting effects

(Pictures from https://ja.wikipedia.org)

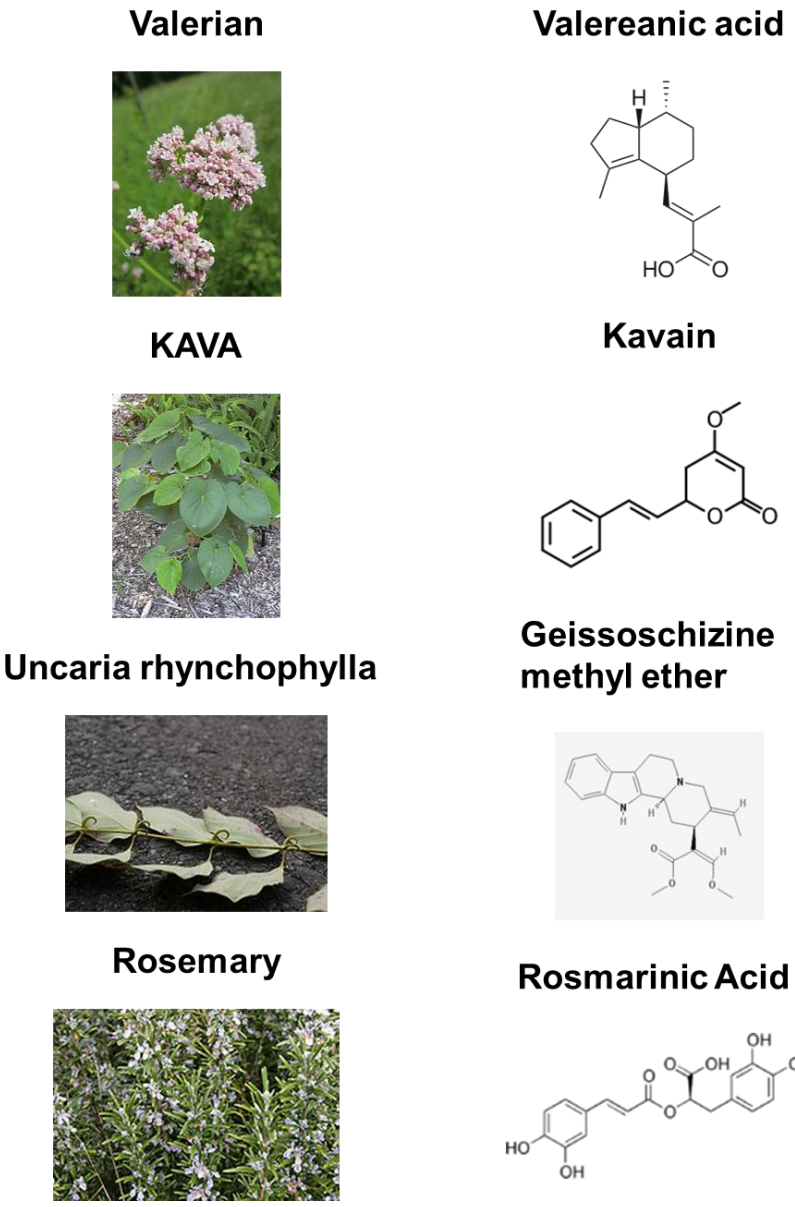

Fig. 1.3 The active components of natural products

(Pictures from https://ja.wikipedia.org)

1.4 Current treatment methods for insomnia

For the treatment of insomnia, several kinds of drugs have been developed. The first were barbiturates. However, barbiturates are not used for insomnia now because of their adverse effects such as low lethal dose, residual sedation tolerance, and dependence. As a tolerable treatment option for insomnia, benzodiazepine (BZD) receptor agonists have been developed. BZD agonists modulate the activity of the gamma aminobutyric acid (GABA) $_{A}$ receptor [17]. Recently, additional drug types were approved by the food and drug administration (FDA). One is a melatonin receptor agonist. The other is an orexin receptor antagonist [18]. Both the orexin and melatonin systems are important for the modulation of sleep/wake homeostasis. Melatonin is produced in the pineal gland of the hypothalamus. Orexin is produced by the lateral hypothalamic area (LHA) of the hypothalamus.

1.5 The orexin system for sleep/wake homeostasis

To develop effective treatment methods, a deep understanding of the neural mechanism of sleep/wake homeostasis is needed. Sleep/wake homeostasis is regulated by complex neural networks. Through recent advances in technology for neuroscience and molecular biology, the molecular mechanism of insomnia has been clarified. The role of the orexin system has been clarified over the past 20 years. Orexin was identified as the ligand of an orphan G protein coupled receptor (GPCR). Orexins contain two peptides derived from one precursor prepro-orexin. Orexin A is a 28 amino acid peptide and orexin B is a 32 amino acid peptide. Orexin peptides bind to orexin receptor 1 (OX1R) and orexin receptor 2 (OX2R), which belong to the GPCR family. Orexin A preferentially binds to OX1R. On the other hand, orexin B binds equally to both OX1R and OX2R [19]. Orexin neuron terminals innervate wide regions of the brain. Dense

projections are observed in serotonergic and adrenergic nuclei, which are involved in sleep/wake homeostasis (Fig. 1.4).

The role of the orexin system in sleep/wake homeostasis has been clarified using Tg/KO mice. OX2R KO mice showed fragmentation of the sleep/wake cycle [20]. *Orexin/Ataxin-3* mice, which express neurodegenerative peptide ataxin-3 specifically in orexin neurons, also showed fragmentation of the sleep/wake cycle [21]. Moreover, a dog strain showing disruption of the sleep/wake cycle was found to have a mutation the in OX2R gene [22]. In human patients of narcolepsy, which is a severe sleep disorder with fragmentation of the sleep/wake cycle, there is a decrease in orexin peptides in the cerebrospinal fluid [23]. Based on this information, the orexin system plays an important role in sleep/wake homeostasis.

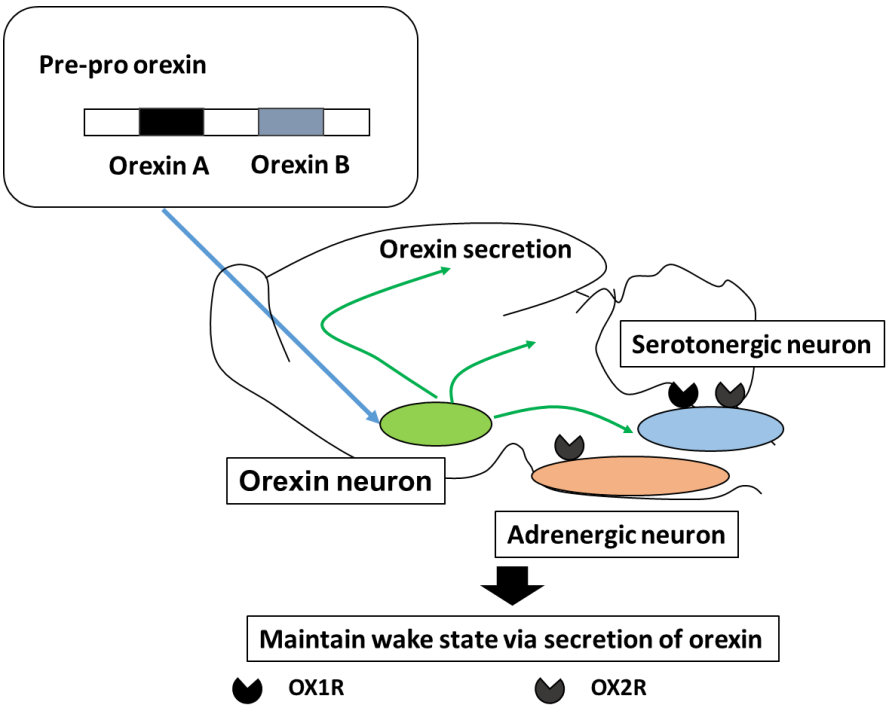

Fig. 1.4 The orexin system

Orexins are produced in orexin neurons in the hypothalamus and secreted to various brain regions to maintain the awake state.

1.6 Analysis of the regulation of the orexin system to understand the mechanism of natural products with sleep promoting effects

Orexin neurons have a key role in sleep/wake homeostasis in spite of their limited numbers. Compounds that can modulate the neuronal activity of orexin neurons can be an effective method for treatment of sleep disorders.

Sleep disorders are associated with environmental factors such as stress and aging. In this study, we focused on the effect of neurotransmitters associated with stress and aging. In Chapter 2, the responsiveness of orexin neurons to neurotransmitters was analyzed using the slice patch clamp method. Moreover, the receptors involved in the responses were analyzed. In Chapter 3, the role of serotonergic and adrenergic modulation of orexin neurons was analyzed to understand their physiological importance. In Chapter 4, the possibility for modulation of orexin neurons by sleep-promoting natural products was analyzed by database analysis to consider the possible modulation of orexin neurons via receptors expressing in orexin neurons (Fig. 1.5).

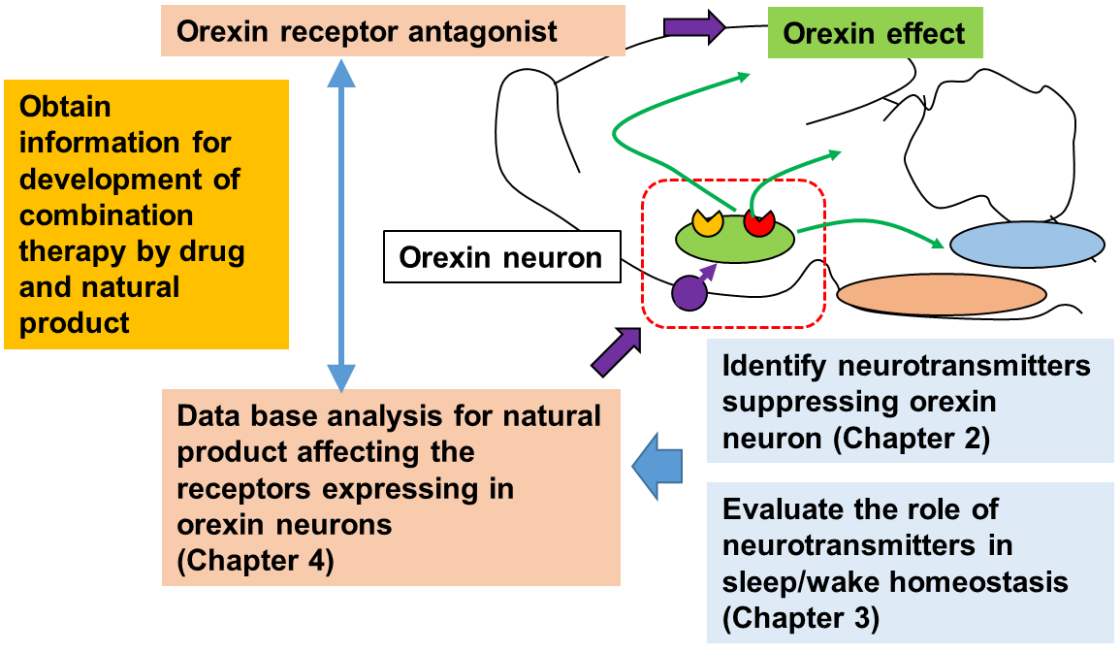

Fig. 1.5 The design of the study

The orexin system is a key system for sleep/wake homeostasis. The effect of neurotransmitters on orexin neurons and how this can be changed by environmental factors was evaluated (Chapter 2). The physiological role of this modulation was clarified to understand the importance of serotonergic and noradrenergic regulation (Chapter 3). The possible treatment of sleep disorders by natural products was evaluated by database analysis (Chapter 4).

Chapter 2 Electrophysiological properties of orexin neurons

2.1 Introduction

Changes to the human environment have an important role in the recent increase in sleep disorders. Human environmental changes such as an increase in stress and aging cause changes in neurotransmitter levels in the brain.

In this chapter, the author focused on the effect of the neurotransmitters serotonin and noradrenaline, which can be affected by stress and aging, on orexin neurons. The levels of serotonin and noradrenaline were decreased in stress conditions [24]. To understand the role of neurotransmitters in sleep/wake homeostasis, their effect on orexin neurons can provide useful information because of the importance of orexin neurons to sleep/wake homeostasis.

Orexin neurons, which produce the wake-promoting neurotransmitters orexins, are located in the LHA, which is traditionally known as a feeding center [25]. The importance of orexin neurons in sleep/wake homeostasis was confirmed by studies using rodent models and clinical samples. For example, the ablation of orexin neurons via expression of a human ataxin-3 fragment with an elongated polyglutamyl stretch under control of the human prepro-orexin promoter caused fragmentation the of sleep/wake cycle in mice and rats [26] [21]. The selective activation of orexin neurons via selective expression of melanopson, which activates neurons in the presence of blue light, caused wakefulness [27]. On the other hand, the selective inhibition of orexin neurons by extrinsic receptor expression and ligand treatment caused an increase in slow wave sleep [28].

Since the activity of orexin neurons can modulate sleep/wake homeostasis, information about intrinsic factors modulating orexin neurons is important. While ex vivo electrophysiological analysis is effective to understand the responsiveness of neurons to neurotransmitters, it has been difficult to identify orexin neurons in slice

samples because the LHA contains several kinds of non-orexin neurons, such as melanin-concentrating hormone (MCH) neurons, and orexin neurons are sparsely distributed [29].

In orexin neurons, the transcription of prepro-orexin gene is mediated by a specific promoter region. Orexin neuron-specific gene expression can be induced using this promoter. To identify orexin neurons, orexin neuron-specific enhanced green fluorescent protein (EGFP)-expressing mice (*orexin/EGFP* mice) were developed to conduct electrophysiological analysis of orexin neurons [30].

In this study, we evaluated the effects of neurotransmitters that can be affected by environmental change on orexin neurons. The author identified that GABA, serotonin, and NA receptor agonists suppress the activity of orexin neurons.

2.2 Materials and methods

2.2.1 Animal usage

All experimental procedures involving animals were approved by the University of Tsukuba Animal Resource Center and were in accordance with National Institutes of Health guidelines. All efforts were made to minimize animal suffering or discomfort and to reduce the number of animals used.

2.2.2 Slice preparation

Male and female *orexin/EGFP* mice, 3-4 weeks old, in which the human prepro-orexin promoter drives expression of EGFP (lines E2 and E7) [30], were used for experiments. The mice were deeply anesthetized with fluothane (Takeda, Osaka, Japan) and then decapitated. The brains were isolated in ice-cold bubbled (100% O_2)

physiological solution containing the following (in mM): 140 choline Cl, 2 KCl, 0.1 $CaCl_2$, 1.9 MgCl2, 10 HEPES, and 10 glucose, pH 7.4 with NaOH; or in sucrose solution (in mM: 234 sucrose, 2.5 KCl, 1.25 $NaHPO_4$, 10 $MgSO_4$, 0.5 $CaCl_2$, 26 $NaHCO_3$, and 10 glucose). Brains were cut coronally into 300 μm slices with a microtome (VTA-1000S; Leica, Nussloch, Germany). Slices containing the LHA were transferred to an incubation chamber filled with extracellular solution containing the following (in mM): 140 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose, pH 7.4, with NaOH at room temperature (24°C –26°C) for at least for 1 hr.

2.2.3 Identification of EGFP-expressing orexin neurons by fluorescent microscopy

For electrophysiological recordings, the slices were transferred to a recording chamber (RC-27L; Warner Instruments, Hamden, CT) at room temperature on a microscope stage (BX51WI; Olympus Optical, Tokyo, Japan). The slices were superfused with room-temperature bubbled (100% O_2) extracellular solution at a rate of 2 ml/min using a peristaltic pump (Dynamax; Rainin, Oakland, CA).

The fluorescence microscope was equipped with an infrared camera (C2741-79; Hamamatsu Photonics, Hamamatsu, Japan) for infrared differential interference contrast imaging and a charge-coupled device camera (IKTU51CU; Olympus Optical) for fluorescent imaging. Each image was displayed separately on a monitor (Gawin; EIZO, Tokyo, Japan) and used to identify neurons expressing EGFP.

2.2.4 Electrophysiological recordings

Patch pipettes were prepared from borosilicate glass capillaries (GC150-10; Harvard Apparatus, Holliston, MA) with a micropipette puller (P-97; Sutter Instruments, Pangbourne, UK). The pipettes were filled with an internal solution containing the

following (in mM): 145 KCl, 1 $MgCl_2$, 1.1 EGTA-Na_3, 10 HEPES, 2 MgATP, 0.5 NaGTP, and 2 Lucifer yellow, pH 7.2 with KOH. Osmolarity of the solution was checked by a vapor pressure osmometer (model 5520; Wescor, Logan, UT). The osmolality of the internal and external solutions was 280–290 and 320–330 mOsm/l, respectively.

Pipette resistance was 4–10 MΩ. The series resistance during recording was 10–25 MΩ and was not compensated. Recording pipettes were advanced toward individual cells in the slice while under positive pressure. After confirmation of tight seals (0.5–1.0 GΩ) by release of positive pressure, the membrane patch was ruptured by suction, Membrane current and potential were monitored using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA). Current pulse was applied to the cell at duration of 200 msec at 20 pA steps at 2-sec intervals to monitor membrane conductance.

The reference electrode was an Ag-AgCl pellet immersed in extracellular solution. All current-clamp recordings were made in Axopatch 200B fast mode. The membrane capacitance was calculated by dividing the time constant by the input resistance. Input resistance was calculated from the slope of the current-voltage relationship. The output signal was low-pass filtered at 5 kHz and digitized at 10 kHz. In the cell-attached and inside-out single-channel recording, the recorded signals were filtered at 2 kHz and digitized at a sampling rate of 10 kHz. Data were recorded on a computer through a Digidata 1322A analog-to-digital converter using pClamp 8.0.1 software (Axon Instruments). The trace was processed for presentation using Origin 6.1 (Microcal Software, Northampton, MA) and Canvas 8.0 (Deneba Systems, Miami, FL) software.

2.2.5 Drugs

The drugs used were tetrodotoxin (TTX), barium chloride (Wako, Osaka, Japan),

serotonin, lucifer yellow dipotassium salt, WAY100635, and 8-OH-DPAT, adrenaline, NA, dopamine (DA), idazoxan, carbachol (CCh), (2R)-amino-5-phosphonopentanoate (AP-5), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), UK-14304 and SKF-96365 (Sigma, St. Louis, MO). In the electrophysiological experiments, drugs were dissolved in extracellular solution and applied by either bath application or local application through a thin polyethylene tube positioned near the cells being recorded.

2.3 Results

2.3.1 Detection of orexin neurons in slice conditions

To detect orexin neurons, *orexin/EGFP* mice were used. EGFP-expressing orexin neurons were easily identified under fluorescent microscopy and charge-coupled device (CCD) camera. About 80% of orexin neurons expressed EGFP and ectopic expression was not observed (Fig. 2.1).

Basic electrophysiological properties were measured by whole cell patch clamp. There was no significant difference between EGFP-expressing orexin neurons and non-EGFP expressing neurons (Table 2.1).

In slices, action potential firing was observed after current injection. Some of the orexin neurons showed burst firing. Generation of action potentials was suppressed by treatment with TTX (Fig. 2.2).

To evaluate adaptations of firing, the relationship between injected current and firing frequency was measured. Correlation between injected current and firing frequency was observed but adaptation was not.

To understand membrane properties, passive membrane properties were measured (Fig. 2.3). By injecting hyperpolarizing current, the activation of ion channels was observed. Cyclic nucleotide-gated ion channels are known to be involved in hyperpolarization-induced current. Hyperpolarization-induced current was inhibited by ZD7288, which is a cyclic nucleotide-gated channel inhibitor.

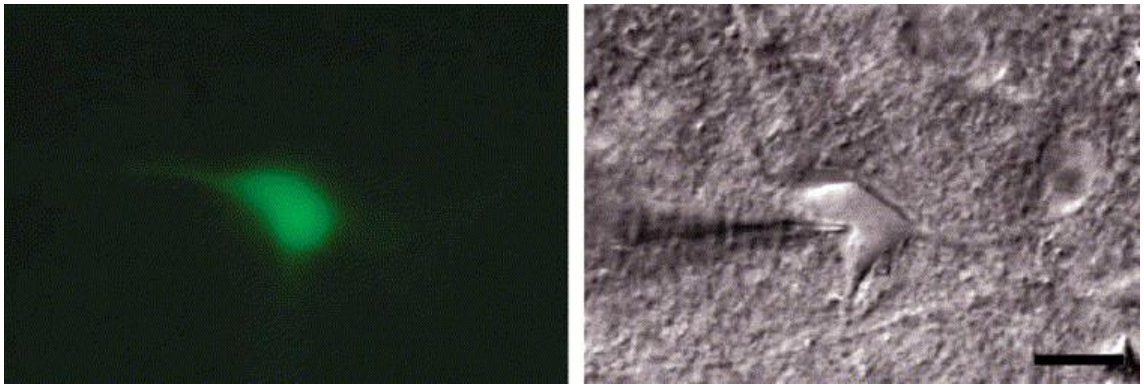

Fig. 2.1 Detection of orexin neurons under the fluorescent microscope.

Fluorescence (left) and IR-DIC (right) images of EGFP-expressing orexin neurons. Scale bar: 20 μm.

Table 2.1 Comparison of electrical properties of orexin neurons and non-orexin neurons

	EGFP-expressing orexin neurons (n = 36)	Non-EGFP-expressing neurons (n = 23)
Resting membrane potential (mV)	−60.5 ± 5.9	−65.3 ± 7.9
Spontaneous firing (Hz)	5.5 ± 3.9	5.8 ± 7.4
Input resistance (MΩ)	390 ± 110	310 ± 220
Threshold (mV)	−30.5 ± 8.9	−39.4 ± 6.0
Peak (mV)	37.1 ± 8.6	33.6 ± 12.3
Afterhyperpolarization (mV)	−59.3 ± 6.2	−59.1 ± 6.7
dV/dt Rise (mV/ms)	132.1 ± 41.3	153.7 ± 50
dV/dt Fall (mV/ms)	79.9 ± 25.4	101.2 ± 37.3
Duration of half amplitude (msec)	0.76 ± 0.17	0.65 ± 0.23

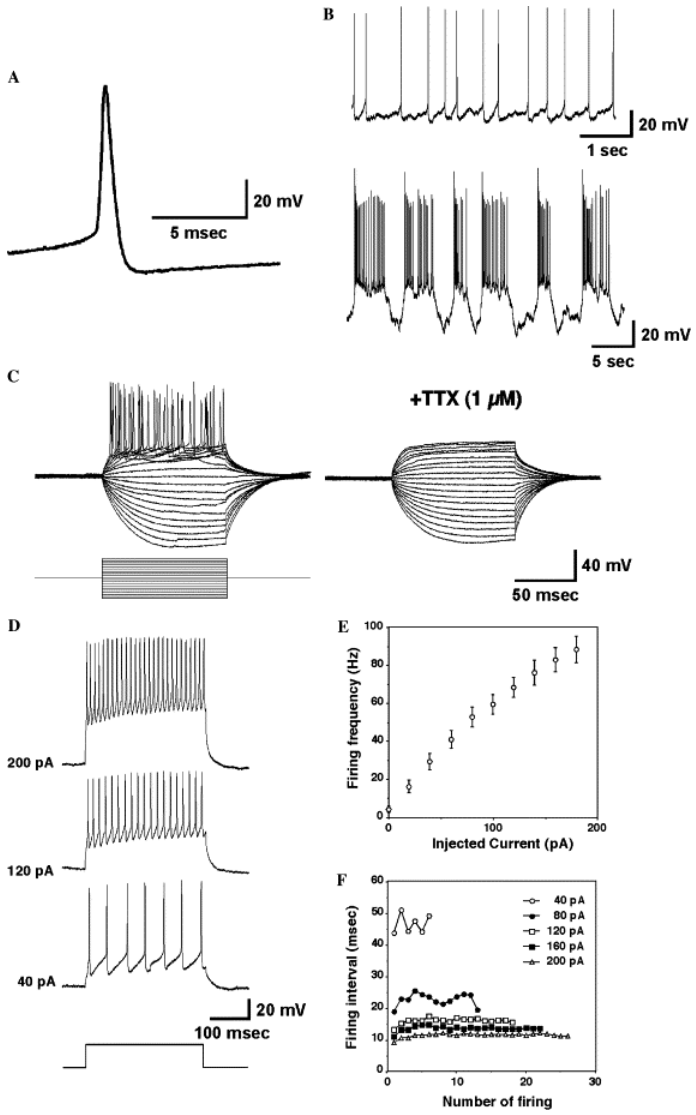

Fig. 2.2 Active membrane properties of orexin neurons

A, The shape of the action potential of orexin neurons. B, Spontaneous action potentials in orexin neurons. Some orexin neurons showed a bursting firing pattern. C, Recordings of membrane potential response to a series of 100 ms current steps (in 20 pA increments) from resting potential (−60 mV) in the absence (left) or presence (right) of TTX (1 μM). D, E, Relationship between injected current and firing frequency. F: Inter-firing interval between events at different current intensities.

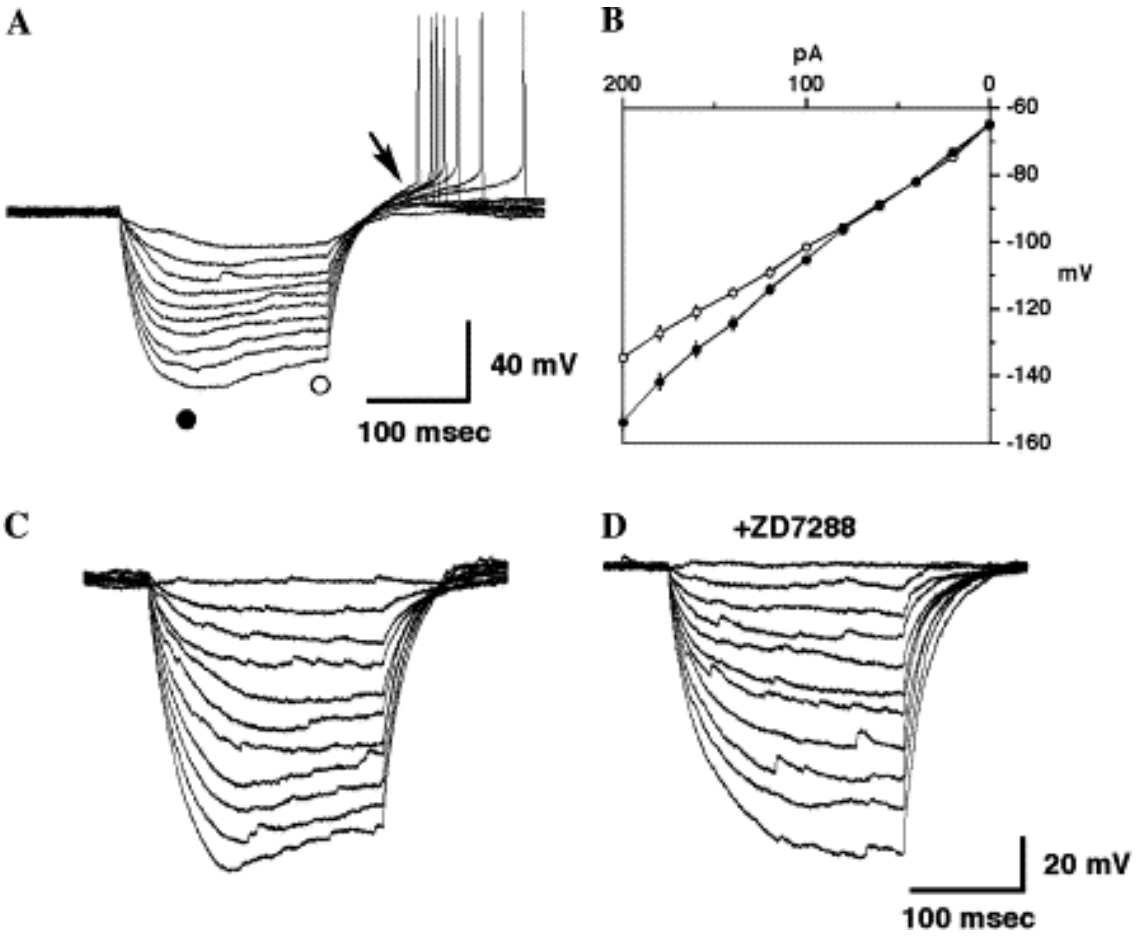

Fig. 2.3 The presence of hyperpolarization-activated current in orexin neurons

A, Response to a series of 200 ms current steps (from −200 pA to −20 pA increments) from resting potential (−60 mV). The arrow shows an action potential triggered via activation of hyperpolarization-induced current. B. I-V relationship. The instantaneous potential (open circle in A) and steady state potential (closed circle in A) are plotted. C, D The effect of ZD7288. After 10 min treatment with ZD 7288, hyperpolarization-induced channel activity was abolished.

2.3.2 The effects of glutamatergic and GABAergic neurotransmitters

To evaluate glutamatergic and GABAergic regulation, α-Amino-2,3-dihydro-5-methyl-3-oxo-4-isoxazolepropanoic acid (AMPA), N-methyl-D-aspartate (NMDA) and muscimol were applied to orexin neurons. AMPA and NMDA depolarized orexin neurons and muscimol hyperpolarized orexin neurons (Fig. 2.4). Orexin neurons express AMPA- and NMDA-type glutamate receptors and $GABA_A$ receptors.

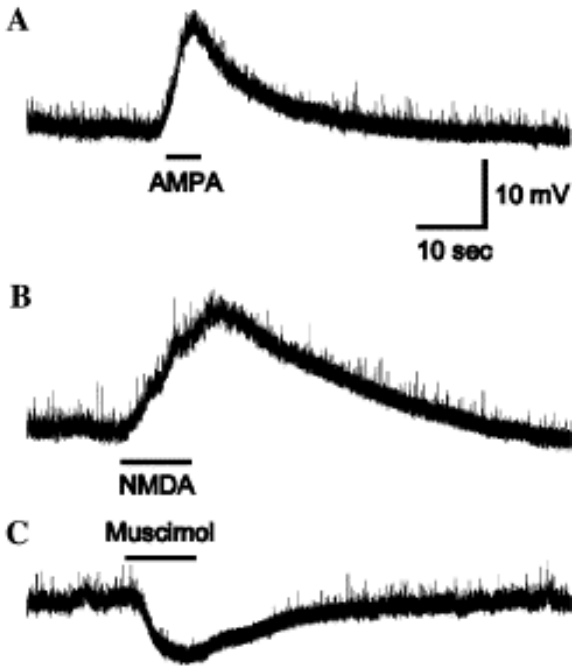

Fig. 2.4 The effect of glutamate receptor and GABA receptor agonists on orexin neurons

A, B: The effects of the glutamate receptor agonists AMPA and NMDA. Membrane potential was set to -60 mV by current injection before compound application. C: The effect of a GABA receptor agonist. Membrane potential was set at −45 mV by current injection.

2.3.3 The effect of monoaminergic, cholinergic, and adrenergic neurotransmitters

To understand the regulation of orexin neurons by monoaminergic and cholinergic system, serotonin, NA, and CCh, which is cholinergic agonist, were applied to orexin neurons (Fig. 2.5). Serotonin (100 μM) and NA (100 μM) strongly hyperpolarized orexin neurons. In the presence of TTX (1 μM), NA and serotonin hyperpolarized the membrane potentials to −14.6 ± 1.5 mV (n = 6) and −15.5 ± 4.5 mV (n = 5), respectively.

On the other hand, CCh depolarized orexin neurons. In the presence of TTX (1 μM), CCh (100 μM) depolarized the membrane potential to 9.0 ± 1.1 mV (n = 5) when the membrane potential was initially set at −60 mV. Atropine (10 μM), an antagonist of the metabolic type of acetylcholine receptor (muscarinic receptor), abolished this effect completely, suggesting that muscarinic receptors are involved in this response (data not shown). The effect of acetylcholine (100 μM) on orexin neurons was the same as that of CCh in the presence of TTX (n = 2).

Orexin neurons express the inhibitory type of serotonin and NA receptors and the excitatory type of cholinergic receptors.

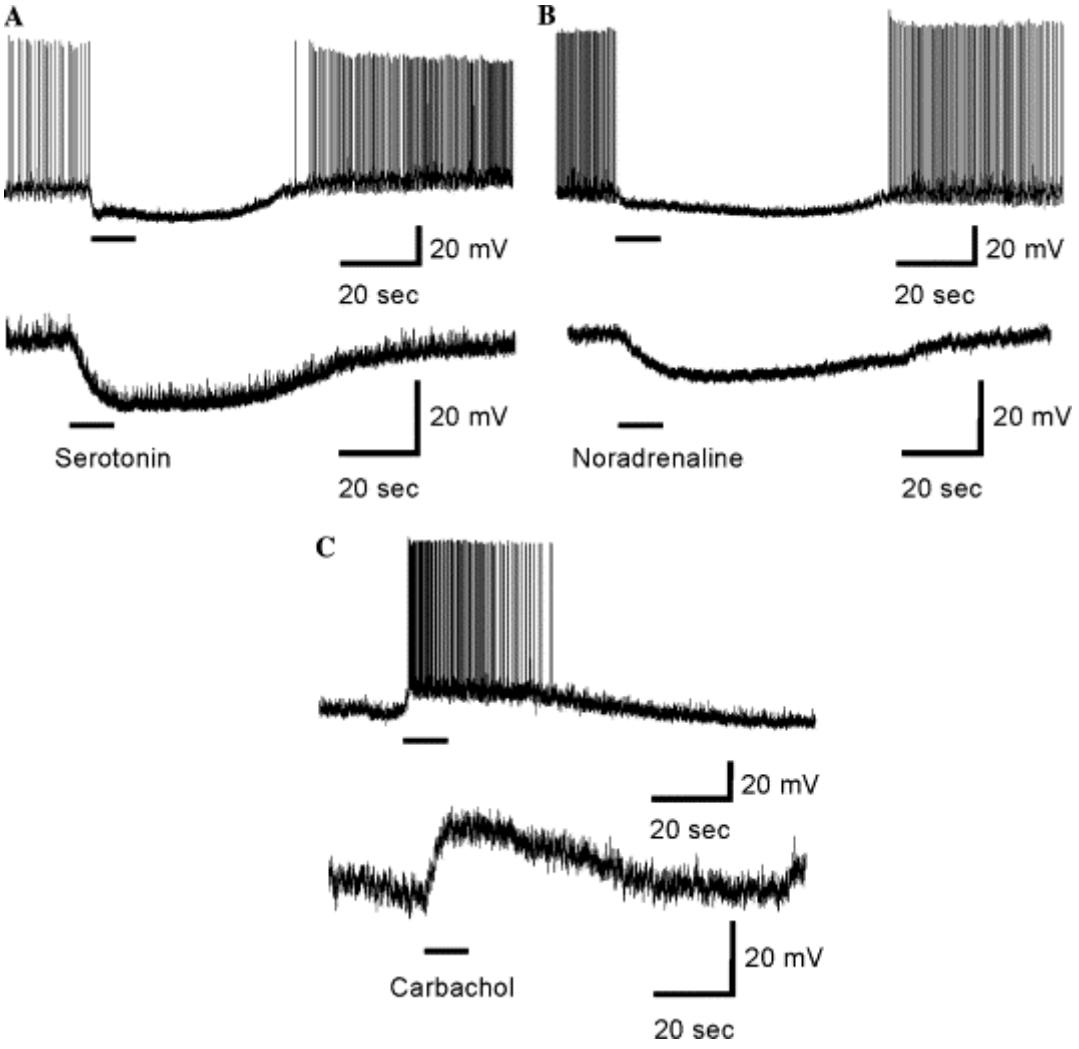

Fig. 2.5 The effects of monoaminergic and cholinergic neurotransmitters on orexin neurons

A: The effect of serotonin (100 μM). B: The effect of NA (100 μM). C: The effect of CCh (100 μM). The membrane potential was set at −45 mV (serotonin and NA) and −60 mV (CCh) by current injection. Experiments were performed in the presence (upper) or absence (lower) of TTX (1 μM).

2.3.4 The mechanism of suppression by serotonin

To understand the mechanism of regulation of orexin neurons by serotonin, detailed electrophysiological analysis was conducted.

All EGFP-positive neurons (orexin neurons) tested were hyperpolarized by serotonin (n = 81), whereas EGFP-negative neurons in the same area (non-orexin neurons and probably a few orexin neurons, because the EGFP fluorescence of 20% of orexin neurons was under the detection limit) showed a variety of responses to serotonin: 33% of the neurons (23 of 70) showed hyperpolarization, 20% (14 of 70) showed depolarization, and 47% (33 of 70) showed no effect [31]. Serotonin hyperpolarized orexin neurons in a concentration-dependent manner. E_{max} was 32.0 ± 1.0 mV and IC_{50} was 0.87 μM (n = 4–6).

A reduction in membrane resistance was observed following treatment with serotonin (Fig. 2.6). The effect of serotonin was measured in the presence of hyperpolarizing current injection. Serotonin hyperpolarized orexin neurons with a reduction in membrane resistance. Membrane resistance was decreased from 551 ± 44 to 328 ± 32 MΩ by treatment of serotonin (100 μM).

The reversal potential was estimated by the I-V relationship by current injection. The estimated reversal potential was −111.2 ± 7.7 mV. The reversal potential was shifted to −74.7 ± 1.6 mV when extracellular potassium concentration was shifted to 10 mM. Both reversal potentials were similar to the reversal potential of the potassium ion. Rectification properties were analyzed by changing the holding potential. Inward rectification was observed. These results suggest that inward rectifier potassium channels were activated by serotonin.

To identify the serotonin receptor subtypes that have important roles in the regulation of orexin neurons, subtype-selective receptor agonist were used (Fig. 2.8). The

serotonin 1A receptor selective agonist, 8-OH-DPAT mimicked the effect of serotonin. The potency of 8-OH-DPAT was comparable with serotonin; the IC_{50} value was 0.3 μM (n = 4–7). On the other hand, the hyperpolarization of orexin neurons by serotonin was suppressed by pre-treatment with WAY-100635, a serotonin 1A receptor selective antagonist. Pretreatment of slices with 1, 10, and 100 nM WAY100635 for 2 min inhibited 10 μM serotonin-induced hyperpolarization to 80% ± 8%, 60% ± 10%, and 4% ± 4%, respectively, compared with the results before antagonist treatment.

Immunohistological analysis was conducted to check the expression of receptors (Fig. 2.8). Serotonin 1A receptor-immunoreactive cells were observed in the lateral hypothalamus. Co-localization of orexin immunoreactivity and serotonin 1A receptor immunoreactivity was confirmed.

For detailed analysis of activated potassium channels, single channel recordings were conducted (Fig. 2.9). Single channel recordings in cell attached mode were conducted in the presence or absence of serotonin (1 μM). To test the effect of serotonin, serotonin was backfilled in a glass pipette to detect the effect of serotonin in a time-dependent manner. Single channel current was increased in the serotonin-containing condition. The average single channel current was 3.4 pA. The single channel conductance was calculated by changing pipette voltage and estimated as 33.8 ± 4.3 pS, which is similar to the conductance of G-protein activated potassium channels.

To test the pharmacological properties of the ion channels, the effect of Ba^{2+}, which is known to suppress G protein-coupled inwardly-rectifying potassium channel (GIRK), was assessed. Ba^{2+} suppressed serotonin-induced hyperpolarization in a dose-dependent manner (Fig. 2.10).

From these data, suppression of orexin neuron activity was mediated by serotonin 1A receptors and subsequent activation of GIRK.

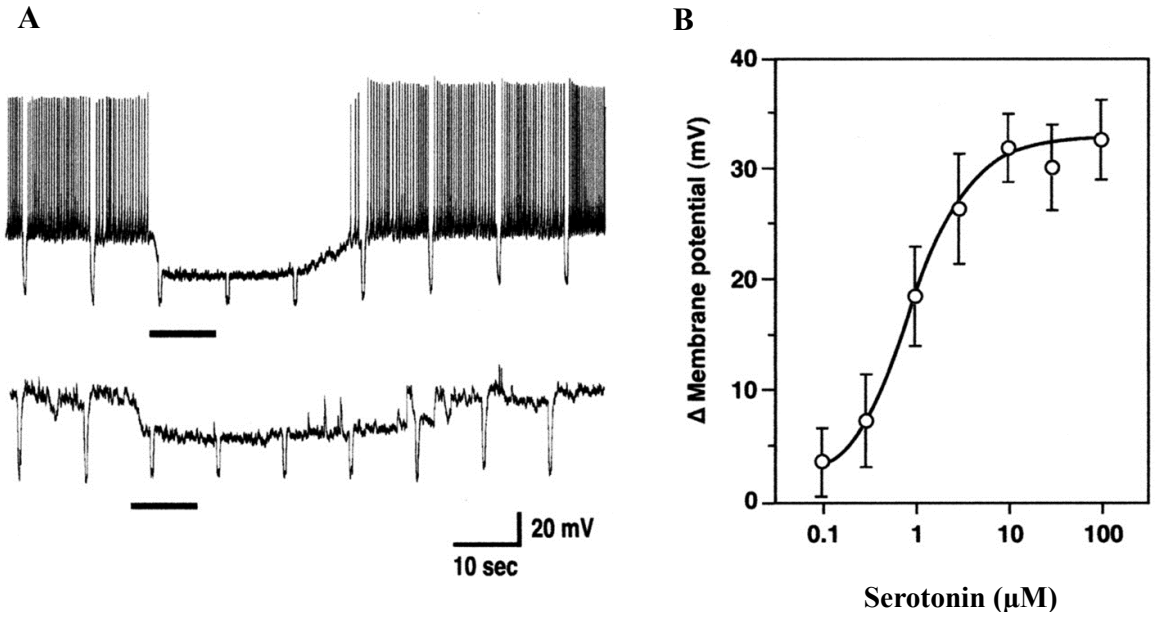

Fig. 2.6 The concentration dependency of serotonin-induced hyperpolarization

A: The decrease in membrane resistance by treatment with serotonin (10 μM). Input resistance was monitored by the change in membrane potential generated by injection of a current pulse (−20 pA, 500 msec, 0.1 Hz). B, The concentration dependence of the serotonin response. IC_{50} was 0.87 ± 0.18 μM. Values are mean ± S.E. (n = 4–7).

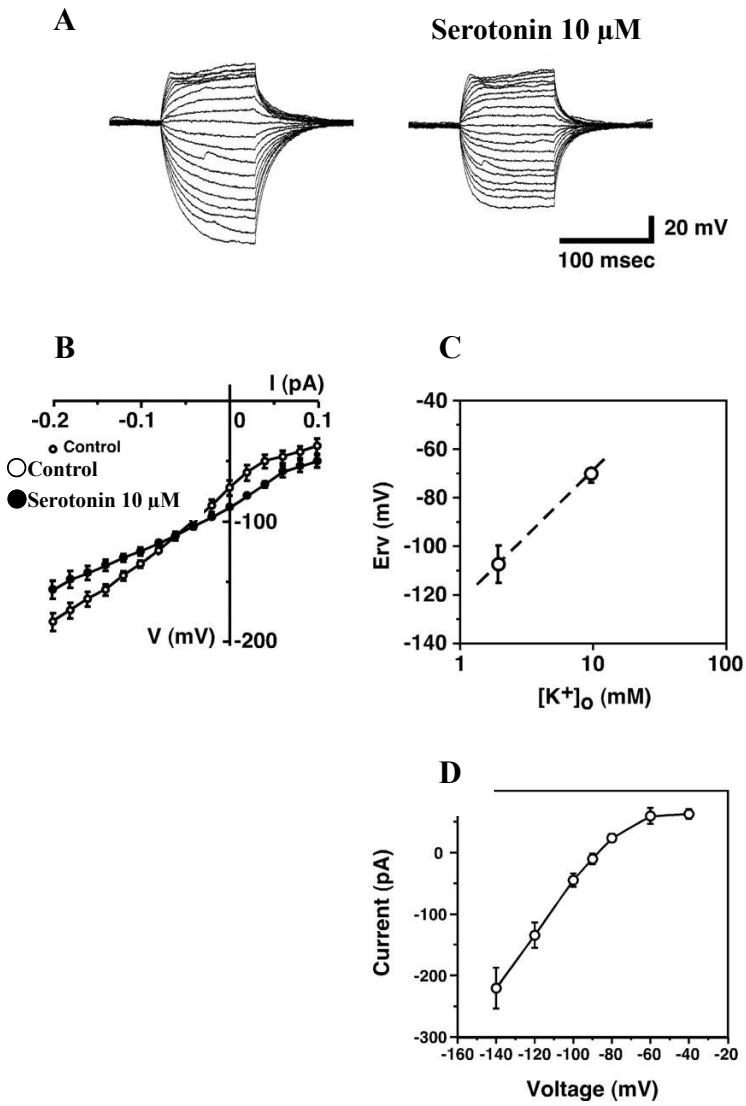

Fig. 2.7 Serotonin increases potassium conductance

A, Recordings of membrane potential in response to a series of 100 ms current steps (in 20 pA increments, −200 pA to 180 pA) from resting potential (−60 mV). Left, absence of serotonin. Right, presence of serotonin (10 µM). B, Current-voltage relationship. Estimated reversal potential was −107.3 mV. C, The shift in reversal potential by the change in extracellular potassium concentration. D, IV relationship in voltage clamp conditions. Serotonin (10 µM) was applied at each holding potential. N = 4–7, mean ± S.E.

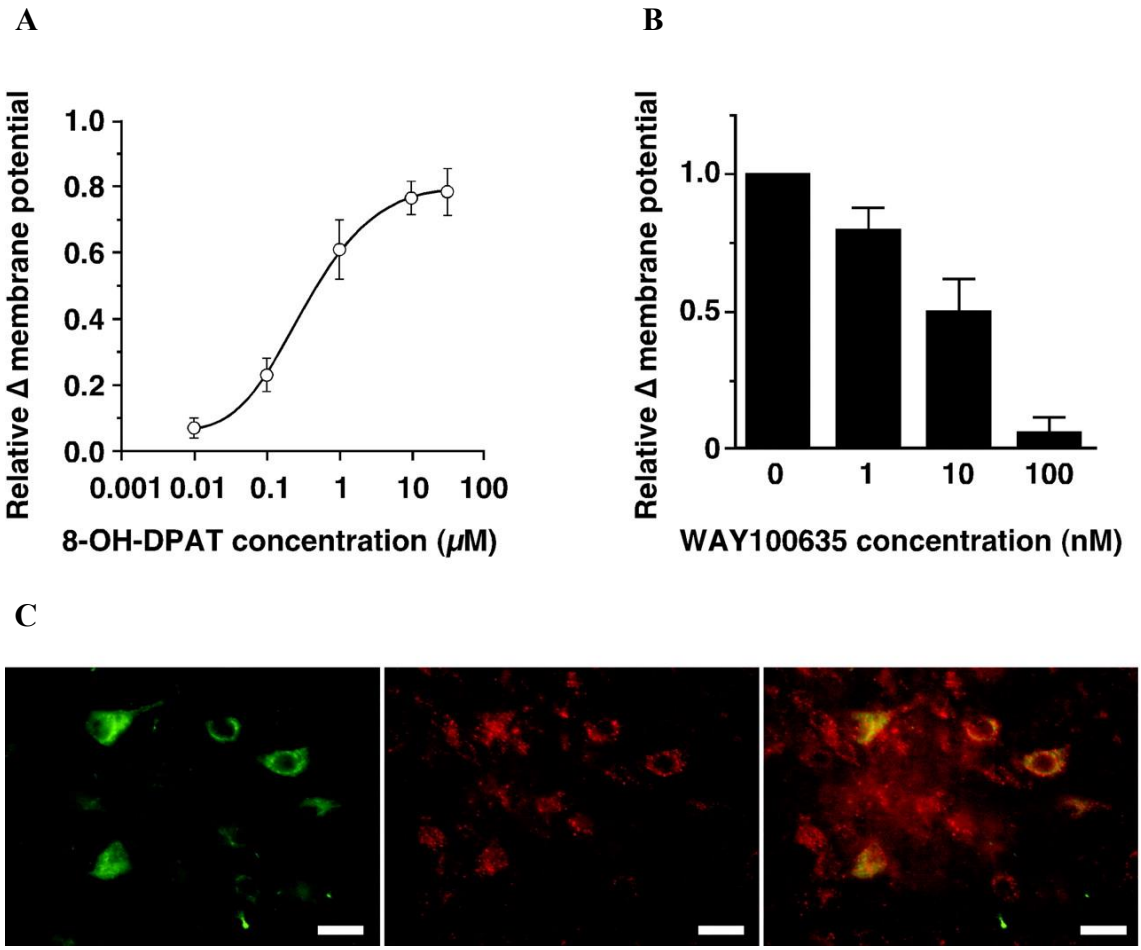

Fig. 2.8 Serotonin 1A receptor expression in orexin neurons

A, Dose relationship of serotonin 1A receptor agonist, 8-OH DPAT. All responses were normalized to 10 μM serotonin applied before treatment. IC_{50} was 0.30 ± 0.002 μM. Mean ± S.E. n = 4–7. B, Inhibition of serotonin-induced hyperpolarization by serotonin 1A receptor antagonist WAY 100635. Mean ± S.E. n = 5–14. C, Immunohistological analysis for serotonin 1A receptor. Left, immunoreactivity for orexin (green, Alexa 488). Middle, immunoreactivity for serotonin 1A receptor (red, Alexa 594). Right, merged image.

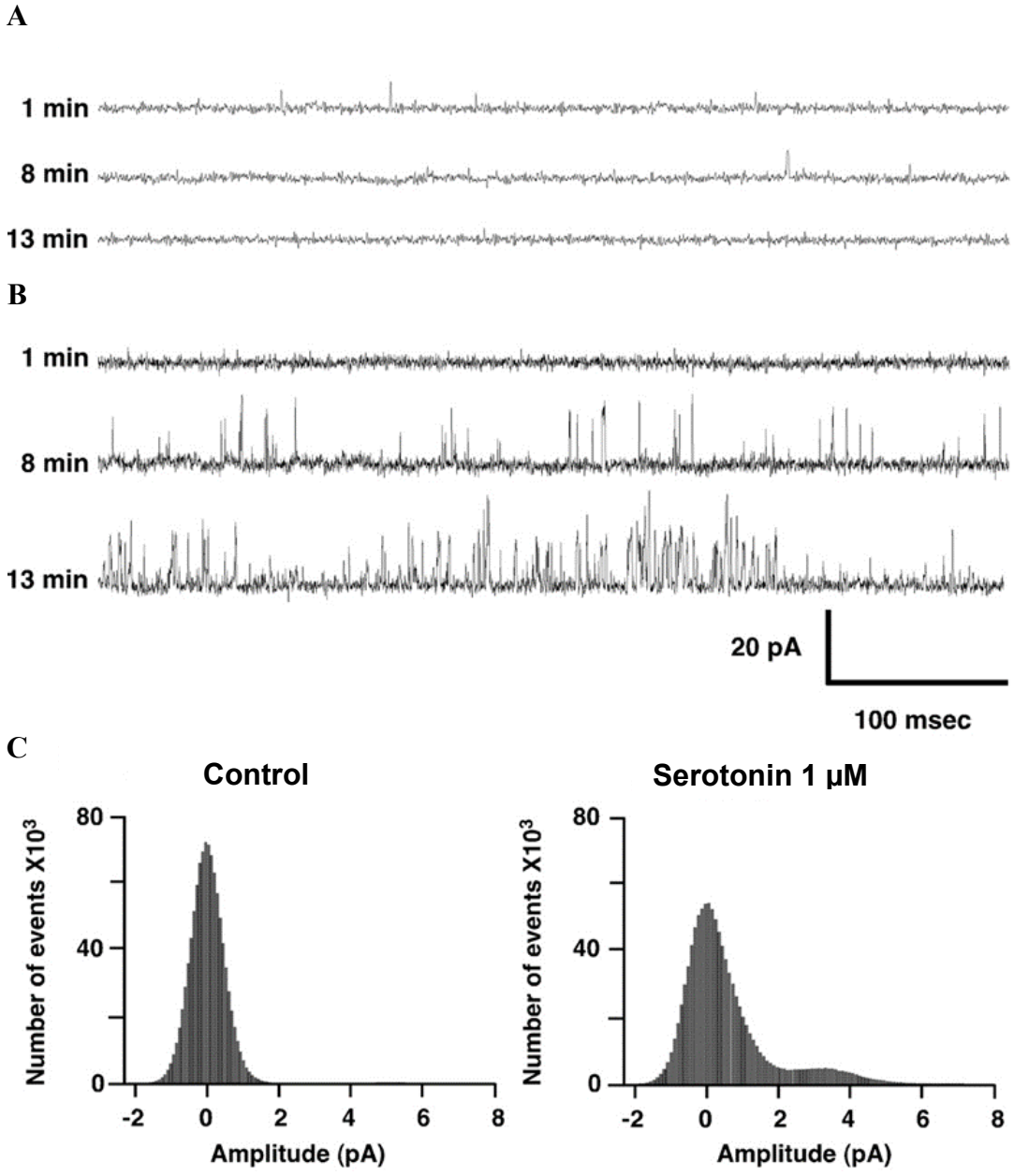

Fig. 2.9 Single channel characteristics of the channels activated by serotonin

A, Cell-attached recording with control pipette. B, Cell attached recordings with serotonin (1 μM) backfilled pipette. C, Histograms of single channel recordings. Left, absence of serotonin. Right, presence of serotonin (1 μM).

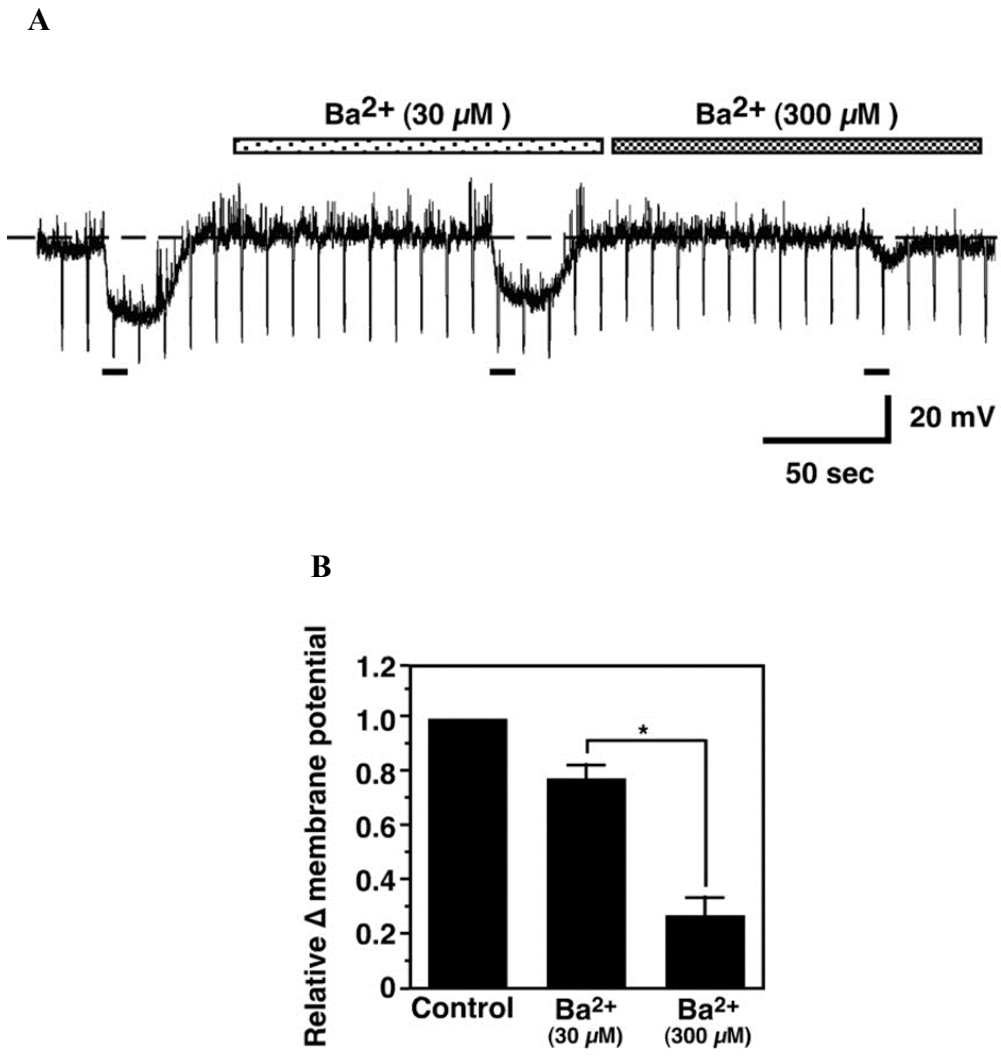

Fig. 2.10 The effect of inhibition of inward rectifier potassium channel

A, Inhibition of serotonin-induced hyperpolarization via Ba^{2+}. Input resistance was monitored by injection of current pulse (-20 pA, 500 msec, 0.1 Hz). B, Relative inhibition of the effect of serotonin. Mean ± S.E. *: $p \leq 0.05$.

2.3.5 The mechanism of suppression of orexin neurons by NA

To clarify the receptors implicated in the response to NA, detailed analysis was conducted. In current clamp mode, NA application hyperpolarized the membrane potential of all EGFP-positive neurons (orexin neurons) tested in the presence or absence of TTX (n = 80).

In the presence of TTX, NA (30 μM) application significantly decreased membrane resistance to 47% of control values; the membrane resistance of orexin neurons before and after NA application was 613.1 ± 24.8 and 289.6 ± 16.9 MΩ (n = 5), respectively (Fig. 2.11). At a holding potential of–60 mV under voltage clamp, NA (30 μM) induced an outward current in orexin neurons in the presence of TTX. NA hyperpolarized orexin neurons in a concentration-dependent manner. E_{max} was 17.3 ± 0.5 mV at 100 μM, IC_{50} was 6.7 ± 0.7 μM (n = 4–6).

Adrenaline also induced hyperpolarization of orexin neurons in a concentration-dependent manner (Fig. 2.11); the effect was more potent than that of NA (E_{max} was 24.2 ± 0.4 mV at 30 μM; IC_{50} was 2.4 ± 0.2 μM, n = 4–6).

On the other hand, DA showed hyperpolarization at a higher concentration than adrenaline and NA (E_{max} = 17.5 ± 0.6 mV, IC_{50} = 141.5 ± 21.9 μM, n = 4–6).

To identify the adrenergic receptor subtype, the effect of selective adrenergic receptor antagonists was evaluated (Fig. 2.12). Idazoxan, which is an α2AR antagonist, suppressed NA (30 μM)-induced hyperpolarization. Pretreatment of slices with 0.01 and 0.1 μM idazoxan for 1.5 min inhibited NA-induced hyperpolarization to 65.5% ± 10.7% (n = 4) and 27.0% ± 15.8% (n = 6), respectively, compared with the values before antagonist treatment. On the other hand, UK-14304, which is an α2AR agonist, mimicked NA-induced hyperpolarization. 1 μM and 10 μM of UK14304 hyperpolarized orexin neurons by 38.3% ± 8.0% (n = 4) and 72.1% ± 6.3% (n = 4), respectively, compared with

prior application of NA (30 μM). Propranolol, which is a beta adrenergic receptor antagonist, did not suppress the effect of NA.

We also found that NA induced a slight depolarization of orexin neurons in the presence of 1 μM idazoxan. NA at concentrations of 1, 10, and 30 μM induced depolarizations of 1.9 ± 0.9, 12.0 ± 2.5, and 17.5 ± 3.0 mV (n = 6), respectively, when membrane potential was adjusted to −70 mV before the experiment. NA-induced depolarization was eliminated by co-application of the selective α1AR antagonist prazosin (n = 6, Fig. 2.12).

The effect of Ba^{2+} on NA-induced hyperpolarization was examined to identify the ion channels involved. Ba^{2+} (3–30 μM) inhibited NA-induced hyperpolarization in a dose-dependent manner. The reversal potentials of NA with 2 mM potassium and 10 mM potassium were −110.2 ± 5.9 mV (n = 5) and –66.6 ± 4.9 mV (n = 5), respectively. The slope of the reversal potential values for a 10-fold change in extracellular potassium ion was 43.6 mV.

Based on these results, hyperpolarization by NA is mediated by α2AR and subsequent activation of inward rectifier potassium channels.

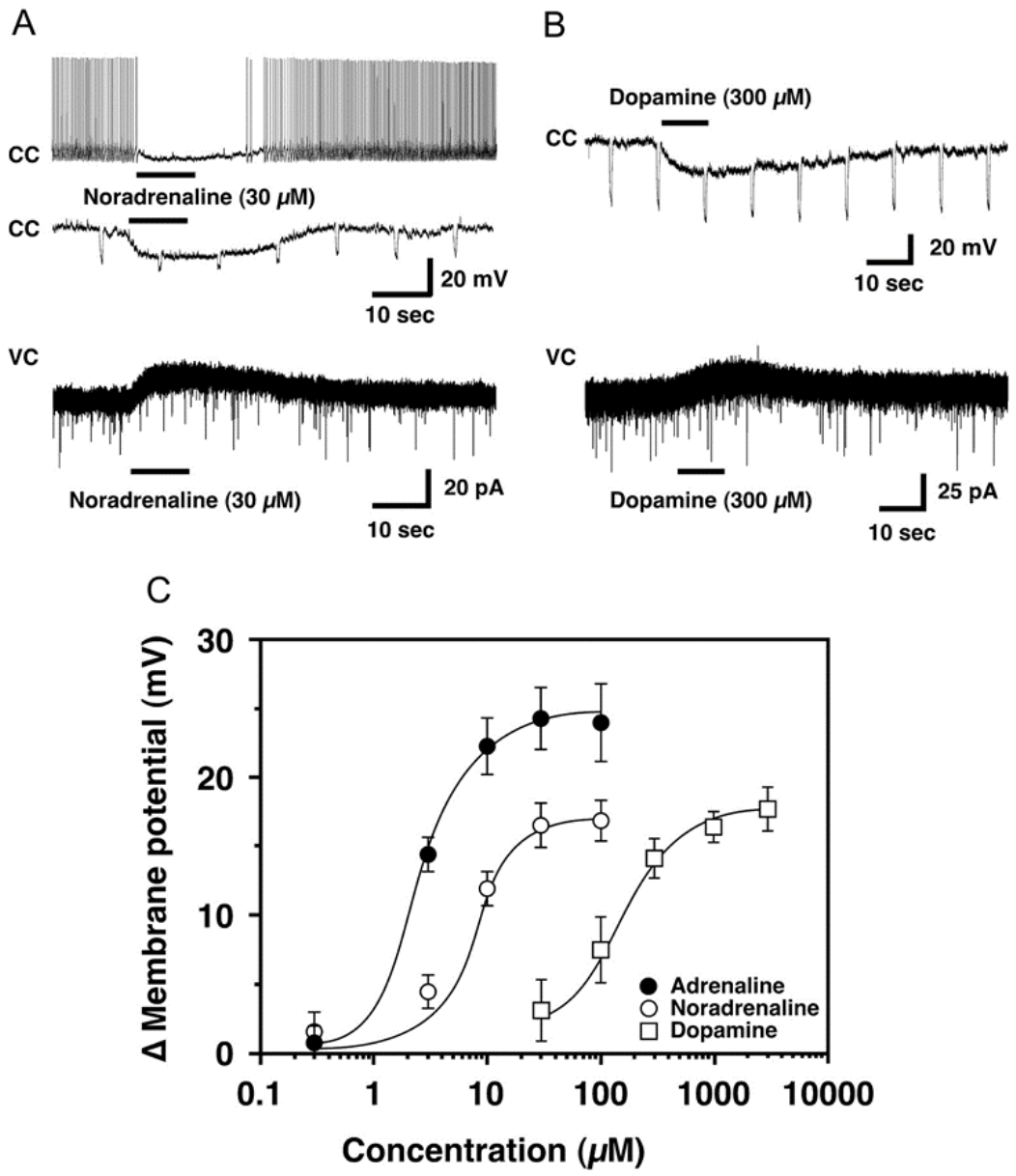

Fig. 2.11 Hyperpolarization of orexin neurons by catecolamine

A, Hyperpolarization by NA. Experiment was conducted in current clamp mode without TTX (upper), with TTX (middle), and in voltage clamp mode (lower). B, Hyperpolarization by DA. Current clamp mode with TTX (upper). Voltage clamp mode (lower). Membrane potential was set at -60 mV in both current clamp mode and voltage clamp mode. C, Concentration relationship to the effect of catecholamine.

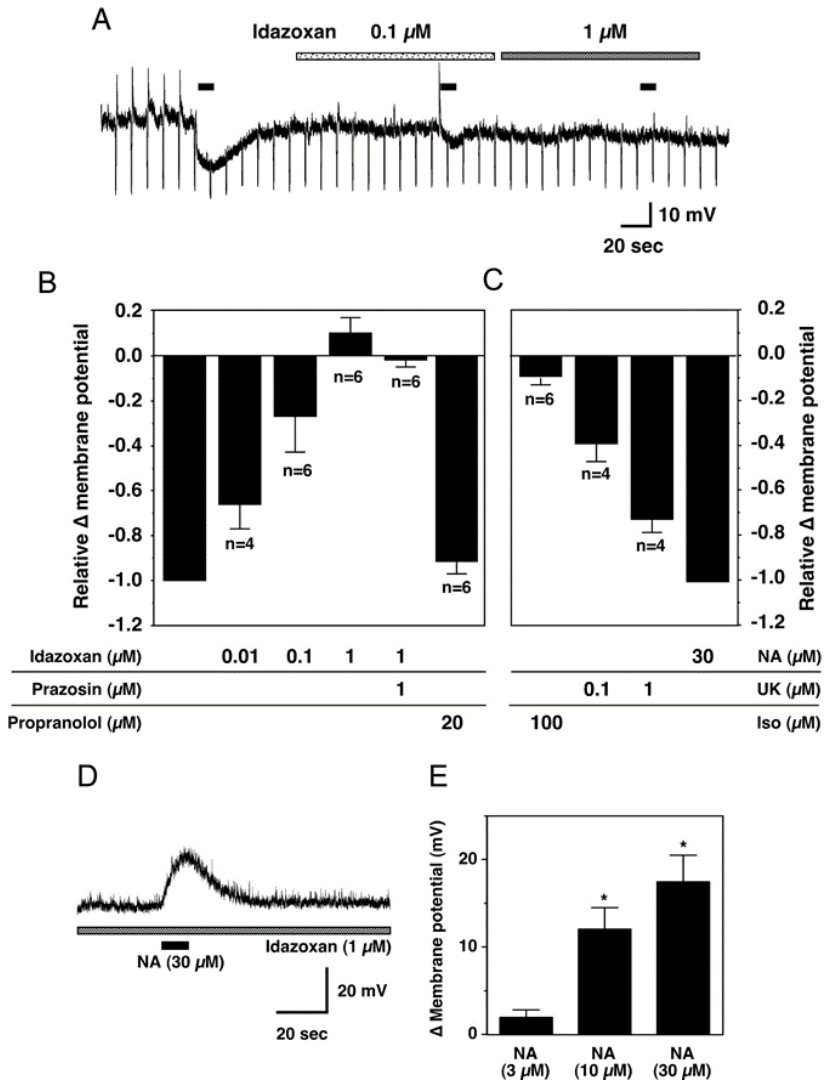

Fig. 2.12 The effect of adrenergic receptor antagonists on NA induced hyperpolarization

Recordings were conducted in current clamp mode. A, Idazoxan, an α2AR antagonist, suppressed hyperpolarization induced by orexin. B, The effect of adrenergic antagonists on NA-induced hyperpolarization. C, The effect of adrenergic agonist on the membrane potential of orexin neurons. B, C, mean ± S.E., Change in membrane potential was normalized to hyperpolarization by NA (30 μM) before treatment with the test compound. D, Weak depolarization in the presence of idazoxan (1 μM). E, Dose response of depolarization by NA in the presence of idazoxan.

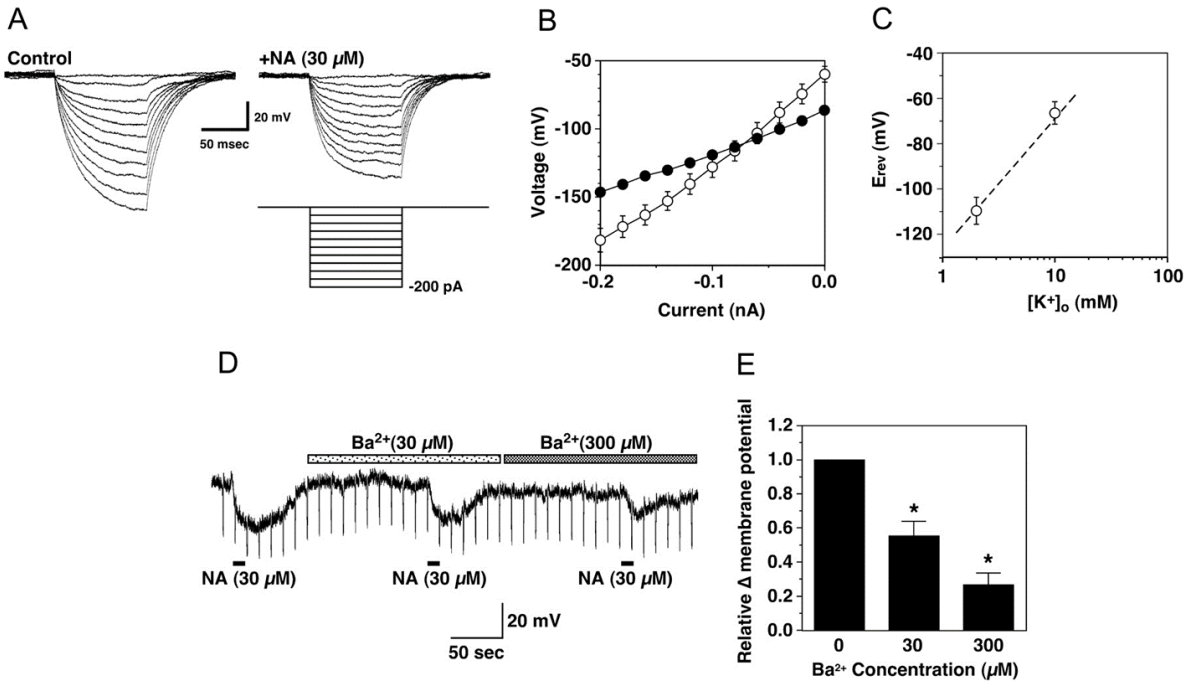

Fig. 2.13 Inward rectifier potassium channels activated by NA

A, Response to a series of 100 ms current steps (in 20 pA increments, −200–0 pA) in the absence (left) or presence (right) of NA. B, IV relationship obtained by current steps. C, Reversal potential at different extracellular potassium concentrations. D, The effects of Ba^{2+}, which is an inhibitor of inward rectifier potassium channel. E, Dose- dependent inhibition of NA-induced hyperpolarization by Ba^{2+}. Mean ± S.E., *: $p \leq 0.05$.

2.3.6. The mechanism of activation of orexin neurons by NA

In the previous section, we showed that NA showed a weak depolarizing effect mediated by alpha 1 receptor activation (Fig. 2.12). The detailed mechanism of depolarization by NA (30 μM) was evaluated.

In voltage clamp conditions (-60 mV), NA dose dependently activated inward current. E_{max} and EC_{50} were 165.6 ± 5.2 pA and 10.7 ± 0.7 μM (n = 6), respectively. Inward current was suppressed by pre-treatment with prazosin, a α1AR selective antagonist. Prazosin inhibited NA-induced inward current to 45.2% ± 9.0% (0.01 μM prazosin, n = 6) and 4.8% ± 1.9% (0.1 μM prazosin, n = 6) of control levels. On the other hand, phenylephrine, a selective α1AR agonist, mimicked inward current produced by NA. The size of the inward current was 16.0% ± 2.2% (10 μM, n = 7) and 46.6% ± 6.1% (100 μM, n = 7) of that induced by 30 μM NA.

To clarify downstream pathways from α1AR activation, activation of ion channels was analyzed (Fig. 2.15). NA (30 μM) caused strong inward current in the absence of extracellular Ca^{2+} ions (composition of extracellular solution, mM: 140 NaCl, 2 CsCl, 1 $MgCl_2$, 1 EGTA, 10 HEPES, and 10 glucose), suggesting NA activates ion channels which are inhibited by extracellular Ca^{2+} ions. The reversal potential of NA using CsCl intracellular solution was 4.5 ± 1.4 mV (n = 5), which is near the estimated value for non-selective cation channels. The activation of inward current was inhibited by SKF96365 (3 μM, 74.4% ± 5.8%, 30 μM, 28.6% ± 5.3% of control levels.), which is an inhibitor of non-selective cation channel.

Based on these results, NA activates orexin neurons via α1ARs and non-selective cation channels expressed in orexin neurons.

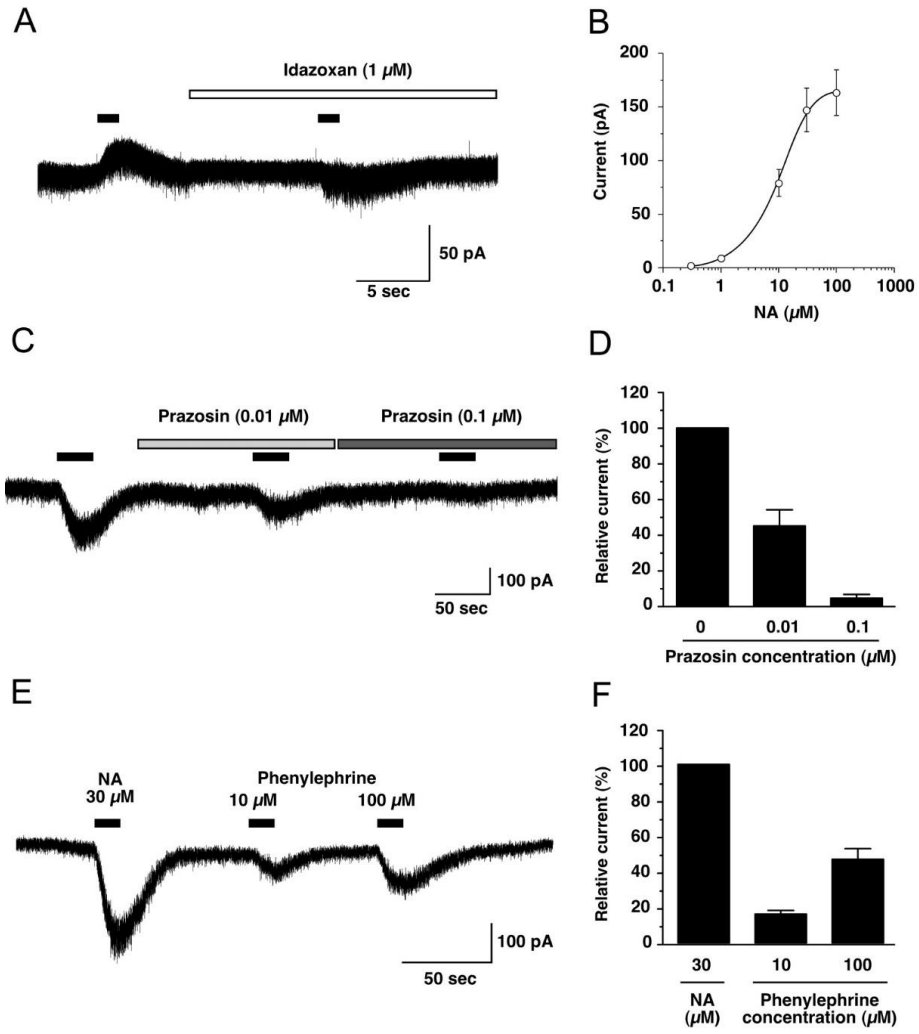

Fig. 2.14 NA-induced inward current in orexin neurons via α1ARs

A, Voltage clamp recording at holding potential at −60 mV. B, Dose relationship of NA-induced inward current. C, D, Inhibition of inward current by prazosin. E, F, Phenylephrine-induced inward current in orexin neurons in a dose-dependent manner. Mean ± S.E. n = 6–7.

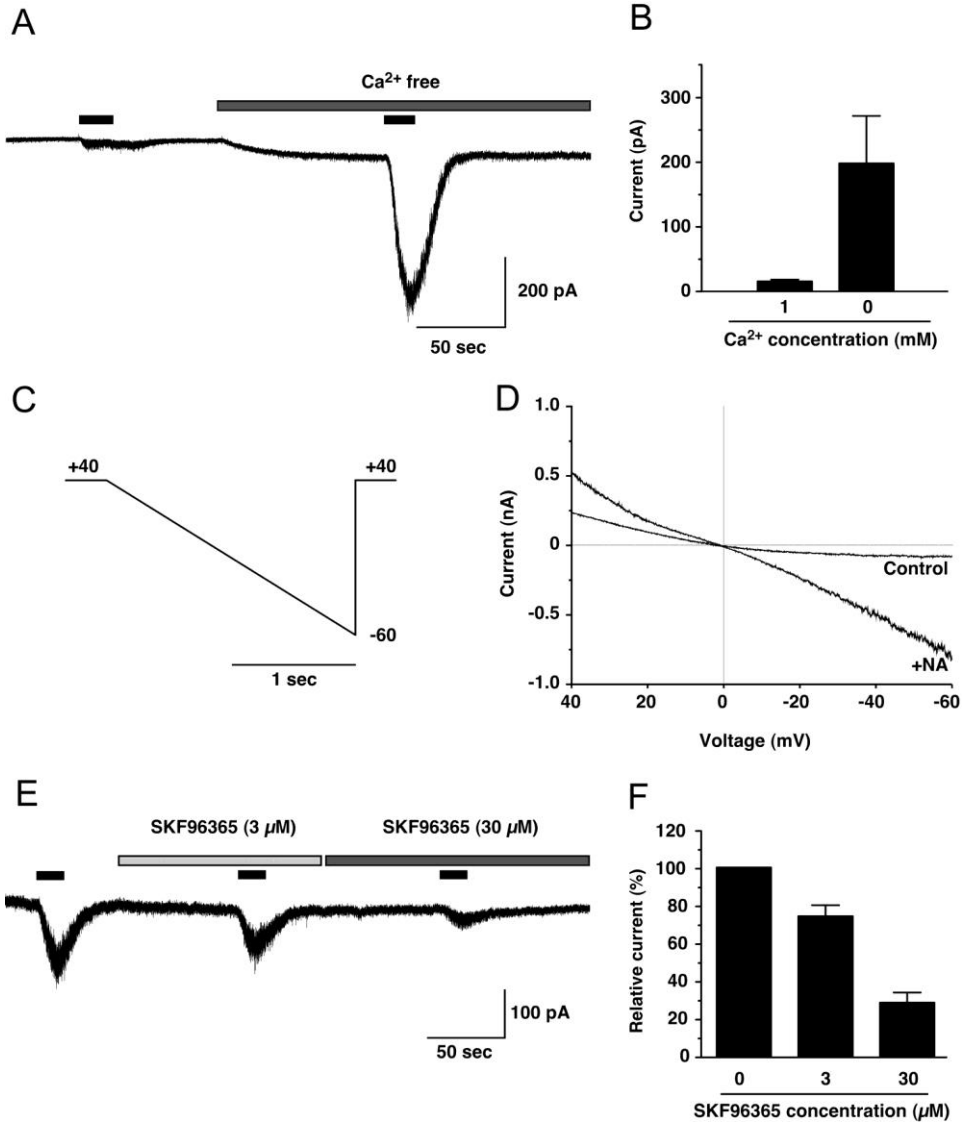

Fig. 2.15 Activation of non-selective cation channels by NA

A, B, The effect of the removal of Ca^{2+} ions from the extracellular solution. C, Ramp protocol for analysis of the IV relationship. D, IV relationship in the control and NA (30 μM)-containing condition. E, F The effect of the non-selective cation channel inhibitor, SKF96365, on inward current. Mean ± S.E., n = 7.

2.3.7. The effect of NA on calcium currents

To examine the effect of NA on Ca^{2+} current, Ca^{2+} current in whole cell patch clamp mode was measured. To measure Ca^{2+} current, Ca^{2+} ions in the extracellular solution were substituted with Ba^{2+} to enhance conductance. AP-5 (50 μM), CNQX (20 μM), picrotoxin (100 μM), and TTX (1 μM) were added to the extracellular solution to block postsynaptic currents and action potentials.

Voltage ramps from −60 mV to 40 mV for 2 s induced −258.9 ± 26.0 pA (n = 6) of inward current. NA (100 μM) decreased Ba^{2+} current by 197 ± 19.0 pA (n = 6), ($P < 0.001$, ANOVA).

These data suggest that NA inhibits Ca^{2+} channels expressed in orexin neurons.

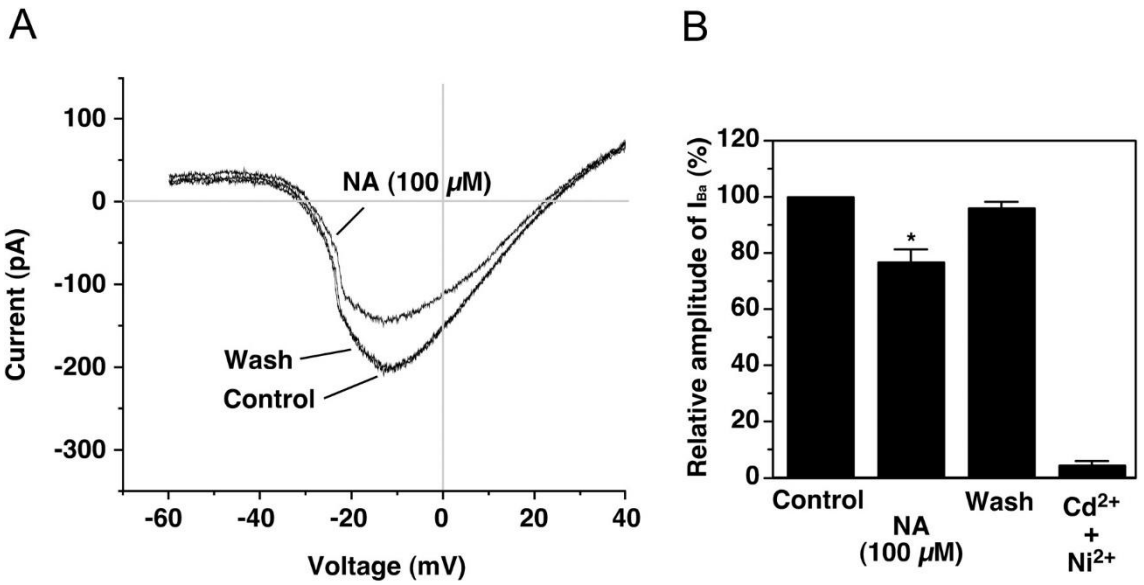

Fig. 2.16 inhibition of Ca^{2+} channel current by NA

A, Ca^{2+} channel current obtained by ramp from -60 mV to 40 mV for 2 sec. To isolate Ca^{2+} channel current from other currents, AP-5 (50 μM), CNQX (20 μM), picrotoxin (100 μM), and TTX (1 μM) were added to the extracellular solution. Decline in amplitude of Ca^{2+} channel current. Cd^{2+} and Ni^{2+} were added to confirm Ca^{2+} channel current.

2.4 Discussion

In this study, the basic electrophysiological properties of orexin neurons were clarified using *orexin/EGFP* mice. The activity of orexin neurons was suppressed by muscimol, which is a $GABA_A$ receptor agonist, NA, and serotonin. Serotonin-induced hyperpolarization was mimicked by the serotonin 1A receptor agonist 8-OH-DPAT and inhibited by the serotonin 1A receptor selective antagonist WAY-100635. NA-induced hyperpolarization was mimicked by the α2AR agonist idazoxan and inhibited by the α2AR antagonist UK-14304. In the presence of idazoxan, NA activated orexin neurons via the α1 adrenergic receptor. On the other hand, the activity of orexin neurons was activated by glutamic acid and cholinergic agonist CCh. AMPA and NMDA receptors are involved in glutamate-induced activation. As the downstream pathway of these receptors, serotonin 1A receptors and α2ARs activated GIRK, and α1ARs activated NSCC. α2AR also activated Ca^{2+} channels. These results are summarized Fig. 2.18.

The author used *orexin/EGFP* mice to identify orexin neurons before patch clamp experiments. Orexin neurons are sparsely distributed in the lateral hypothalamic area. Basic electrophysiological properties were not different from non-orexin neurons located in LHA. Thus, identification of orexin neurons using *orexin/EGFP* mice is an effective method to identify orexin neurons and evaluate the effects of neurotransmitters on these neurons.

Serotonin strongly hyperpolarized orexin neurons. In this study, receptors were identified using selective agonists, antagonists, and immunohistochemistry using antibodies. 8-OH-DPAT has agonistic activity for serotonin 1A and serotonin 7 receptors [32]. On the other hand, WAY-100635 is a serotonin 1A receptor-selective antagonist. Serotonin activates GIRK potassium channels, which are regulated via Gi-coupled GPCRs. Serotonin 7 receptors are known to be Gs-coupled GPCRs. Based

on our results, serotonin 1A receptors have a major role in serotonergic regulation of orexin neurons. However, several studies showed that serotonin 1A receptors and serotonin 7 receptors make heterodimers and crosstalk in downstream signaling [33]. There is the possibility of involvement of serotonin 7 receptors in serotonergic signaling.

NA, adrenaline, and DA hyperpolarized orexin neurons. However, the IC_{50} was different between NA/adrenaline and DA. A high concentration of DA activates α2ARs [34]. The hyperpolarization could be mediated by activation of the adrenaline receptor. In this study, we found strong hyperpolarization via α2ARs and weak depolarization via the alpha 1 receptor. Alpha 1-mediated suppression is a major effect because depolarization was not observed in the absence of the α2AR antagonist idazoxian. However, there is the possibility that these neurons receive different inputs between synapses containing α2AR and α2AR. We found Ca^{2+} channels were inhibited by NA. Ca^{2+} channels in synaptic terminals have important roles in the secretion of neurotransmitters. NA can suppress the release of orexin from orexin neurons.

The activity of orexin neurons is associated with sleep/wake state. Activation of orexin neurons leads to wakefulness. The receptors expressed in orexin neurons are related to sleep/wake state. $GABA_A$ receptor modulators such as BZD drugs promote sleep [35]. Serotoinin 1A receptor agonists, such as tandospirone, cause sleep [36]. The α2AR agonist dexmedetomidine causes sedation [37]. The α1AR antagonist prazosin is reported to ameliorate sleep disorder [38]. The modulation of orexin neurons by serotonergic and adrenergic receptors is consistent with their role as modulators in human.

Serotonin and noradrenaline are decreased by stress and aging caused by environmental changes. This suppressive effect of serotonin and noradrenaline for

orexin neurons indicates that these neurotransmitters have sleep-promoting effects, and reduction of these neurotransmitters can inhibit sleep. The results of this study indicate that reduction of serotonin and noradrenaline can suppress wake-promoting orexin neurons.

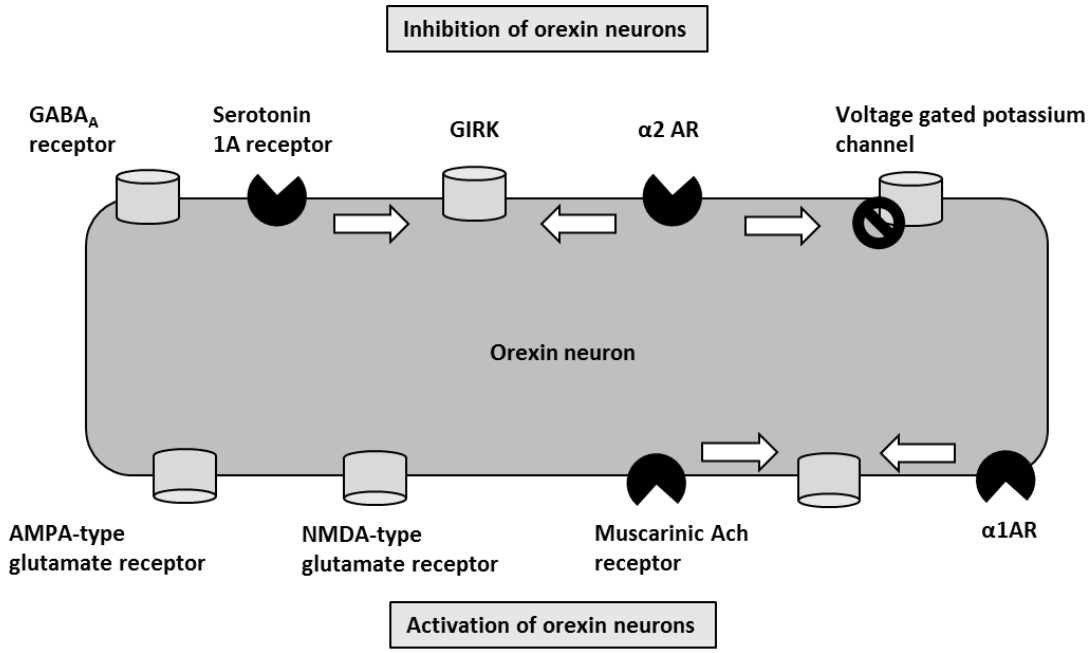

Fig. 2.17 Summary of receptors and ion channels identified in orexin neurons

Orexin neurons were suppressed via $GABA_A$, α2AR, and serotonin 1A receptors and activated by AMPA, NMDA, muscarinic, and α1ARs.

Chapter 3 The importance of the regulation of orexin neurons by sleep/wake homeostasis-related pathways

3.1 Introduction

In Chapter 2, the regulation of orexin neurons by neurotransmitters was evaluated. These results suggested that orexin neurons are regulated by neurotransmitters such as glutamate, GABA, serotonin, NA, and acetylcholine. Stress and aging, which are affected by environmental changes, are related to the neurotransmitters serotonin and noradrenaline that suppressed the activity of orexin neurons. In Chapter 3, the author will focus on the physiological importance of the effect of neurotransmitters in sleep/wake homeostasis.

The transition from wake to sleep is mediated by GABAergic and monoaminergic/serotonergic systems. The orexin system stabilizes the waking state by activation of the monoaminergic cholinergic system.

GABA neurons located in the ventrolateral preoptic area (VLPO) are considered as inducers of sleep [39]. The firing pattern of VLPO GABA neurons is sleep active. VLPO neurons project their axons into wake active nuclei such as the histaminergic tuberomamillary nucleus (TMN), serotonergic dorsal and medial raphe nuclei, cholinergic laterodorsal tegmental nucleus (LDT)/ pedunculopontine nucleus (PPN) and adrenergic locus coeruleus (LC). GABA neurons promote sleep via suppression of the activity of monoaminergic/cholinergic neurons.

Serotonergic neurons are located in the midbrain dorsal and medial raphe nuclei. Neurons in both regions are active in the waking state. The dorsal and medial raphe nuclei have ascending projections to the cerebral cortex, limbic system, and basal ganglia. Orexin neurons activate serotonergic dorsal and medial raphe neurons via the activation of orexin 1 and 2 receptors [40] [41]. Raphe serotonergic neurons show wake-active properties.

Noradrenergic neurons in the LC are considered to have an important role in

sleep/wake homeostasis. LC adrenergic neurons are activated by OX1R. LC adrenergic neurons show wake active properties and innervate the neocortex. Inhibition of LC adrenergic neuron via optogenetic methods causes the induction of sleep.

Histaminergic and cholinergic neurons in the LDT/PPN are also activated in the waking period [39]. Orexin activates both cholinergic and histaminergic neurons [42] [43].

The response of orexin neurons to GABA, NA, and serotonin suggests that orexin neurons receive innervation from these neurotransmitter-containing neurons. In this chapter, the presence of physiological input pathways for orexin neurons via GABA, serotonin, and NA is evaluated. The input from GABA neurons was evaluated by the slice patch clamp method. The presence of noradrenergic and serotonergic inputs was evaluated by immunohistochemistry. Moreover, in vivo pharmacological studies were conducted to clarify the physiological importance of serotonergic input pathways for orexin neurons. The physiological importance of GABAergic, serotoninergic, and noradrenergic inputs is discussed using obtained experimental results and literature information.

3.2 Materials and methods

3.2.1 Recording of postsynaptic current

Postsynaptic currents were recorded using a sodium channel blocker QX-314 (1 mM)-containing pipette to suppress action potentials in recorded neurons. TTX (1 μM) was added to measure miniature postsynaptic currents. Excitatory postsynaptic current was measured by adding picrotoxin (100 μM) and inhibitory postsynaptic current was measured by adding AP-5 (50 μM) and CNQX (20 μM). To measure evoked postsynaptic current, electrical stimuli (100-200 μA, 0.1 msec, 0.1 Hz) were generated using a bipolar

stimulation electrode placed in the LHA.

3.2.2. Immunohistochemistry

Adult male C57BL/6J mice (20–25 gm; Charles River Laboratories, Kanagawa, Japan) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused sequentially with 10 ml of saline and 20 ml of 4% paraformaldehyde in 0.1 M phosphate buffer for 20 min. The brains were removed, trimmed, and immersed in the same fixative solution for 12 hr and then immersed in 30% sucrose solution for 2 d at 4°C. The brains were quickly frozen in embedding solution with OCT compound (Sakura Finetechnical, Tokyo, Japan). The frozen brains were cut into 40-μm-thick coronal sections on a cryostat (MICROM HM 500; MICROM International, Walldorf, Germany). These slices were washed in Tris-buffered saline (TBS) containing 0.25% Triton X-100 (TBS-TX) and incubated in 1% bovine serum albumin fraction V in TBS-TX for 30 min. For orexin and serotonin transporter double staining, sections were incubated with rabbit anti-orexin antiserum (1:2000) [44] and goat anti-serotonin transporter antiserum (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA) for 24 hr at 4°C. Sections were then incubated with Cy3-labeled donkey anti-goat IgG antibody (1:800; Molecular Probes, Eugene, OR) for 1 hr at room temperature and with fluorescein isothiocyanate-labeled donkey anti-rabbit IgG (1:800; Molecular Probes) for 1 hr at room temperature. For orexin and serotonin 1A receptor double staining, sections were incubated with rabbit anti-orexin antiserum (1:2000) [44] and guinea pig anti-serotonin 1A receptor antiserum (1:1000) (Chemicon, Temecula, CA) for 48 hr at 4°C. These sections were simultaneously incubated with Alexa 488-labeled goat anti-rabbit IgG antibody (1:800; Molecular Probes) and with Alexa 594-labeled goat anti-guinea pig IgG (1:800; Molecular Probes) for 1 hr at room temperature. The sections were mounted and examined with a fluorescence microscope

(AX-70; Olympus Optical). To confirm the specificity of antibodies, incubations without primary antibody were conducted as a negative control in each experiment, and no signal was observed.

3.2.3. Intracerebroventricular administration

Male C57BL/6J wild type mice (20–25 g; Charles River Laboratories) or orexin/ataxin-3 hemizygous mice (25–27 g, N7 backcross to C57BL/6J) were housed under controlled lighting (12 hr light/dark cycle; lights on from 8:00 A.M. to 8:00 P.M.) and temperature (22°C) conditions. Food and water were available ad libitum. Mice were anesthetized with pentobarbital (50 mg/kg, i.p.) and positioned in a stereotaxic frame (David Kopf Instruments, Tujunga, CA), and a guide cannula was implanted into the third ventricle under sterile conditions. Mice were then housed separately for a recovery period of at least 7 d. The position of the cannula was verified by central administration of human neuropeptide Y (0.3 nmol in sterile water) to test for a positive response. Mice that ate at least 0.5 g of food over a 1 hr period after injection were used for experiments. WAY100635, a serotonin 1A receptor antagonist, was delivered in saline at a volume of 3 μl over 60 sec, and the injector was left in position for an additional 60 sec to ensure complete dispersal of the drug. Saline alone was injected in the vehicle control experiment. Intracerebroventricular injection was initiated at 7:30 P.M. and was completed by the end of the light period (8:00 P.M.). The locomotor activity of individual mice during the dark period after intracebroventricular injection was assessed with an infrared activity monitor (Supermex; Muromachi Kikai, Tokyo, Japan) in Plexiglas cages to which mice had been well habituated. The chambers were light-controlled (12 hr light/dark cycle; lights on from 8:00 A.M. to 8:00 P.M.) and were sound-attenuated. The infrared activity monitor was a sensor mounted above the cage to detect changes in heat

across multiple zones of the cage through an array of Fresnel lenses.

3.2.4 Statistical analysis

Data were analyzed by two-way ANOVA followed by post hoc analysis of significance by Fisher's protected least significant difference test using the Stat View 4.5 software package (Abacus Concepts, Berkeley, CA). p values less than 0.05 were considered statistically significant.

3.3 Results

3.3.1. The excitatory and inhibitory input to orexin neurons

In slice preparations, the synaptic input to orexin neurons can be measured. To determine whether orexin neurons actually received glutamatergic or GABAergic input in the brain, spontaneous excitatory postsynaptic current (EPSC) and inhibitory postsynaptic current (IPSC) were recorded. EPSC was measured using extracellular solution containing bicuculline (50 μM) to suppress IPSC, and IPSC was measured using extracellular solution containing AP-5 (50 μM) and NBQX (50 μM) to suppress EPSC. Membrane potential was held at −30 mV in voltage clamp condition.

Under this condition, spontaneous EPSC and IPSC were observed. Simultaneous application of AP-5 (50 μM), NBQX (50 μM), glutamate receptor antagonists, and bicuculline (50 μM), a $GABA_A$ receptor antagonist, largely blocked EPSCs and IPSCs (Fig. 3.1).

These results suggest that orexin neurons receive glutamatergic excitatory and GABAergic inhibitory input in the brain.

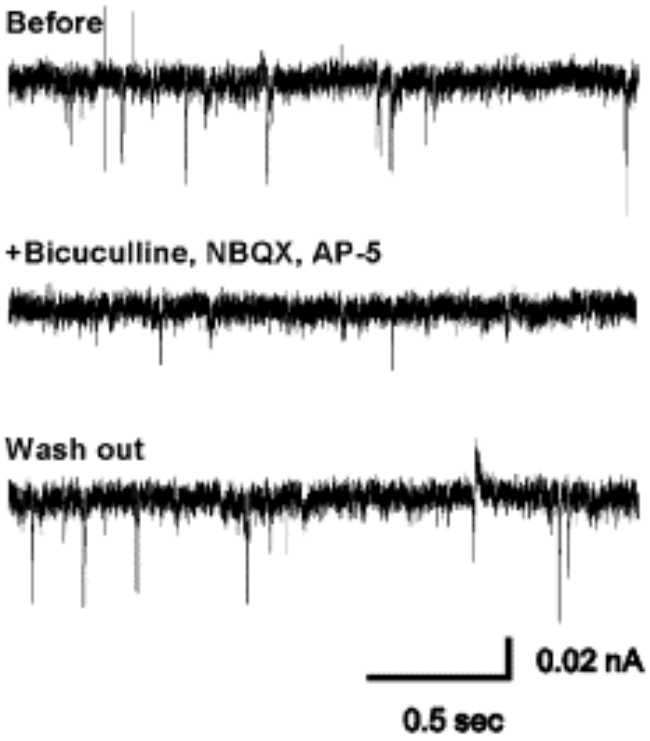

Fig. 3.1 Spontaneous PSC in orexin neurons

Membrane potential was set at −30 mV. EPSC and IPSC were completely blocked by treatment with bicuculline (50 μM), NBQX (50 μM) and AP-5 (50 μM). EPSC and IPSC were recovered by removal of inhibitors.

3.3.2. TH-ir neurons are in apposition to orexin-ir neurons

Immunohistological analysis was conducted to understand the distribution of TH immunoreactive axons. The majority of orexin-ir neurons were located within a field of dense TH-ir axons. TH-ir varicosities were closely opposed to orexin-ir cell bodies and TH-ir axons appeared to be located adjacent to orexin-ir neurons (Fig. 3.2).

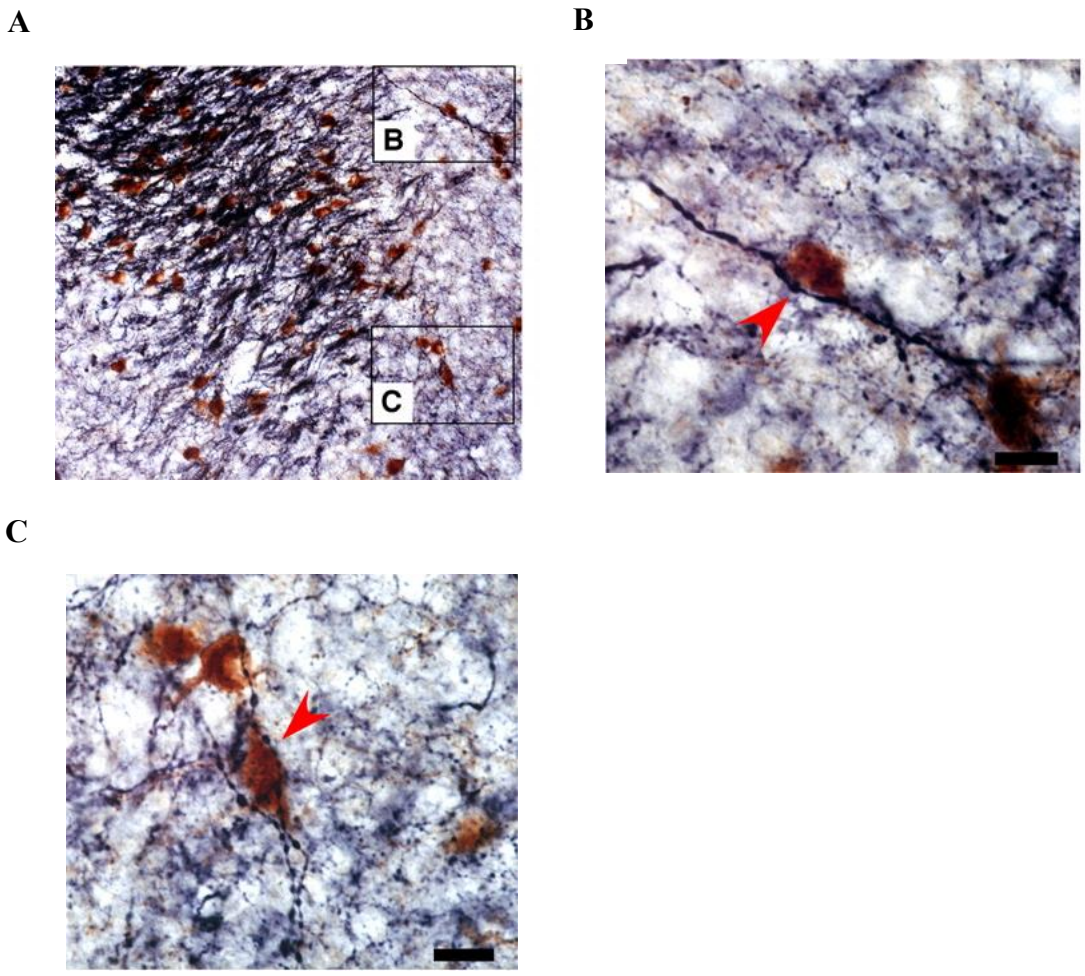

Fig. 3.2 TH-ir nerve endings in the lateral hypothalamic area

A, Immunoreactivity for orexin neurons (brown) and TH (black). B, C Magnified view marked in A. Arrowheads indicate varicosities which are localized near the orexin neurons. Scale bar, 20 μm

3.3.3. The influence of NA on excitatory and inhibitory synaptic inputs

Because TH-ir neurons were observed in the lateral hypothalamic area, the possibility for the modulation of synaptic inputs of orexin neurons by NA was evaluated. Postsynaptic current was measured using QX-314 (1 mM)-containing intracellular solution to suppress action potentials in orexin neurons. Spontaneous excitatory postsynaptic current (sEPSC) was measured in the presence of picrotoxin (100 μM) to suppress IPSC and spontaneous inhibitory postsynaptic current was measured in the presence of AP-5 (50 μM) and CNQX (20 μM) to suppress EPSC (Fig. 3.3). NA (30 μM) application significantly decreased sEPSC frequency to 38.2% ± 6.7% of control values ($n = 6$, $P < 0.0001$, ANOVA). sEPSC frequency recovered partially after NA washout. Reduction of sEPSC by NA was abolished by pre-treatment of idazoxan, antagonist for α2AR (1 μM, $n = 6$, $P < 0.05$). On the other hand, NA increased IPSC frequency by 398.1% ± 78.6% ($n = 6$, $P < 0.0001$). Spontaneous inhibitory postsynaptic currents (sIPSC) amplitude was also increased by 340% ± 100% ($n = 6$) of control value. The increase in sIPSCs was abolished in the presence of the α1-AR antagonist prazosin (1 μM, $n = 6$, $P < 0.0001$, ANOVA)

To reveal the role of adrenergic receptor expression in the presynaptic terminal, the effect of NA on the miniature excitatory postsynaptic current (mEPSC)s and miniature inhibitory postsynaptic current (mIPSC)s were studied in the presence of TTX (1 μM) (Fig. 3.4). NA decreased mEPSC frequency to 38.5% ± 8.9% of control levels ($n = 6$, $P < 0.001$, ANOVA). In contrast to the effect on sIPSCs, NA decreased mIPSC frequency to 32.6% ± 4.1% of control levels ($n = 7$, $P < 0.0001$, ANOVA) (Fig. 3.3, B–D). Reduction of mEPSCs or mIPSCs was suppressed in the presence of idazoxan (1 μM).

The role of presynaptic adrenergic receptors was also evaluated using evoked excitatory postsynaptic current (eEPSC) and evoked inhibitory postsynaptic current

(eIPSC), generated by electrical stimuli (100–200 μA, 0.1 ms, 0.1 Hz) using bipolar stimulation electrodes placed within the LHA (Fig. 3.5). Recordings were conducted under voltage clamp condition (−60 mV). In the presence of picrotoxin (100 μM), eEPSCs with an amplitude of 226.0 ± 22.6 pA (n = 5) were recorded. NA (30 μM) reduced the eEPSC amplitude to 41.3% ± 8.0% (n = 5, $P < 0.0001$, ANOVA) compared to values before NA application. eEPSCs recovered within 10–18 min after NA washout. Recovered eEPSCs were completely abolished by co-application of AP-5 (50 μM) and CNQX (20 μM) in the bath solution, which suggests that they were attributable to the activation of ionotrophic glutamate receptors. In the presence of AP-5 (50 μM) and CNQX (20 μM), we observed eIPSCs with an amplitude of 589.8 ± 277.7 pA (n = 5). NA application decreased the eIPSCs to 27.8% ± 10.9% (n = 5) as compared to values before application. eIPSCs partially recovered after NA washout.

Based on these results, NA can suppress the activity of orexin neurons via indirect mechanisms. NA activated GABAergic neurons innervating orexin neurons via alpha 1 receptors and suppressed glutamatergic neurons innervating orexin neurons. Synaptic transmission to orexin neurons was suppressed by α2AR.

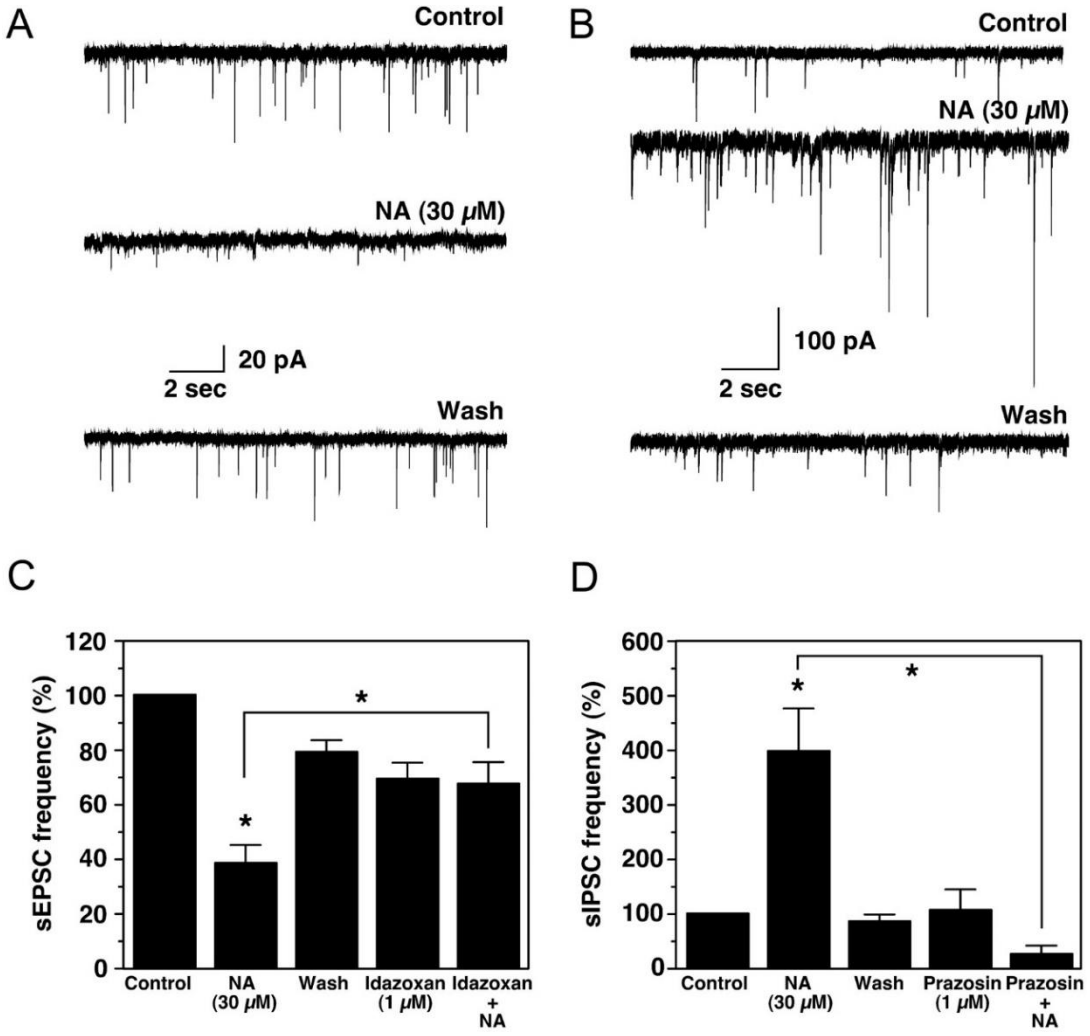

Fig. 3.3 Reduction of sEPSC and induction of sIPSC by NA

A, Reduction in frequency of sEPSC. B, Induction of sIPSC. C, The effect of idazoxan on sEPSC reduction D, The effect of prazosin on sIPSC induction by NA. PSC frequency was normalized to basal PSC frequency. Mean ± S.E., n = 6, *: $p \leq 0.05$

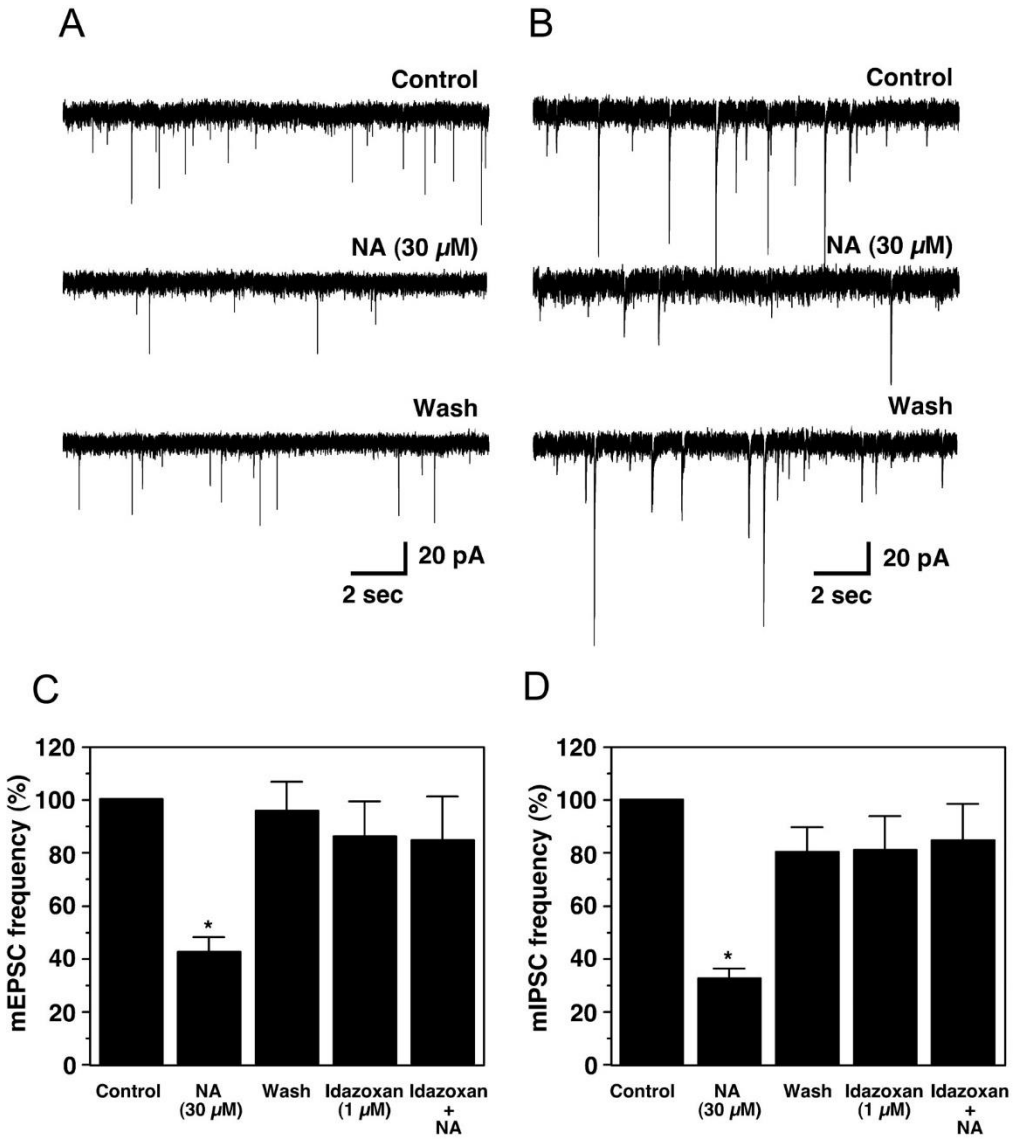

Fig. 3.4 Reduction of mEPSCs and mIPSCs by NA

A, B: Reduction of mEPSCs and mIPSCs by NA. C and D: Summaries of the data in A and B, respectively. Frequency of PSCs were normalized to the frequency before NA treatment. Data are means ± S.E. *, $p \leq 0.05$, ANOVA. Wash, washout.

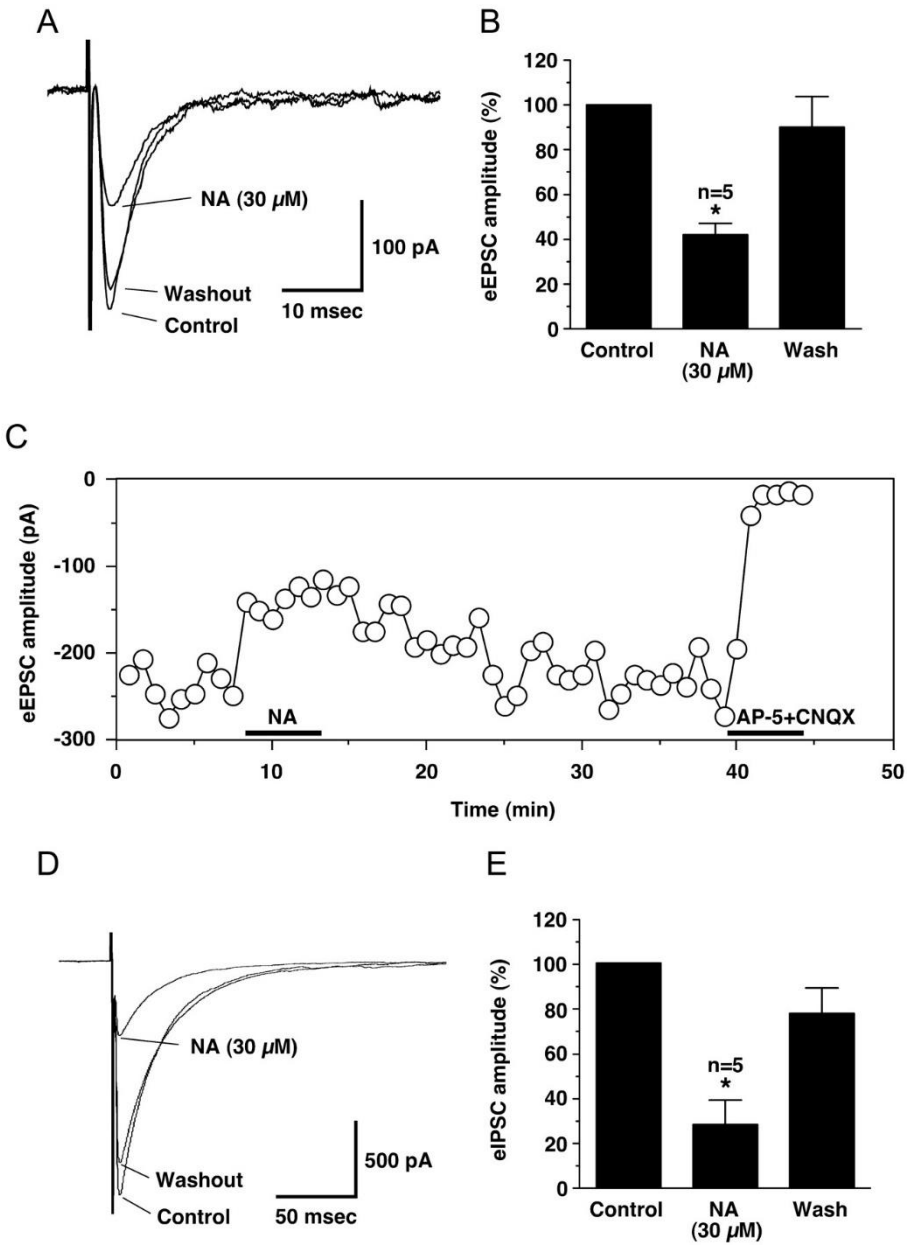

Fig. 3.5 The effect of NA on eEPSC and eIPSC

A, B, Inhibition of eEPSC by NA (30 μM). C, Time course of NA-induced inhibition of eEPSC. D, E, Inhibition of eIPSC by NA. Traces represent the mean of ten recordings. Mean ± S.E. n = 5, *: $p \leq 0.05$.

3.3.4. Orexin-ir neurons are in apposition to serotonin transporter-ir nerve endings

To determine whether serotonergic neurons innervate orexin neurons, sections of hypothalamus were double stained immunohistochemically for serotonin transporter, which is expressed in serotonergic presynaptic membranes, and orexin.

Serotonin transporter-ir nerve endings were observed in the LHA (Fig. 3.6). The nerve endings were distributed near the somata and dendrites of orexin-ir neurons. All orexin-ir neurons were surrounded by serotonin transporter-ir nerve endings.

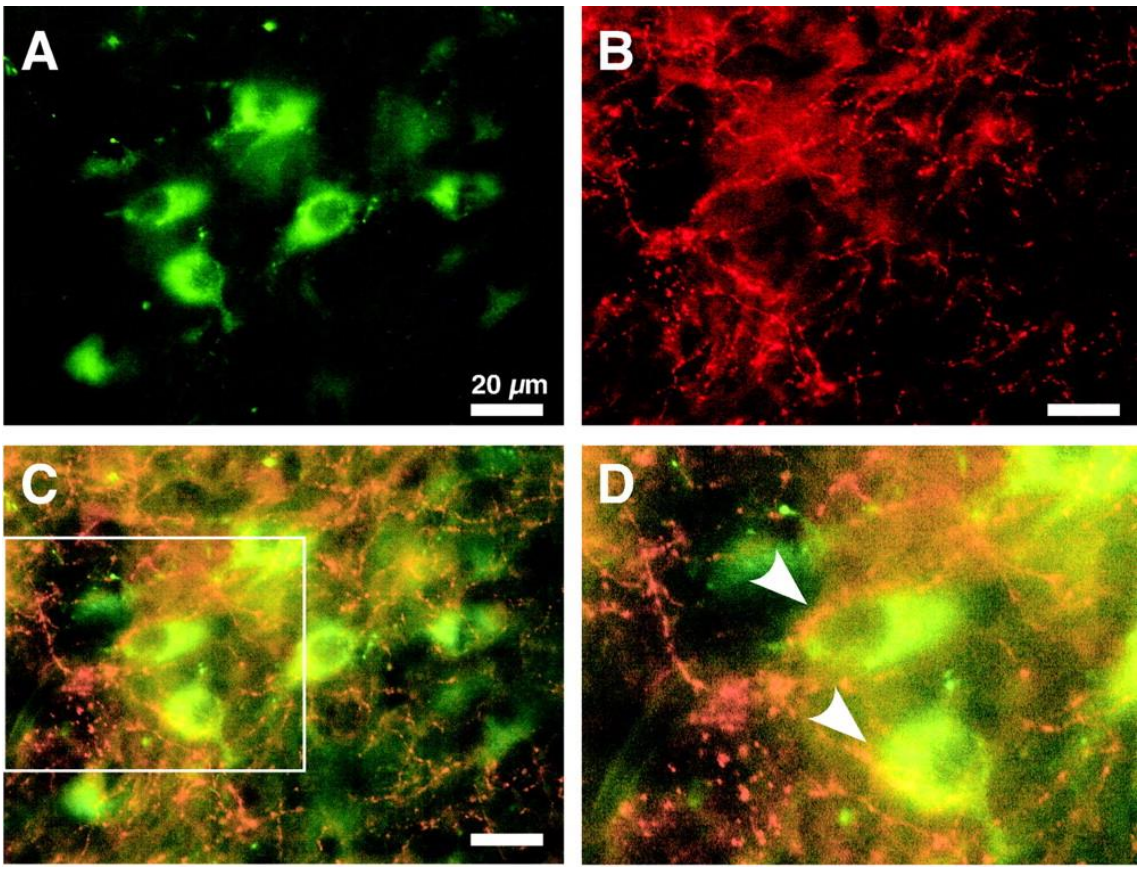

Fig. 3.6 Serotonin transporter-ir nerve ending in the lateral hypothalamic area

A, Immunoreactivity for orexin (fluoresceinisothiocyanate (FITC), green). B, Immunoreactivity for serotonin transporter (red, Cy3). C, D, The merged image. D, Magnified view marked in C. Arrowheads indicate varicosities located near orexin-ir neurons.

3.3.5. Modulation of locomotor activity by serotonin 1A receptor antagonists in wild type mice and orexin-neuron-deficient mice

To understand the physiological importance of serotonergic inhibitory inputs to orexin neurons, the serotonin 1A receptor antagonist WAY-100635 was injected into the third ventricle of wild type mice and orexin-neuron-deficient orexin/ataxin-3 mice.

WAY-100635 increased locomotor activity in wild type mice in the late dark phase (Fig. 3.7). On the other hand, the increase was not observed in orexin/ataxin-3 mice. The activation of orexin neurons can increase locomotor activity. These results suggest that inhibitory input for orexin neurons by serotonergic neurons exists in the late dark phase.

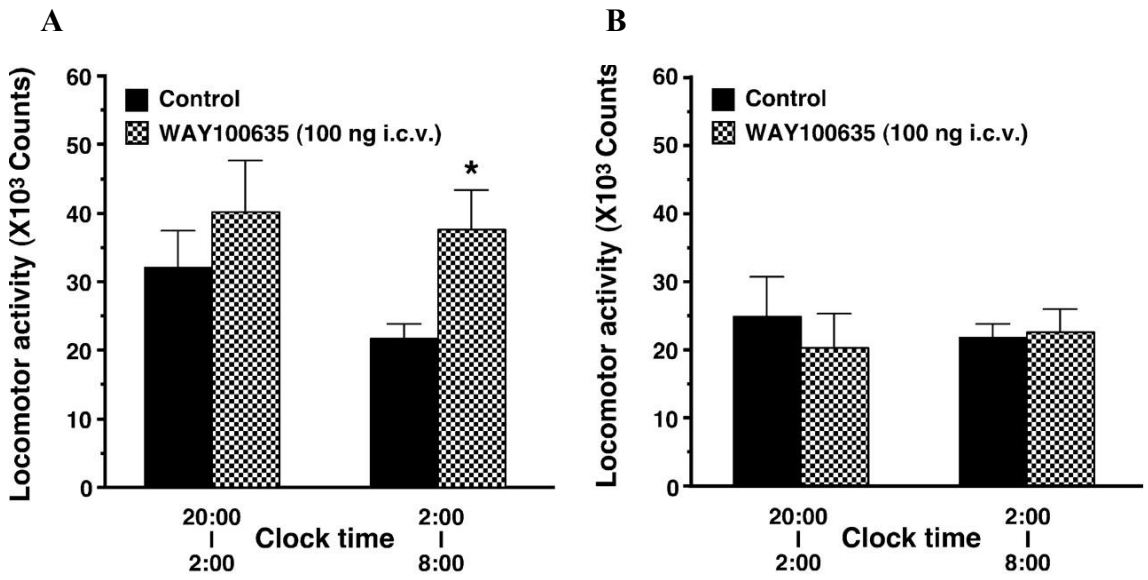

Fig. 3.7 The effect of the serotonin receptor antagonist on locomotor activty

A, Locomotor activity after administration of a serotonin 1A receptor antagonist into the third ventricle in wild type mice. B, Locomotor activity in orexin/ataxin-3 mice. Values are mean ± S.E.

3.4. Discussion

In this chapter, we showed that glutamatergic and GABAergic input was observed in orexin neurons by electrophysiological study of slice samples. Serotonergic and adrenergic nerve terminals were observed in the lateral hypothalamic area near the orexin neurons. NA increased the frequency of IPSC via α1AR and decreased the frequency of EPSC via α2AR. α2AR suppressed the release of glutamate and GABA in synaptic terminals innovating orexin neurons. Inhibition of serotonergic input increased spontaneous locomotor activity. The increase was not observed in orexin-neuron-deficient mice. Experimental results are summarized in Fig. 3.8.

This study confirmed the presence of GABAergic, serotonergic, and adrenergic input to orexin neurons. For further understanding of sleep/wake homeostasis, information about the origin is important. In one study, transgenic mice that express a fusion protein between the nontoxic C-terminal fragment of tetanus toxin (TTC) and GFP (TTC::GFP) exclusively in orexin neurons, acting as a retrograde tracer, were used to identify the origin of inputs [45]. Based on this retrograde tracer study, orexin neurons receive input from GABAergic sleep active neurons and medial raphe serotonergic wake active neurons.

GABAergic neurons in the rostral hypothalamic area, especially VLPO, are known as sleep promoting neurons. A study using neuronal unit recording showed that VLPO neurons are active during sleep. A recent study showed that sleep deprivation increases the amplitude of GABA-induced hyperpolarization in orexin neurons. GABAergic input plays an important role in sleep promotion via suppression of orexin neurons.

A study using TTC::GFP mice showed that orexin neurons receive serotonergic innervation from the medial raphe nucleus. The dorsal raphe nucleus projects to the

medial raphe nucleus. Because serotonin neurons in the raphe nucleus are wake-active, inhibitory input from the medial raphe can contribute to negative feedback to stabilize the activity of orexin neurons in the waking state and transition to sleep state. Serotonin 1A receptor overexpression in orexin neurons causes increased slow wave sleep. By these results, suppression of orexin neurons by activation of serotonin 1A receptor can promote sleep [46].

A study using TTC::GFP mice showed that there is not clear evidence of synaptic innervation for orexin neurons by adrenergic neurons in the locus coeruleus. However, NA inhibited orexin neurons not only directly but also indirectly via activation of GABAergic and glutamatergic interneurons in an indirect feedback loop from adrenergic neurons to stabilize sleep/wake homeostasis.

The hypothesis based on this study and the literature is shown in Fig. 3.9. Orexin neurons receive direct inhibitory input from serotonergic neurons in the median raphe nucleus (MnR) and direct/indirect inhibitory input from the LC. A closed loop between orexin neurons and serotonergic and adrenergic systems can exist. This loop can contribute to sleep wake homeostasis. Modulation of input pathways to orexin neurons can be a method to induce sleep.

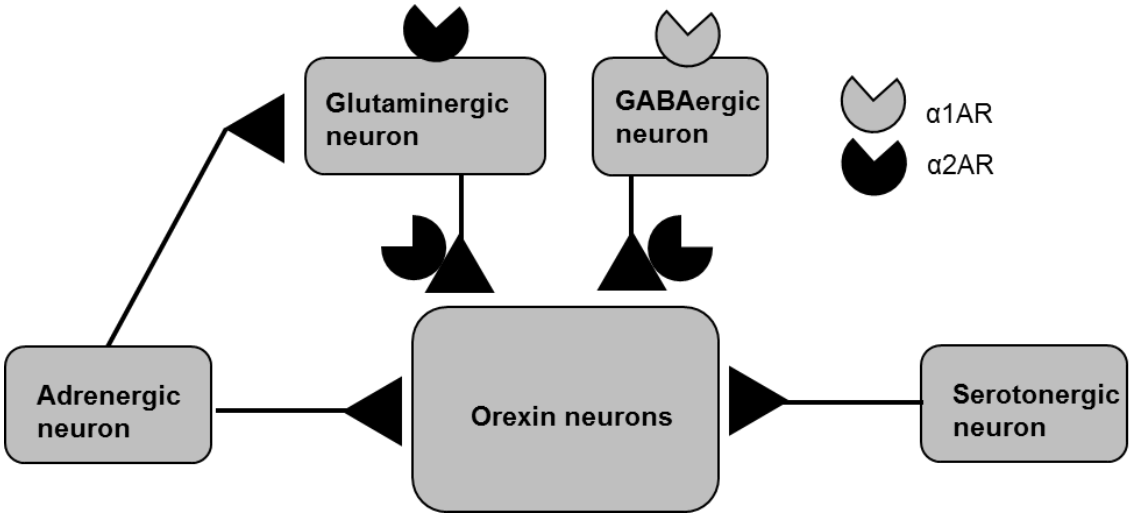

Fig. 3.8 Summary of results in chapter 3

Glutamatergic and GABAergic neurons innervate orexin neurons. Neuronal terminals of serotonin neurons are observed near the orexin neurons in the LHA. Neuronal terminals and varicosities of adrenergic neurons were observed in the LHA.

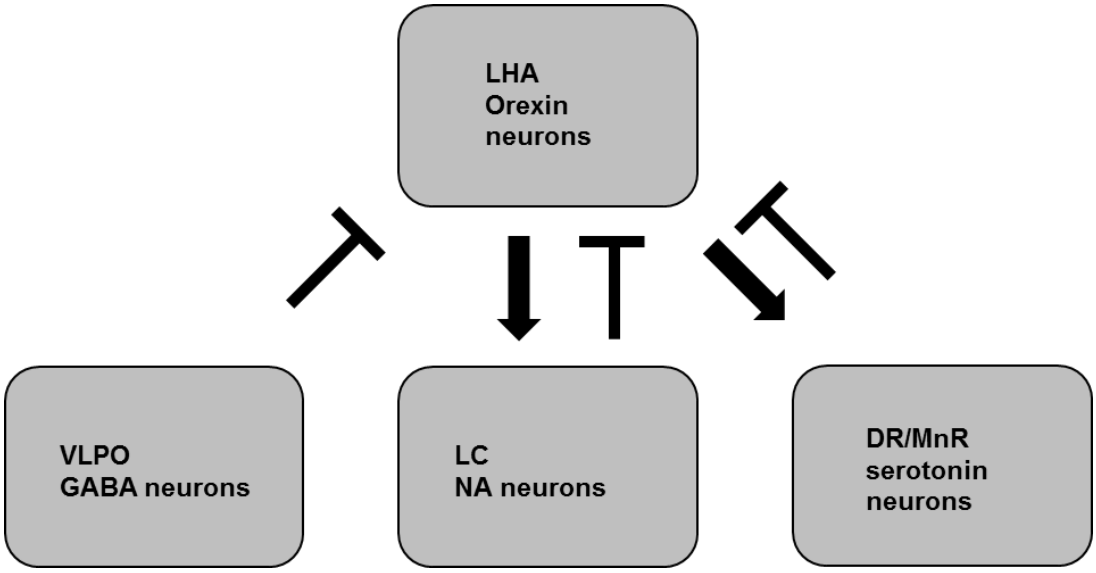

Fig. 3.9 Feedback loop between orexin and serotonin/NA neurons

Orexin neurons activate dorsal raphe serotonin neurons via orexin 1 and 2 receptors and serotonergic inhibitory input from the medial raphe nucleus. Orexin neurons activate adrenergic neurons in the LC via OX1R and receive inhibitory input in a direct/indirect manner from the adrenergic system. Orexin neurons receive GABAergic input from sleep-promoting VLPO GABA neurons.

Chapter 4 The possible treatment of sleep disorders via regulation of input pathways for the orexin system

4.1 Introduction

In the previous chapters, the suppressive effect of NA and serotonin on orexin neurons and receptors involved in this response were identified. Moreover, the physiological importance of serotonergic and adrenergic inhibition of the orexin system in sleep/wake homeostasis was examined using a preclinical method. The information about receptors expressed in orexin neurons can provide information for the understanding of the mechanism of sleep-promoting compounds and discovery of sleep-promoting methods.

For treatment of patients with insomnia, a general sleep disorder, drugs that suppress wake/active neurons are widely used. There are three categories of anti-insomnia drugs: GABA receptor modulators, melatonin receptor agonists, and orexin receptor antagonists. While $GABA_A$ modulators have strong sleep-promoting effects, several kinds of side effects are observed because $GABA_A$ receptors are expressed in wide areas of the brain and neuronal pathways that are not involved in sleep/wake homeostasis. In 2006, a melatonin receptor antagonist was approved. The melatonin pathway can cause promotion of sleep. Moreover, an orexin receptor antagonist was approved. The orexin receptor antagonist is considered an effective treatment method for insomnia because the mechanism is based on modulation of the physiological sleep/wake pathway.

Non-drug treatment can be considered as important for the treatment of insomnia because insomnia is chronic disease and side effects can be a problem for aged patients. There are several kinds of natural products which are known to have sleep-promoting effects in humans. For example, 5 kanpo medicines have indications for insomnia. In Europe, several kinds of herbs have been used for the treatment of insomnia based on long-term experience of human use. The author identified that orexin

neurons are inhibited by $GABA_A$ receptors, serotonin 1A receptors, and α2AR. In this chapter, database analysis was conducted to evaluate the possibility for modulation of orexin neurons by natural products. Based on the collected information, the author discusses the possibility for the development of new treatment methods for insomnia via the orexin system and possible new treatment methods are discussed.

4.2. Materials and methods

Target molecules of natural products written in guidelines for sleep disorder were examined using literature information. The active component and their chemical structure were examined using the PubChem and Pubmed database.

4.3 Results

4.3.1 The natural products used for treatment of insomnia

Natural products that are used to modulate sleep disorders and can modulate receptors expressed in orexin neurons are shown in Table 4.1. Valerian is used as an herbal medicine in Europe. Extracts of this root show improvement in insomnia in human experience. The active component is considered to be valerenic acid. Valerenic acid can activate $GABA_A$ receptors. Kava is the dry root extract of *Pioer methysticum.* Kava has been used for the treatment of insomnia by human experience. The active component is considered to be Kavain, which activates $GABA_A$ receptors.

In Chinese and Japanese herbal medicine, kanpo has an indication for insomnia. Yoku-kan-san and Suan Zao Ren Tang also have indications for insomnia based on long-term experience in human use. Yoku-kan-san is made by seven kinds of herbs. Uncaria Hook, which is hook of *Uncaria rhynchophylla*, is considered to have an important role in sleep-promoting effects and the main component, Geissoschizine

methyl ether, can activate serotonin 1A receptors. Suan Zao Ren Tang contains the seed of *Zizyphus jujube Mill* var, which contain sanjoine A as an active component. $GABA_A$ receptors and serotonin 1A receptors are activated by extract from the seeds of *Zizyphus jujube Mill* var.

Rosemary is considered to improve insomnia by human experience. Rosmarinic acid is considered the active component. Rosmarinic acid activates the GABAergic pathway in preclinical models.

Table 4.1 Natural products modulating sleep/wake homeostasis

Name of plant	Name as herbal medicine	Possible active compound	Possible mechanism
Valeriana officinalis L.	Valerian	Valerenic acid	Activation of $GABA_A$ receptors [47]
Piper methysticum	Kava	Kavain	Activation of $GABA_A$ receptors[48]
Uncaria rhynchophylla	Yoku-kan-san	Geissoschizine methyl ether	Activation of serotonin 1A receptors[49]
Zizyphus jujube Mill var. *spinosa*	Suan Zao Ren Tang	Sanjoinine A	Activation of serotonin 1A receptors Activation of GABAergic pathway [50]
Perilla frutescens	Rosmarinic acid	Rosmarinic acid	Activation of GABAergic pathway [51]

4.3.2 The effects of natural products for insomnia

The clinical study of natural products for insomnia is summarized in Table 4.2. Preclinical studies of natural products are summarized in Table 4.3. The effect of valerian on insomnia was confirmed by a two-week clinical study. Valerian improved sleep quality in insomnia patients. In a preclinical study, a single administration of valerian shortened sleep latency in rodents. Kava (200 mg) improved insomnia after 4 weeks of treatment in humans. In rodents, Kava shortened sleep latency after a single administration. Yoku-kan-san increased the total amount of sleep time in humans. In rodents, the induction of sleep was reported after a single administration. Suan Zao Ren improved sleep quality in patients treated with methadone. Rosmarinus officinalis increased sleep time in opiate-withdrawal patients. In rodents, rosmarinic acid shortened sleep latency.

Table 4.2 Clinical trials of natural products for insomnia

Herbal medicine	Study design	Treatment period	Result
Valerian	Valerian vs. placebo	4 weeks	Improvement of sleep quality [52]
Kava	Kava vs. placebo	4 weeks	Improvement of sleep quality score [52]
Yoku-kan-san	Yokukan-san vs. Anchu-san	3 days	Increase of total sleep time[15]
Suan Zao Ren Tang	1. Placebo 2. Suan Zao Ren (n = 45) (during methadone treatment)	4 weeks	Improvement of sleep score Average sleep efficiency [53]
Rosmarinus Officinalis	Opiate withdrawal syndrome patients	14 days	Increased sleep time [54]

Table 4.3 Preclinical studies of the effects of herbal medicine on insomnia

Natural products	Study Model	Treatment period	Result
Valerian	Sleep disturbed rat	Single administration (1000 mg/kg)	Shortening in sleep latency [55]
Kava	Sleep disturbed rat	Single administration (1000 mg/kg)	Shortening in sleep latency [56]
Yoku-kan-san	Normal mice	Single administration (300 mg/kg)	Sleep promotion (reduction by skin temperature) [57]
Sanjoinine A	Pentobarbital induced sleep model	Single administration of sanjoinine A (2 mg/kg)	Shortening in sleep latency [58]
Rosmarinic acid	Pentobarbital induced sleep model	Single administration (0.5–2 mg/kg)	Shortening in sleep latency Increase of total sleep time [51]

4.3.3 The relationship between the mechanism of insomnia-modulating natural products and orexin neuron modulation

Based on the expression of receptors in orexin neurons analyzed in Chapter 2 and the information for target pathways of natural products that can ameliorate insomnia, possible effecst on orexin neurons are shown in Fig. 4.2.

Varelian, KAVA, Suan Zao Ren Tang, and rosmarinic acid can suppress the activity of orexin neurons via activation of the GABAergic pathway. Suan Zao Ren Tang and Yoku-kan-san can activate serotonin 1A receptors and suppress activity of orexin neurons. In the sleep-promoting effect of these herbal medicines, suppression of orexin neurons may be one of possible mechanism. Rosmarinic acid also suppresses orexin neurons via increased synthesis of noradrenaline [24].

The analysis of properties of orexin neurons can provide information which can be useful for understanding the mechanisms of herbal drugs for sleep/wake disorders.

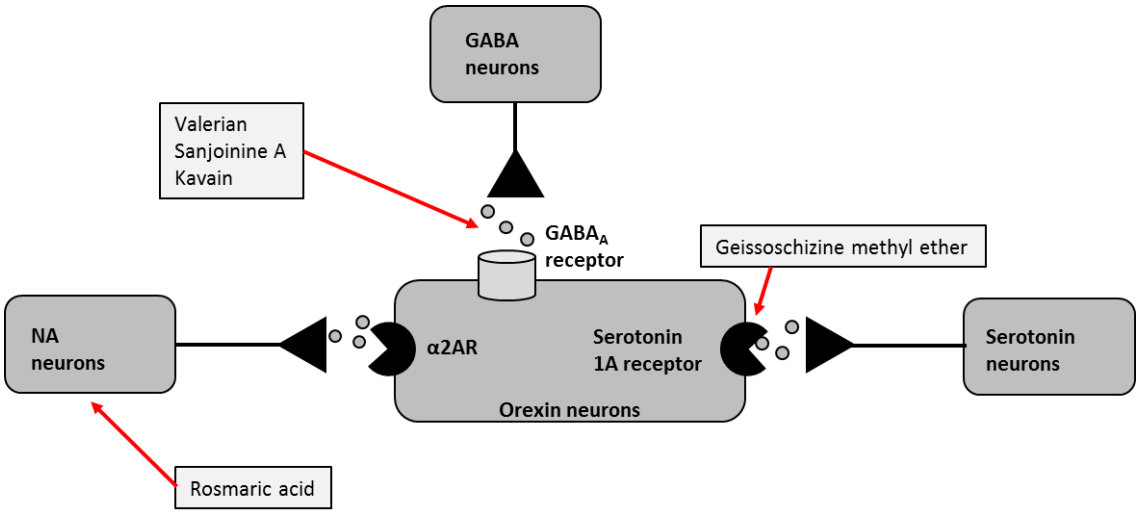

Fig. 4.1 Possible modulation of orexin neurons via herbal medicines that ameliorate sleep/wake disorders

4.4 Discussion

In this chapter, the possible regulation of orexin neurons by herbal medicines known to modulate sleep/wake homeostasis was examined. Moreover, based on the mechanism of the approved orexin receptor antagonist drug and the herbal medicine, combination therapy could be an effective treatment method for insomnia.

The effect of herbal medicine was mainly identified by human use. Moreover, anti-insomnia effects can be evaluated using pre-clinical models. Natural products showing an anti-insomnia effect in humans would show sleep promoting effects in rodent models.

Combination therapy using drugs with different mechanisms of action is useful because of additive or synergistic effects. Now, an orexin receptor antagonist is approved as an anti-insomnia drug. The combined use of the orexin receptor antagonist and natural products that are expected to modulate orexin neurons could provide effective treatment methods for insomnia (Fig. 4.2).

Valerian, KAVA, Suan Zao Ren Tang, and rosmarinic acid are reported to modulate $GABA_A$ receptors. In our experimental data, we showed that orexin neurons express functional $GABA_A$ receptors. The combined use of those compounds and orexin receptor antagonists may be an effective treatment method for insomnia.

Our study showed that serotonin suppressed the activity of orexin neurons via serotonin 1A receptors. A serotonin 1A receptor antagonist locally administrated to the hypothalamus increases locomotor activity in an orexin neuron-dependent manner. Combination therapy using orexin receptor antagonists and Yoku-kan-san could be an effective treatment method for insomnia.

The activity of orexin neurons was suppressed by α2AR. In herbal medicine often used for insomnia, adrenergic alpha 2 receptor agonists were not observed.

However, activation of α2ARs has the potential to increase sleep time [59]. This information suggests that screening natural products for an effect on adrenergic receptors can lead to identifying new natural products with anti-insomnia effects.

In this chapter, the possible modulation of orexin neuron activity by several herbal medicines with sleep-promoting effects was discussed. This information could contribute to future development of new approaches for the treatment of insomnia by combination therapy by approved orexin receptor antagonists and herbal medicine.

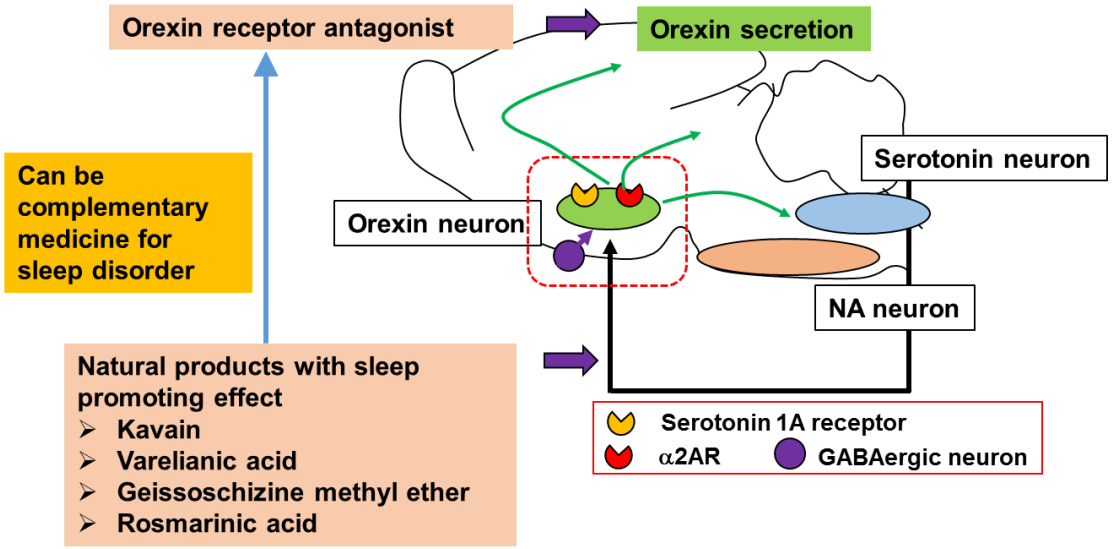

Fig. 4.2 The possibility of combination therapy using approved drugs and natural products as complementary medicine

Chapter 5 General conclusions

Insomnia is a common disease and the change in our environment provides risk factors such as aging, irregular work, and jetlag caused by internationalization. In this study, the author evaluated the responsiveness of orexin neurons to neurotransmitters. GABA, serotonin, and NA suppressed the activity of orexin neurons. The receptor subtype related to responses was identified using selective agonists and antagonists. The $GABA_A$ receptor, serotonin 1A receptor, and α2AR were involved in the suppression of orexin neurons. α1ARs are involved in the activation of orexin neurons although their impact was lower than that of α2AR. These experimental results were summarized in Chapter 1.

Sleep/wake homeostasis is regulated by the relationship between orexin neurons and monoaminergic, cholinergic, and GABAergic neurons. In Chapter 2, the author evaluated input pathways to orexin neurons from serotonergic, GABAergic, and adrenergic systems. In slice patch clamp experiments, GABAergic inputs to orexin neurons were observed. GABAergic input was increased by treatment with NA. By immunohistochemical analysis, the authors identified that serotonergic and noradrenergic neural fiber are apparent near orexin neurons in the LHA. Treatment with the serotonin 1A receptor selective antagonist in the third ventricle increased locomotor activity in wild type mice, and the effect was not observed in orexin/ataxin-3 mice. Orexin neurons receive physiological input from GABAergic, serotonergic, and adrenergic systems. The physiological role of these inputs was discussed based on the experimental data and literature information. Orexin neurons receive serotonergic input from the medial raphe nucleus and GABAergic input from the VLPO. In the induction of sleep, orexin neurons receive GABAergic input from other orexin neurons. The serotonergic and adrenergic inhibitory input can act as negative feedback pathway to stabilize the sleep/wake cycle. The importance of serotonergic input to orexin neurons

was confirmed by reported studies using orexin neuron-selective serotonin 1A receptor-overexpressing Tg mice. By overexpression of serotonin 1A receptor in orexin neurons, the duration of slow wave sleep was increased. Serotonergic input to orexin neurons has a sleep promoting effect.

In Chapter 4, the possible regulation of orexin neurons by natural products was discussed. Several natural products are known to modulate sleep/wake homeostasis and have been used for treatment of insomnia based on human experience. From the literature and public database information, valerianic acid and sanjoinin A promote sleep in humans via activation of $GABA_A$ receptors. Rosmarinic acid, which is a component of *Perilla frutescens*, activates GABAergic and NA pathways. Yoku-kan-san, which is a Chinese and Japanese herbal medicine, has been used to manage insomnia. Geissoschizine methyl ether, which has serotonin 1A receptor agonistic activity, has also been used. Seeds of the *Zizyphus jujube* Mill *var. spinose,* which are used to make Suan Zao Ren Tang, activate serotonin 1A receptors and the GABAergic pathway. Modulation of GABAergic, adrenergic, and serotonergic pathways for orexin neurons can modulate sleep/wake homeostasis in whole animals. Herbal medicines which activate GABA, NA, and serotonin pathways can modulate the activity of orexin neurons.

Sleep disorders are common, and complementary medicine, such as herbal medicine, is widely used. On the other hand, effective drugs based on a biological understanding of sleep/wake homeostasis have been developed such as orexin receptor antagonists. The author considers that combination therapy using approved drugs and complementary medicine could be an effective treatment method. Strong efficacy via combination of different drug mechanisms is expected. Moreover, adverse effects of the drug could be reduced by reduction of the dosage in combination therapy. The

combined use of herbal medicine that suppresses the activity of orexin neurons and orexin receptor antagonists is expected to have synergistic effects because of dual inhibition upstream and downstream in the same pathway.

In this study, the author used *orexin/EGFP* mice and identified the response of orexin neurons to neurotransmitters. By database searching, herbal medicines that affect receptors expressed by orexin neurons were listed. This information can be useful for understanding current treatment methods using herbal medicine and the development of future new treatment options for insomnia.

Summary

Orexin neurons are modulated by GABAergic, serotonergic, and noradrenergic pathways. These pathways have important roles in sleep/wake homeostasis. Several herbal medicines that are used for insomnia can affect GABAergic, serotonergic, and noradrenergic signaling. An understanding of the modulation of orexin neurons can be useful to understand the mechanism of action of herbal medicineis. Moreover, the information may contribute to the establishment of new treatment options for insomnia using approved drugs and herbal medicines.

Acknowledgements

I would like to express my gratitude to Professor Hiroko Isoda for great help in writing this thesis. I am also grateful to Isoda lab members who gave helpful advice.

I also appreciate Professor Akihiro Yamanaka (Nagoya University) and Dr. Natsuko Kanda for their advice on conducting electrophysiological and pharmacological research. I appreciate Research Manager Masatoshi Hazama and Associated Director Takanori Matsuo (supervisors of the author) to approve to study in University of Tsukuba. I would like to thank Enago (www.enago.jp) for the English language review.

References

1. Tsou, M.-T., *Prevalence and risk factors for insomnia in community-dwelling elderly in northern Taiwan.* Journal of Clinical Gerontology and Geriatrics, 2013. **4**(3): p. 75-79.
2. Bannai, A., S. Ukawa, and A. Tamakoshi, *Long working hours and sleep problems among public junior high school teachers in Japan.* J Occup Health, 2015. **57**(5): p. 457-64.
3. Anothaisintawee, T., et al., *Sleep disturbances compared to traditional risk factors for diabetes development: Systematic review and meta-analysis.* Sleep Med Rev, 2015. **30**: p. 11-24.
4. de Almondes, K.M., et al., *Insomnia and risk of dementia in older adults: Systematic review and meta-analysis.* J Psychiatr Res, 2016. **77**: p. 109-15.
5. Conden, E. and A. Rosenblad, *Insomnia predicts long-term all-cause mortality after acute myocardial infarction: A prospective cohort study.* Int J Cardiol, 2016. **215**: p. 217-22.
6. Imaizumi, H., et al., *The Association between Sleep Duration and Non-Alcoholic Fatty Liver Disease among Japanese Men and Women.* Obes Facts, 2015. **8**(4): p. 234-42.
7. Banerjee, S., T.K. Ghosh, and M.K. Poddar, *Carnosine reverses the aging-induced down regulation of brain regional serotonergic system.* Mech Ageing Dev, 2015. **152**: p. 5-14.
8. Mei, Y., et al., *Aging-associated formaldehyde-induced norepinephrine deficiency contributes to age-related memory decline.* Aging Cell, 2015. **14**(4): p. 659-68.
9. Roth, T., *Insomnia: definition, prevalence, etiology, and consequences.* J Clin Sleep Med, 2007. **3**(5 Suppl): p. S7-10.

10. Grewal, R. and K. Doghramji, *Epidemiology of Insomnia.* 2010: p. 13-22.

11. Ministry of Health, L.a.W., *Patient Survey.* 2014.

12. Bannai, M. and N. Kawai, *New therapeutic strategy for amino acid medicine: glycine improves the quality of sleep.* J Pharmacol Sci, 2012. **118**(2): p. 145-8.

13. Shi, Y., et al., *Herbal Insomnia Medications that Target GABAergic Systems: A Review of the Psychopharmacological Evidence.* Curr Neuropharmacol, 2014. **12**(3): p. 289-302.

14. Butterweck, V. and M. Schmidt, *St. John's wort: role of active compounds for its mechanism of action and efficacy.* Wien Med Wochenschr, 2007. **157**(13-14): p. 356-61.

15. Aizawa, R., et al., *Effects of Yoku-kan-san-ka-chimpi-hange on the sleep of normal healthy adult subjects.* Psychiatry Clin Neurosci, 2002. **56**(3): p. 303-4.

16. Yeung, W.F., et al., *Chinese herbal medicine for insomnia: a systematic review of randomized controlled trials.* Sleep Med Rev, 2012. **16**(6): p. 497-507.

17. Roehrs, T. and T. Roth, *Insomnia pharmacotherapy.* Neurotherapeutics, 2012. **9**(4): p. 728-38.

18. Kumar, A., P. Chanana, and S. Choudhary, *Emerging role of orexin antagonists in insomnia therapeutics: An update on SORAs and DORAs.* Pharmacol Rep, 2016. **68**(2): p. 231-42.

19. Sakurai, T., et al., *Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior.* Cell, 1998. **92**(5): p. 1 page following 696.

20. Chemelli, R.M., et al., *Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation.* Cell, 1999. **98**(4): p. 437-51.

21. Hara, J., et al., *Genetic ablation of orexin neurons in mice results in narcolepsy,*

hypophagia, and obesity. Neuron, 2001. **30**(2): p. 345-54.

22. Lin, L., et al., *The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene.* Cell, 1999. **98**(3): p. 365-76.
23. Nishino, S., et al., *Hypocretin (orexin) deficiency in human narcolepsy.* Lancet, 2000. **355**(9197): p. 39-40.
24. Sasaki, K., et al., *Rosmarinus officinalis polyphenols produce anti-depressant like effect through monoaminergic and cholinergic functions modulation.* Behav Brain Res, 2013. **238**: p. 86-94.
25. Zhu, Y., et al., *Orexin-mediated feeding behavior involves both leptin-sensitive and -insensitive pathways.* Physiol Behav, 2002. **77**(2-3): p. 251-7.
26. Beuckmann, C.T., et al., *Expression of a poly-glutamine-ataxin-3 transgene in orexin neurons induces narcolepsy-cataplexy in the rat.* J Neurosci, 2004. **24**(18): p. 4469-77.
27. Tsunematsu, T., et al., *Ectopic expression of melanopsin in orexin/hypocretin neurons enables control of wakefulness of mice in vivo by blue light.* Neurosci Res, 2013. **75**(1): p. 23-8.
28. Sasaki, K., et al., *Pharmacogenetic modulation of orexin neurons alters sleep/wakefulness states in mice.* PLoS One, 2011. **6**(5): p. e20360.
29. Sakurai, T., et al., *Structure and function of human prepro-orexin gene.* J Biol Chem, 1999. **274**(25): p. 17771-6.
30. Yamanaka, A., et al., *Hypothalamic orexin neurons regulate arousal according to energy balance in mice.* Neuron, 2003. **38**(5): p. 701-13.
31. Muraki, Y., et al., *Serotonergic regulation of the orexin/hypocretin neurons through the 5-HT1A receptor.* J Neurosci, 2004. **24**(32): p. 7159-66.
32. Hedlund, P.B., et al., *8-OH-DPAT acts on both 5-HT1A and 5-HT7 receptors to*

induce hypothermia in rodents. Eur J Pharmacol, 2004. **487**(1-3): p. 125-32.

33. Renner, U., et al., *Heterodimerization of serotonin receptors 5-HT1A and 5-HT7 differentially regulates receptor signalling and trafficking.* J Cell Sci, 2012. **125**(Pt 10): p. 2486-99.

34. Cornil, C.A. and G.F. Ball, *Interplay among catecholamine systems: dopamine binds to alpha2-adrenergic receptors in birds and mammals.* J Comp Neurol, 2008. **511**(5): p. 610-27.

35. Dundar, Y., et al., *Newer hypnotic drugs for the short-term management of insomnia: a systematic review and economic evaluation.* Health Technol Assess, 2004. **8**(24): p. iii-x, 1-125.

36. Tsutsui, R., et al., *Effects of the 5-HT(1A) Receptor Agonist Tandospirone on ACTH-Induced Sleep Disturbance in Rats.* Biol Pharm Bull, 2015. **38**(6): p. 884-8.

37. Cruickshank, M., et al., *Alpha-2 agonists for sedation of mechanically ventilated adults in intensive care units: a systematic review.* Health Technol Assess, 2016. **20**(25): p. v-xx, 1-117.

38. Khachatryan, D., et al., *Prazosin for treating sleep disturbances in adults with posttraumatic stress disorder: a systematic review and meta-analysis of randomized controlled trials.* Gen Hosp Psychiatry, 2016. **39**: p. 46-52.

39. Sorooshyari, S., R. Huerta, and L. de Lecea, *A Framework for Quantitative Modeling of Neural Circuits Involved in Sleep-to-Wake Transition.* Front Neurol, 2015. **6**: p. 32.

40. Mieda, M., N. Tsujino, and T. Sakurai, *Differential roles of orexin receptors in the regulation of sleep/wakefulness.* Front Endocrinol (Lausanne), 2013. **4**: p. 57.

41. Liu, R.J., A.N. van den Pol, and G.K. Aghajanian, *Hypocretins (orexins) regulate*

serotonin neurons in the dorsal raphe nucleus by excitatory direct and inhibitory indirect actions. J Neurosci, 2002. **22**(21): p. 9453-64.

42. Kim, J., et al., *Electrophysiological effects of orexins/hypocretins on pedunculopontine tegmental neurons in rats: an in vitro study.* Peptides, 2009. **30**(2): p. 191-209.

43. Yamanaka, A., et al., *Orexins activate histaminergic neurons via the orexin 2 receptor.* Biochem Biophys Res Commun, 2002. **290**(4): p. 1237-45.

44. Nambu, T., et al., *Distribution of orexin neurons in the adult rat brain.* Brain Res, 1999. **827**(1-2): p. 243-60.

45. Sakurai, T., et al., *Input of orexin/hypocretin neurons revealed by a genetically encoded tracer in mice.* Neuron, 2005. **46**(2): p. 297-308.

46. Tabuchi, S., et al., *Influence of inhibitory serotonergic inputs to orexin/hypocretin neurons on the diurnal rhythm of sleep and wakefulness.* Sleep, 2013. **36**(9): p. 1391-404.

47. Luger, D., et al., *Identification of the putative binding pocket of valerenic acid on GABAA receptors using docking studies and site-directed mutagenesis.* Br J Pharmacol, 2015. **172**(22): p. 5403-13.

48. Chua, H.C., et al., *Kavain, the Major Constituent of the Anxiolytic Kava Extract, Potentiates GABAA Receptors: Functional Characteristics and Molecular Mechanism.* PLoS One, 2016. **11**(6): p. e0157700.

49. Nishi, A., et al., *Geissoschizine methyl ether, an alkaloid in Uncaria hook, is a potent serotonin (1)A receptor agonist and candidate for amelioration of aggressiveness and sociality by yokukansan.* Neuroscience, 2012. **207**: p. 124-36.

50. Han, H., et al., *Anxiolytic-like effects of sanjoinine A isolated from Zizyphi Spinosi Semen: possible involvement of GABAergic transmission.* Pharmacol Biochem

Behav, 2009. **92**(2): p. 206-13.

51. Kwon, Y.O., J.T. Hong, and K.W. Oh, *Rosmarinic Acid Potentiates Pentobarbital-Induced Sleep Behaviors and Non-Rapid Eye Movement (NREM) Sleep through the Activation of GABAA-ergic Systems.* Biomol Ther (Seoul), 2016.

52. Jacobs, B.P., et al., *An internet-based randomized, placebo-controlled trial of kava and valerian for anxiety and insomnia.* Medicine (Baltimore), 2005. **84**(4): p. 197-207.

53. Chan, Y.Y., et al., *Clinical Efficacy of Traditional Chinese Medicine, Suan Zao Ren Tang, for Sleep Disturbance during Methadone Maintenance: A Randomized, Double-Blind, Placebo-Controlled Trial.* Evid Based Complement Alternat Med, 2015. **2015**: p. 710895.

54. Solhi, H., et al., *Beneficial Effects of Rosmarinus Officinalis for Treatment of Opium Withdrawal Syndrome during Addiction Treatment Programs: A Clinical Trial.* Addict Health, 2013. **5**(3-4): p. 90-4.

55. Tokunaga, S., et al., *Effect of valerian extract preparation (BIM) on the sleep-wake cycle in rats.* Biol Pharm Bull, 2007. **30**(2): p. 363-6.

56. Shinomiya, K., et al., *Effects of kava-kava extract on the sleep-wake cycle in sleep-disturbed rats.* Psychopharmacology (Berl), 2005. **180**(3): p. 564-9.

57. Ogawa, Y., et al., *The role of the seven crude drug components in the sleep-promoting effect of Yokukansan.* J Ethnopharmacol, 2016. **177**: p. 19-27.

58. Ma, Y., et al., *Sanjoinine A isolated from Zizyphi Spinosi Semen augments pentobarbital-induced sleeping behaviors through the modification of GABA-ergic systems.* Biol Pharm Bull, 2007. **30**(9): p. 1748-53.

59. Wu, X.H., et al., *Low-dose Dexmedetomidine Improves Sleep Quality Pattern in*

Elderly Patients after Noncardiac Surgery in the Intensive Care Unit: A Pilot Randomized Controlled Trial. Anesthesiology, 2016. **125**(5): p. 979-991.