Reverse translational research to investigate the mechanisms underlying insomnia with depression and to develop an algorithm for selecting hypnotics

２０２１

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Preface

The World Health Organization estimates that ~350 million individuals worldwide suffer from major depressive disorder (MDD) (1). Among them, 70-80% of the MDD patients complain of sleep disturbances such as sleep-onset insomnia and nocturnal awaking (2-4).

Although, benzodiazepines are widely prescribed hypnotics used for treating insomnia in MDD patients (5-7), some MDD patients can experience insomnia resistant to benzodiazepine treatment (8). In fact, in a previous retrospective study on patients with psychiatric disorders at Kyoto University Hospital, it was found that "nocturnal awaking", which is one of the endpoints of the Hamilton depression rating scale (HAMD), was not improved in many patients despite receiving treatment with benzodiazepines. These findings indicate the important need for a drug that can be used as an alternative to benzodiazepines for treating insomnia that is highly comorbid with depression. However, there is no convincing evidence demonstrating the mechanisms underlying insomnia and decreased efficacy of benzodiazepines under depression. Furthermore, treatment guidelines do not state a defined strategy to treat with alternatives to benzodiazepines in MDD patients with insomnia (5). To address these issues, I conducted a neuropharmacological study to explore the brain mechanisms of insomnia in a mouse model with chronic social defeat stress (CSDS), which is a well-studied model developing a wide range of depressive-like behaviors, and a prospective study on the efficacy of alternative treatments to benzodiazepines in MDD patients.

In Chapter 1, I showed orexinergic neurons are hyperactivated in the lateral hypothalamus (LH) of CSDS mice, but also show that these neurons were less responsive to a benzodiazepine, resulting in a reduced hypnotic effect that was likely caused by a decrease in γ-aminobutyric acid (GABA) levels in the ventrolateral preoptic nucleus (VLPO). By contrast, the hypnotic effects of an orexin receptor antagonist suvorexant was not altered in these mice.

In Chapter 2, I found that suvorexant or eszopiclone (non-benzodiazepine GABA receptor agonists) are beneficial alternatives to benzodiazepines for treating residual insomnia in MDD patients despite receiving more than 2 weeks of benzodiazepine treatment. In addition, switching to both drugs is unlikely to increase the risk of adverse events or worsen rebound insomnia.

These results are described in detail below.

Abbreviations

BDI-II	beck depression inventory-II
CSDS	chronic social defeat stress
DSM-5	the diagnostic and statistical manual of mental disorder, fifth edition
DST	digit span test
DSST	digit symbol substitution test
EEG	electroencephalogram
EMG	electromyogram
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GAD-7	generalized anxiety disorder-7
GAT	GABA transporter
ISI-J	insomnia severity index Japanese version
LH	lateral hypothalamus
LOCF	last observation carried forward
MCH	melanin-concentrating hormone
MDD	major depressive disorder
OX_1R	orexin receptor 1
OX_2R	orexin receptor 2
PPO	prepro-orexin
PSQI-J	Pittsburgh sleep quality index Japanese version
REM	rapid eye movement
SSRI	serotonin reuptake inhibitor
TMN	tuberomammillary nucleus
VLPO	ventrolateral preoptic nucleus

Chapter 1: Decreased inhibitory effect of brotizolam on hyperactivated orexinergic neurons in the lateral hypothalamus is responsible for its reduced hypnotic potency in a mouse chronic social defeat stress model of depression

INTRODUCTION

To date, many research on patients with depression and mouse model with CSDS, which is a well-studied model developing a wide range of depressive-like behaviors, have been performed to understand changes in the sleep pattern in depressive state. Electroencephalogram (EEG) and electromyogram (EMG) studies revealed that patients with MDD exhibit decreased slow-wave sleep (regarded as deep sleep), increased total rapid eye movement (REM) sleep (referred to as light sleep) time and diminished REM sleep latency (9,10). Similar arousal and sleep disturbances, such as elevated high-frequency EEG activity during non-REM sleep, have been observed in mice subjected to CSDS (11). However, the mechanisms underlying insomnia accompanied by depression are not fully understood.

An excitatory neuropeptide orexin mediates arousal, appetite and cognition (12,13). It is generally accepted that the transition between arousal and sleep is modulated by mutual inhibition of sleep- and wake-inducing neurotransmitters: the flip-flop switch model (14). During sleep, firing of GABA-ergic inhibitory neurons projected from the VLPO increases, and consequently inhibits the downstream arousal-promoting orexinergic neurons in the LH (15,16). By contrast, upon arousal, orexinergic neurons projected from the LH activate histaminergic neurons in the tuberomammillary nucleus (TMN), serotonergic neurons in the dorsal raphe nucleus and noradrenergic neurons in the locus coeruleus (17).

Benzodiazepines are one of the most widely prescribed therapeutic agents for the treatment

of insomnia as well as anxiety disorders or catatonia in patients with depression (5-7). These drugs increase the GABA-induced influx of Cl^- ions via $GABA_A$ receptors to strengthen the inhibitory postsynaptic potential in the central nervous system. However, a growing body of evidence suggests that GABA neurotransmission is attenuated in several brain regions (e.g., the hippocampus, occipital cortex, and anterior cingulate cortex) in patients with MDD (18-20). These findings raise the possibility that GABAergic neurons projected from the VLPO to the LH are functionally disturbed under the depressive state, contributing to the insomnia in these patients. If so, benzodiazepines would have less potent hypnotic efficacy in patients with MDD, as their pharmacological action requires sufficient amounts of GABA (21). However, at present, there is no reliable and undisputed evidence to support this hypothesis.

In this Chapter, the study was conducted to investigate the impact of CSDS on spontaneous firing activity of orexinergic neurons in the LH and GABA level in the VLPO in the CSDS mouse model of depression with sleep disturbance. In addition, the effects of hypnotic drugs, including a barbiturate (pentobarbital), a benzodiazepine analog (brotizolam), a selective dual orexin receptor antagonist (suvorexant), and an antiepileptic drug (valproate), were assessed.

EXPERIMENTAL PROCEDURES

Animals

All animal care and experimental procedures were in complied with the ethical guidelines of the Kyoto University Animal Research Committee (permission numbers: Med Kyo 16527, 17104 and 18104). Male C57BL6/J mice (7 weeks old; 20–25 g) and male ICR mice (50–70 g) were purchased from Nihon SLC (Shizuoka, Japan). They were maintained on a 12-h light/dark cycle (lights on at 8:00 a.m.) with water and food available *ad libitum* and kept at a constant temperature

of 24 ± 1°C.

Drugs

Pentobarbital sodium, bovine serum albumin, dimethyl sulfoxide (DMSO) and methanol were purchased from Nacalai Tesque (Kyoto, Japan). Pentobarbital sodium was dissolved in a solution consisting of 49.5% distilled water, 40% propylene glycol and 10.5% ethanol, and diluted with saline (5 mg/mL). Brotizolam and sodium valproate were purchased from Wako Pure Chemical (Osaka, Japan) and Sigma-Aldrich (St. Louis, MO, USA), respectively, and dissolved in saline. Suvorexant was obtained from Toronto Research Chemicals (North York, ON, Canada), dissolved in 10% DMSO, and diluted in saline. 3-Amino-1-propanesulfonic acid was obtained from Kanto Chemical Co. Inc. (Tokyo, Japan). Other chemicals were from Wako Pure Chemical.

CSDS and social interaction and (SI) test

The CSDS paradigm was used as described previously with minor modifications (22-24). Briefly, C57BL6/J mice were first housed individually for 7 days and then exposed to an aggressive ICR mouse for 5 min of social defeat stress each day for 10 consecutive days.

The SI test was performed within 1 week from the final defeat session to select susceptible mice. For this, the individually housed mice were first acclimated to testing rooms with a dim red light for at least 30 min. Then, each mouse was allowed 150 s to explore the open field test chamber (50 × 50 × 50 cm) containing an empty wire mesh cage (10 × 6.5 cm) at one end. Then, an unfamiliar ICR mouse was placed in the wire mesh cage (to prevent physical interaction with the defeated mouse), and mouse behaviors were video monitored for 150 s. The trajectory of mouse ambulation was determined and recorded by ANY-maze (Stoelting Co., Wood Dale, IL, USA). The time that the defeated mice spent in the interaction zone (the area [10 × 24 cm] surrounding the wire mesh cage) and avoidance zones (corners of the chamber [9 × 9 cm]) was

measured in the presence and absence of the target (ICR) mouse. The ratio of time spent during the observation period (150 s) in the interaction zone in the presence of the target mouse to that in the absence of the target mouse was defined as the SI ratio, an index for the level of social avoidance. Mice with a SI ratio of <1.0 were considered CSDS susceptible and selected for further experiments within 2 weeks after the SI test.

Elevated plus maze test

The elevated plus maze consists of two opposing open arms (30 × 5 cm) and two opposing enclosed arms (30 × 5 cm) with 15 cm-high transparent walls connected by a central platform (5 × 5 cm; illumination level, 100 lux). The arms and central square are made of wood and elevated 42 cm above the floor. To minimize the likelihood of animals falling from the apparatus, 3 mm-high plastic ledges were provided for the open arms. Each mouse was placed on the central platform, facing one of the closed arms. Mouse behavior was recorded during a 15 min test period. The numbers of entries into and time spent in the open arms were recorded and analyzed with video tracking software (ANY-maze; Stoelting Co., Wood Dale, IL, USA).

24 h sleep-wake behavior test

Six small cages (15 × 21 × 21 cm) were positioned 77.5 cm below an infrared video camera (JN-2305C; Shenzhen JIN Technology Co., Shenzhen, Guangdong, China) secured to a horizontal pole (100 cm) supported by two vertical stands. The light condition was controlled by two light bulbs fixed at either end of the pole. Black paper was inserted between cages to prevent visual interactions between mice. Mice were placed in the cages with food and HydroGel (ClearH_2O, Westbrook, ME, USA) under a 12 h light/dark cycle (lights on at 8:00 a.m.). After 1 day of acclimation, their locomotor activity was recorded for 24 h. To shorten the time of analysis, 24 h

video data were converted at 8 × speed into 3 h video data by Windows Movie Maker (Microsoft, Redmond, WA, USA) and analyzed by ANY-maze (version 5.1; Stoelting Co.). On the basis of a previous report (25), immobility-defined sleep was measured as a period of extended immobility (>40 s) during which 95% or more of the area of the animal is stationary. Then, the total time of immobility-defined sleep, latency (time [min] from the beginning of the sleeping period to the first appearance of immobility-defined sleep), and sleep bout duration in each 30 min period were analyzed. The sleep bout duration was calculated as the total duration of immobility-defined sleep in 30 min segments divided by the number of immobility-defined sleep episodes in 30 min segments.

Pentobarbital-induced sleeping test

The hypnotic assessment method is based on the prolongation of sleep induced by pentobarbital, which is a widely used method to analyze the potency of hypnotic drugs, as previously described with slight modifications (26-28). The hypnotic effect of brotizolam, valproate, or suvorexant was evaluated based on the degree of potentiation of pentobarbital-induced sleeping. Brotizolam (0.03, 0.1, 0.17, 0.3, and 1.0 mg/kg), valproate (30, 100, 150, and 200 mg/kg), suvorexant (10, 13.3, 17.8, 23.7, and 30 mg/kg), or vehicle (saline for brotizolam and valproate and 10% DMSO diluted in saline for suvorexant) was injected intraperitoneally (i.p.) 30 min before the i.p. injection of pentobarbital (40 mg/kg). The onset time of sleep was defined by the time the righting reflex was lost after the pentobarbital injection. The duration of sleep was defined as the time from being placed in a supine position until the righting reflex was regained. The ED_{50} was calculated based on the dose-response line for the potentiating effect of pentobarbital-induced sleeping according to the method of Litchfield and Wilcoxon (29). As previously reported (30), the effective sleep rate (%) was calculated as the number of mice whose

sleep duration after pentobarbital injection was extended >2-fold that of control/total number of mice × 100.

Stereotaxic surgery and microinjection into the LH

Under pentobarbital anesthesia (50 mg/kg, i.p.), each mouse was placed in a stereotaxic apparatus (Narishige, Tokyo, Japan) with bregma and lambda landmarks in a horizontal plane. The mice were implanted with two guide cannulas (Eicom Co., Kyoto, Japan) in the left and right LH (AP, -1.5 mm; ML, ±2.2 mm; DV, 5.0 mm; 10°), according to a stereotaxic atlas of the mouse brain (31). After surgery, the animals were returned to cages and housed individually. Two to four days later, brotizolam (0.3 μg/μL/side) was injected into the left and right LH for 3 min (flow speed, 1 μL/3 min) with a syringe pump (model 11 Plus; Harvard Apparatus, Holliston, MA, USA). Thirty minutes later, the mice were injected i.p. with pentobarbital (40 mg/kg body weight) and subjected to the pentobarbital-induced sleeping test. After the experiment, 0.01% Evans blue in phosphate-buffered saline (PBS) was microinjected into the LH to validate the injection technique. Then, I collected the brain from the individual mice, and confirmed the success by checking diffuse of Evans blue around injection site in the LH. Only data from mice with accurately inserted probes were used for subsequent statistical analysis.

Electrophysiological recordings

Electrophysiological recordings were performed as previously described (32). After 10 days of CSDS, susceptible mice were deeply anesthetized with isoflurane and decapitated. The brains were rapidly collected in ice-cold cutting solution (120 mM *N*-methyl-D-glucamine-Cl, 2.5 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 0.5 mM $CaCl_2$, 7 mM $MgCl_2$, 15 mM D-glucose, and 1.3 mM ascorbic acid, pH 7.2). Coronal slices containing the hypothalamus (200 μm thick) were

prepared with a vibratome (VT1000S; Leica, Wetzlar, Germany). Slices were recovered in oxygenated artificial CSF (ACSF; 124 mM NaCl, 3 mM KCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 2.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 10 mM D-glucose, pH 7.3) at 32°C for at least 1 h before recording. Individual slices were transferred to a recording chamber with continuous perfusion of oxygenated ACSF with continuous perfusion of oxygenated ACSF at a flow rate of 1–2 mL/min. ACSF were warmed to keep the recording chamber at 27 ± 1˚C.

Whole-cell current clamp recordings were performed with an EPC9 amplifier (HEKA, Pfalz, Germany), and the data were recorded using Patchmaster software (HEKA). Individual neurons were visualized with a microscope equipped with a 40× water-immersion objective lens (Carl Zeiss, Jena, Germany) and a charge-coupled device camera. The resistance of the electrodes was 3–7 MΩ when filled with the internal solution (140 mM K-gluconate, 5 mM KCl, 10 mM HEPES, 2 mM Na-ATP, 2 mM $MgCl_2$, and 0.2 mM EGTA, pH 7.3 adjusted with KOH). Spontaneous firing was examined with the current held at 0 pA. Orexinergic neurons were identified by hyperpolarization-induced voltage sag elicited by negative voltage steps (-200 and -150 pA), spontaneous firing, and uniphasic pronounced afterhyperpolarization (AHP) as previously reported (33).

To compare the spontaneous activity between different neurons, spontaneous firing activity was recorded for 30 s after stabilization. To examine the effects of brotizolam, the average firing rate in the first 30 s (without brotizolam) was considered the basal firing rate. After drug perfusion for 150 s, firing activity was recorded for 30 s and compared with basal activity.

Single-cell RT-PCR

Single-cell RT-PCR was performed as previously described (32). After whole-cell recording, the contents of the cell were aspirated into the recording pipette and harvested in a sampling tube.

The collected samples were reverse-transcribed using a ReverTra Ace RT kit (TOYOBO, Tokyo, Japan) and amplified with Blend Taq (TOYOBO, Tokyo, Japan). The oligonucleotide primers were designed for targeting *prepro-orexin* (sense: 5'-CTG CGG GTA TCC TGA CTC TG-3', antisense: 5'-TGG TTA CCG TTG GCC TGA AG-3'), *pro-melanin-concentrating hormone* (*Pmch*, sense: 5'-GCA CTC TTG TTT GGC TTT ATG C-3', antisense: 5'-AGC CAG CAT TAA CAT GTA GGA-3'), *glutamic acid decarboxylase 1* (*Gad1*, sense: 5'-ATA CAA CCT TTG GCT GCA TGT-3', antisense: 5'-TTC CGG GAC ATG AGC AGT-3'), *glutamic acid decarboxylase 2* (*Gad2*, sense: 5'-ACC GTG TAT GGG GCT TTT GA-3', antisense: 5'-ATC AGT AAC CCT CCA CCC CA-3') and *enolase 2* (*Eno2*, sense: 5'-CCG CTG ATC CTT CCC GAT AC-3', antisense: 5'-CGA CGT TGG CTG TGA ACT TG-3') as a neuronal marker. PCR products were analyzed by agarose gel electrophoresis.

qRT-PCR

Mice were sacrificed by cervical dislocation. The brains were quickly removed and sliced into 1 mm-thick coronal sections using a Brain Blocker (Muromachi Kikai Co., Ltd., Tokyo, Japan) on ice. Then, brain tissue punches (2–3 mm diameter) containing the LH, VLPO, or TMN were harvested with a microscalpel according to the atlas of the mouse brain (31); the punches of the VLPO and TMN may have contained other preoptic nuclei and posterior hypothalamic areas, respectively. Then, total RNA was extracted from the brain tissues using the SV Total RNA Isolation system (Promega, Madison, WI, USA), according to the manufacturer's instructions. Purified total RNA was quantified in a spectrophotometer at 260 nm (NanoDrop 2000; Thermo Fisher Scientific, Waltham, MA, USA). To prepare first-strand cDNA, 0.5 μg of RNA was incubated in 40 μL of buffer containing a dNTP mixture, a reverse transcription random primer, and reverse transcriptase (High Capacity cDNA reverse transcription kit; Thermo Fisher

Scientific), according to the manufacturer's instructions. cDNA was amplified in 20 μL of a PCR solution containing 10 μL of Power SYBR green PCR Master Mix (Thermo Fisher Scientific) and primers targeting *glutamic acid decarboxylase 1* (*GAD1*, sense: 5′-CCG TGT ATG GGG CTT TTG AT-3′, antisense: 5′-GGG GAC ACC CAT CAT CTT GT -3′), *glutamic acid decarboxylase 2* (*GAD2*, sense: 5′-CCA GAA AAC TGG GCC TGA AG-3′, antisense: 5′-TTT GCT CCT CCC CGT TCT TA-3′), GABA transporter 1 (*GAT1*, sense: 5′-TTT GCG GGT GTT CCT CTC TT-3′, antisense: 5′-TCA GTG TTC CAC GGG TTG TC-3′), *prepro-orexin* (sense: 5′-AAG ACG TGT TCC TGC CGT CT-3′, antisense: 5′-GGG TGC TAA AGC GGT GGT AG-3′), *orexin* receptor 1 (*OXR1*, sense: 5′-AGT GGG GAA CCC TTC CAT CT-3′, antisense: 5′-ACA TCT GCC AGG GAC AGG TT-3′), *orexin* receptor 2 (*OXR2*, sense: 5′-CCT CAA GCC ATT GTC ATG GA-3′, antisense: 5′-GAA GTT CCG GGA ATC TGT CG-3′), and *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*, sense: 5′-GTT ACC AGG GCT GCC TTC TC-3′, antisense: 5′-TGA TGA CCA GCT TCC CAT TC-3′). PCR was performed using the StepOnePlus system (Thermo Fisher Scientific) under the following cycling conditions: 95°C for 10 min and 60°C for 1 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Fluorescence was detected after each extension step. GAPDH was used as a normalization control, and relative mRNA levels were calculated using a comparative C_T method and StepOnePlus software (Thermo Fisher Scientific).

HPLC

Brain tissue punches containing the VLPO or amygdala were dissected as described above and placed in a 10-fold volume of 100% methanol (Nacalai Tesque) containing 10 μL of 3-amino-1-propanesulfonic acid (0.1 M; Kanto Chemical Co. Inc., Tokyo, Japan) as an internal standard. These samples were homogenized with a Polytron homogenizer (PT 1300 D; Kinematica AG, Luzern, Switzerland), put on ice for >15 min, and then centrifuged at 13,000 × *g* for 15 min. The

supernatants were filtered through a Falcon 40 µm cell strainer (Corning Inc., NY, USA). GABA concentrations were measured using an electrochemical detection system (ECD-300; Eicom Co.) with an FA-3ODS column (Eicom Co.). The standard solution contained 10 µL of 3-amino-1-propanesulfonic acid, 10 µL of GABA (0.1 M, Wako Pure Chemical), and 980 µL of 100% methanol. After derivatization with 4 mM *ortho*-phthalic aldehyde (Wako Pure Chemical Industries), the samples (diluted 500 times with 10% methanol) were applied to HPLC. The mobile phase containing $NaH_2PO_4 \cdot 2H_2O$ (10.76 g/L; Wako Pure Chemical), $Na_2HPO_4 \cdot 12H_2O$ (3.95 g/L, Wako Pure Chemical), 13% acetonitrile (Wako Pure Chemical), and 7% methanol (Nacalai Tesque) was delivered at a flow rate of 500 µL/min. GABA was identified according to the retention time of the GABA standard, and amounts of GABA were quantified based on calculations using peak areas. GABA content was expressed as micromoles GABA per milligram wet tissue weight.

Immunohistochemical analysis

Mice were deeply anesthetized with pentobarbital (50 mg/kg, i.p.) and perfused through the ascending aorta with 0.1 M PBS, followed by 4% paraformaldehyde in 0.1 M PBS. The brains were removed quickly after perfusion, and 2 mm-thick coronal sections containing the preoptic area were initially dissected using a Brain Blocker. The coronal sections were postfixed in paraformaldehyde for 4 h at 4°C, transferred to 15% sucrose overnight, and then transferred to 30% sucrose overnight. The sections were frozen in an embedding compound (Sakura Finetek USA, Torrance, CA, USA) and stored at -80°C. The frozen coronal sections (40 µm thick) containing the preoptic area were cut with a freezing cryostat (Leica CM 1850; Leica) and thaw-mounted on MAS-coated glass slides (Matsunami Glass Ind, Osaka, Japan). The sections were washed with PBS three times, blocked in blocking buffer (PBS containing 0.1% Tween 20 and

5% BSA [Nacalai Tesque]) for 1 h at room temperature, and then incubated for 24 h with following primary antibodies: guinea pig anti-GABA antibody (1:100, Abcam, Cambridge, UK) and rabbit anti-microtubule associated protein 2 (MAP2) antibody (1:250, Merck Millipore Co., Darmstadt, Germany) overnight at 4˚C. After washing twice with PBS, the sections were incubated with the secondary antibodies, goat anti-guinea pig IgG labeled with Alexa 488 and goat anti-rabbit IgG labeled with Alexa 594 (1:500, Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at room temperature. After washing, the sections were mounted using Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA). The position of the VLPO was determined with reference to the mouse brain atlas (31), and images were acquired under a laser scanning confocal microscope (Fluoview FV10i Confocal Microscope, Olympus Corp., Tokyo, Japan).

Data analysis and statistics

Data were analyzed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) and expressed as the means ± SEMs. Differences between two groups were compared using Student's *t* tests. Data from more than two groups were compared using one-way or two-way ANOVAs, followed by Bonferroni's multiple comparison tests. In all cases, differences with a p value of <0.05 were considered statistically significant. The numbers (n) of mice or cells used in each experiment are given in the figure legends.

RESULTS

Sleep disturbance in CSDS mice

The ambulation trajectories of mice susceptible to 10 days of CSDS were largely concentrated

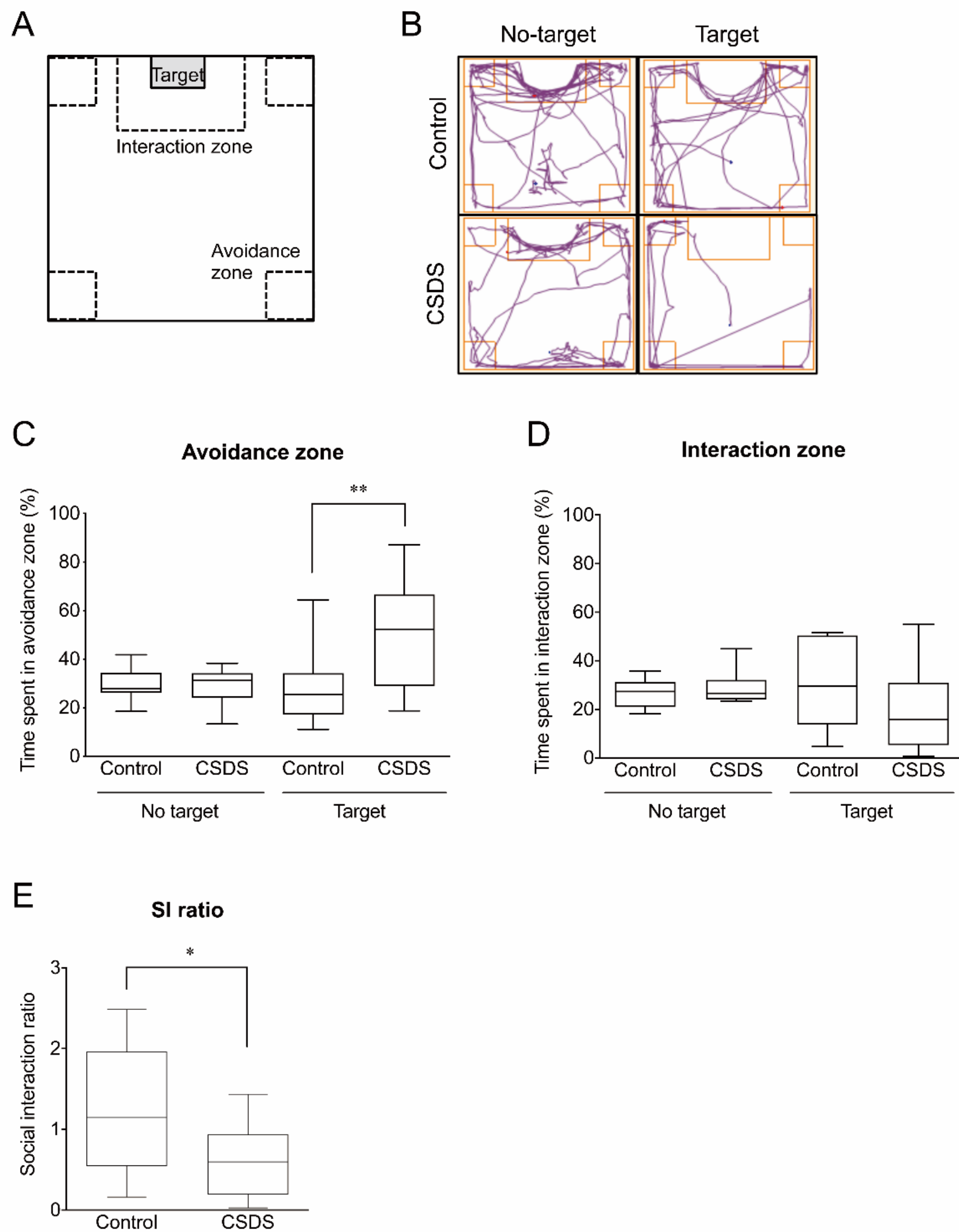

Fig. 1-1. Depression-like social avoidance behavior induced by CSDS. (A) Schematic illustration of social interaction (SI) test. (B) Representative movement traces of unstressed (control) and CSDS mice in the absence (no target) and presence (target) of an unfamiliar ICR mouse in the SI test. (C and D) The ratio of time spent in the avoidance (C) and interaction (D) zones by control (n = 10) and CSDS (n = 14) mice during the observation period (150 s) in the presence and absence of a target mouse. $^{**}p < 0.01$ (Bonferroni's *post hoc* test). (E) SI ratios in control (n = 10) and CSDS (n = 14) mice. SI ratios (time spent in the interaction zone in the presence of an unfamiliar ICR mouse/time spent in the interaction zone in the absence of an ICR mouse). $^{*}p < 0.05$ (unpaired t test). The lower and upper edges of each box show the 25th–75th percentiles, respectively, whereas the horizontal line within the box shows the median. The whiskers span all data points included within each point.

around the avoidance zones in the presence of a target mouse, with minimal activity around the interaction zone (Fig. 1-1A–B). CSDS mice spent significantly more time in avoidance zones (Fig. 1-1C; $F_{1,44} = 6.487$, $p = 0.014$, *post hoc* $p < 0.01$ vs. control in the presence of a target mouse) and tended to spend less time in the interaction zone (Fig. 1-1D; $F_{1,44} = 1.519$, $p = 0.224$, $p = 0.014$, *post hoc* $p < 0.01$ vs. control in the presence of a target mouse) than unstressed control mice. The SI ratios of CSDS mice were significantly lower than those of control mice (Fig. 1-1E; $t_{22} = 2.341$, $p = 0.029$). The times spent in the interaction and avoidance zones in control mice did not differ when a social target was present or absent. In the elevated plus maze test to assess anxiety-like behavior, CSDS mice entered the open arms fewer times than control mice (Fig. 1-2A) and spent significantly less time in the open arms (Fig. 1-2B; $t_{18} = 2.672$, $p = 0.016$).

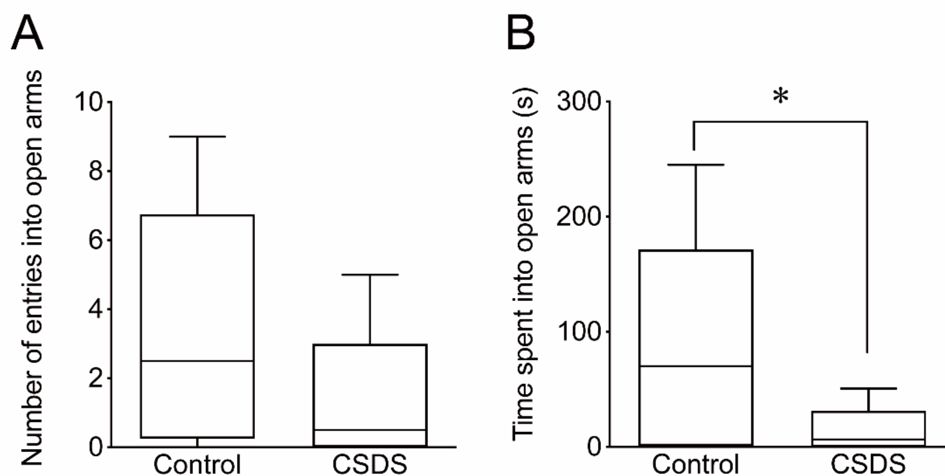

Fig. 1-2. Elevated plus maze test to assess anxiety-like behavior induced by CSDS. (A) The number of entries into the open arms and (B) the time spent in open arms during a 15 min test period in control ($n = 8$) and CSDS ($n = 12$) mice. The lower and upper edges of each box show the 25th–75th percentiles, respectively, whereas the horizontal line within the box shows the median. The whiskers span all data points included within each point. *$p < 0.05$ (unpaired t test).

To investigate the influence of CSDS on the circadian sleep-wake cycles in mice, I assessed immobility behaviors of mouse across 24 h period. Whereas control mice exhibited typical

circadian *patterns of* immobility-defined sleep (i.e., nocturnal activity and diurnal immobility), CSDS mice had significantly shorter times of immobility-defined sleep in a 24 h period (Fig. 1-3A; $F_{1,22} = 4.737$, $p = 0.041$, repeated two-way ANOVA). In regard to immobility-defined sleep onset after the beginning of sleeping period, the latency tended to be longer in CSDS mice than in control mice (Fig. 1-3B), although the difference was not significant. Further analyses revealed that the average sleep bout duration was significantly longer in CSDS mice than in control mice (Fig. 1-3C; $F_{1,22} = 6.928$, $p = 0.015$, repeated two-way ANOVA).

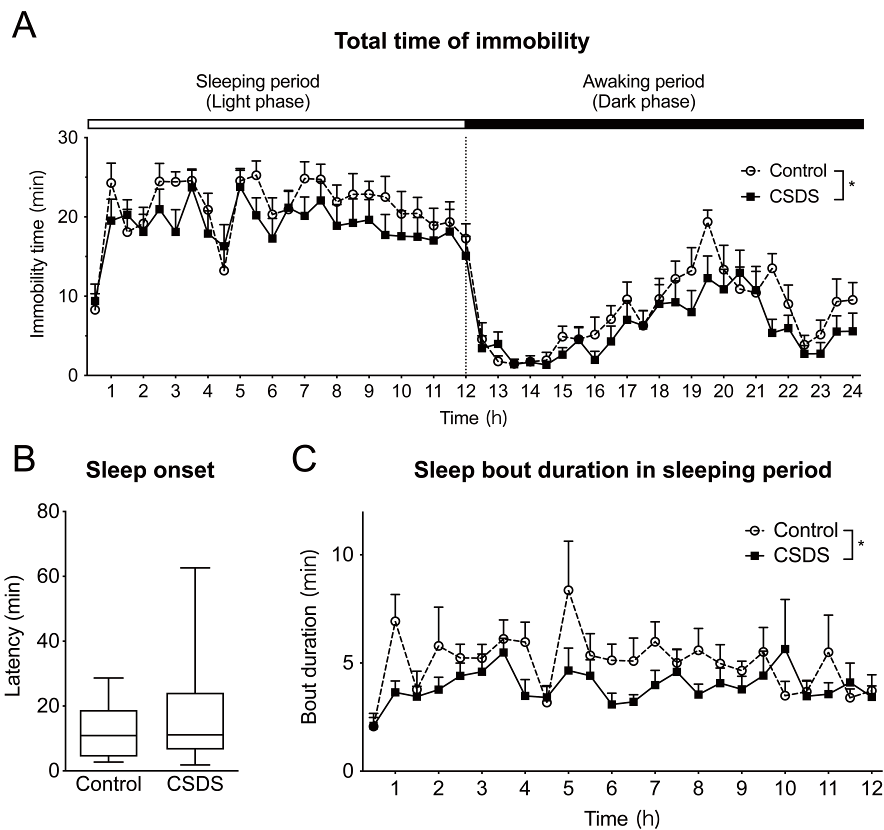

Fig. 1-3. CSDS induces sleep disturbance. (A) Average immobility-defined sleep every 30 min in control and CSDS mice determined by 24 h video monitoring of sleep-wake behavior under a 12 h light/dark cycle ($n = 12$). *$p < 0.05$ (repeated two-way ANOVA). Data are expressed as means ± SEMs. (B) Latency from the beginning of the sleeping period to the first appearance of immobility-defined sleep ($n = 12$) The lower and upper edges of each box show the 25th–75th percentiles, respectively, whereas the horizontal line within the box shows the median. The whiskers span all data points included within each point. (C) Sleep bout durations (total duration of immobility-defined sleep in 30 min segments/the number of immobility-defined sleep episodes in 30 min segments) ($n = 12$). *$p < 0.05$ (repeated two-way ANOVA). Data are expressed as means ± SEMs.

Increased spontaneous firing activity of orexinergic neurons in the LH in CSDS mice

To determine whether the activity of orexinergic neurons in the LH was altered in CSDS mice, I performed whole-cell patch-clamp recordings of spontaneous firing in acute LH slices. Although the LH also contains melanin-concentrating hormone (MCH) neurons and GABA interneurons (33), orexinergic neurons can be distinguished by hyperpolarization-induced voltage sag, spontaneous firing, and uniphasic pronounced AHP (33) (Fig. 1-4A top). The identity of the orexinergic neurons was confirmed by their expression of prepro-orexin (PPO) by single-cell RT-PCR, whereas cells that did not exhibit hyperpolarization-induced voltage sag expressed *Pmch* or GAD1/GAD2 mRNA (encoding glutamic acid decarboxylase 1 and 2, respectively) and thus were considered MCH neurons or GABA interneurons, respectively (Fig. 1-4A bottom). The LH orexinergic neurons showed spontaneous firing even in the control slices. The spontaneous firing of orexinergic neurons in LH slices from CSDS mice was significantly increased compared with that in LH slices from control mice (Fig. 1-4B–C; $t_{15} = 2.185$, $p = 0.045$). However, expression levels of mRNA for PPO in the LH and for orexin receptor 1 (OX_1R) and orexin receptor 2 (OX_2R) in the TMN did not differ between control and CSDS mice (Fig. 1-4D).

Decreased GABA content in the VLPO in CSDS mice

As orexinergic neurons in the LH are regulated by GABAergic inhibition from the VLPO (15,16), I hypothesized that the hyperactivity of these neurons in CSDS mice was a result of decreased GABA transmission within the LH. To test this, I performed HPLC to measure GABA amounts in the LH tissue samples. The GABA content in CSDS mice was significantly lower than in controls (Fig. 1-5A; $t_{11} = 2.365$, $p = 0.045$). In contrast, there was no significant difference in GABA level in the amygdala between control and CSDS mice. To confirm the difference in GABA levels in the VLPO (as the tissue punches likely contained cells from

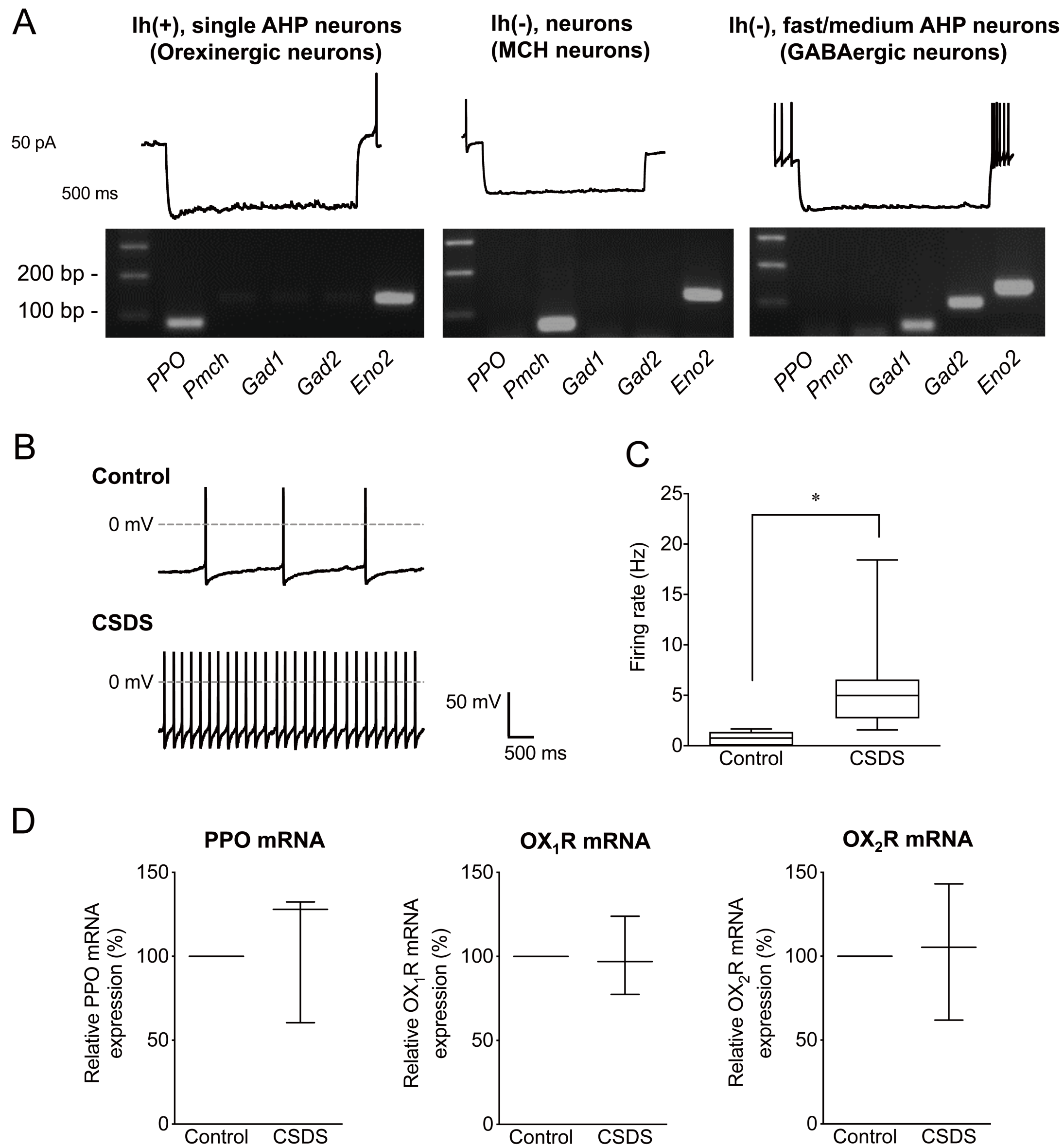

Fig. 1-4. CSDS induces increased spontaneous firing of orexinergic neurons in the lateral hypothalamus (LH). (A, top) Representative traces of hyperpolarization-induced voltage sag and afterhyperpolarization (AHP) in LH neurons. Orexinergic, melanin-concentrating hormone (MCH), and GABAergic neurons were identified by the presence/absence of hyperpolarization-induced voltage sag [Ih(+)/Ih(-)], uniphasic or fast/medium AHP, and spontaneous firing, as previously reported (Linehan et al., 2015). (Bottom) Representative single-cell RT-PCR for prepro-orexin (PPO), pro-melanin-concentrating hormone (*Pmch*), glutamic acid decarboxylase 1 (*Gad1*), glutamate decarboxylase 2 (*Gad2*), and gamma-enolase (*Eno2*) mRNAs in the LH. (B) Representative traces of spontaneous firing of LH orexinergic neurons from unstressed (control) and CSDS mice. (C) Spontaneous firing rates recorded for 30 s in LH slices of control ($n = 7$) and CSDS ($n = 8$) mice. Data are expressed as means ± SEMs. *$p < 0.05$ (unpaired t test). (D) qRT-PCR analysis of mRNAs for PPO in the LH and for orexin receptor 1 (OX_1R) and orexin receptor 2 (OX_2R) mRNA in the tuberomammillary nucleus (TMN) of control and CSDS mice. ($n = 3$). The lower and upper edges of each box show the 25th–75th percentiles, respectively, whereas the horizontal line within the box shows the median. The whiskers span all data points included within each point.

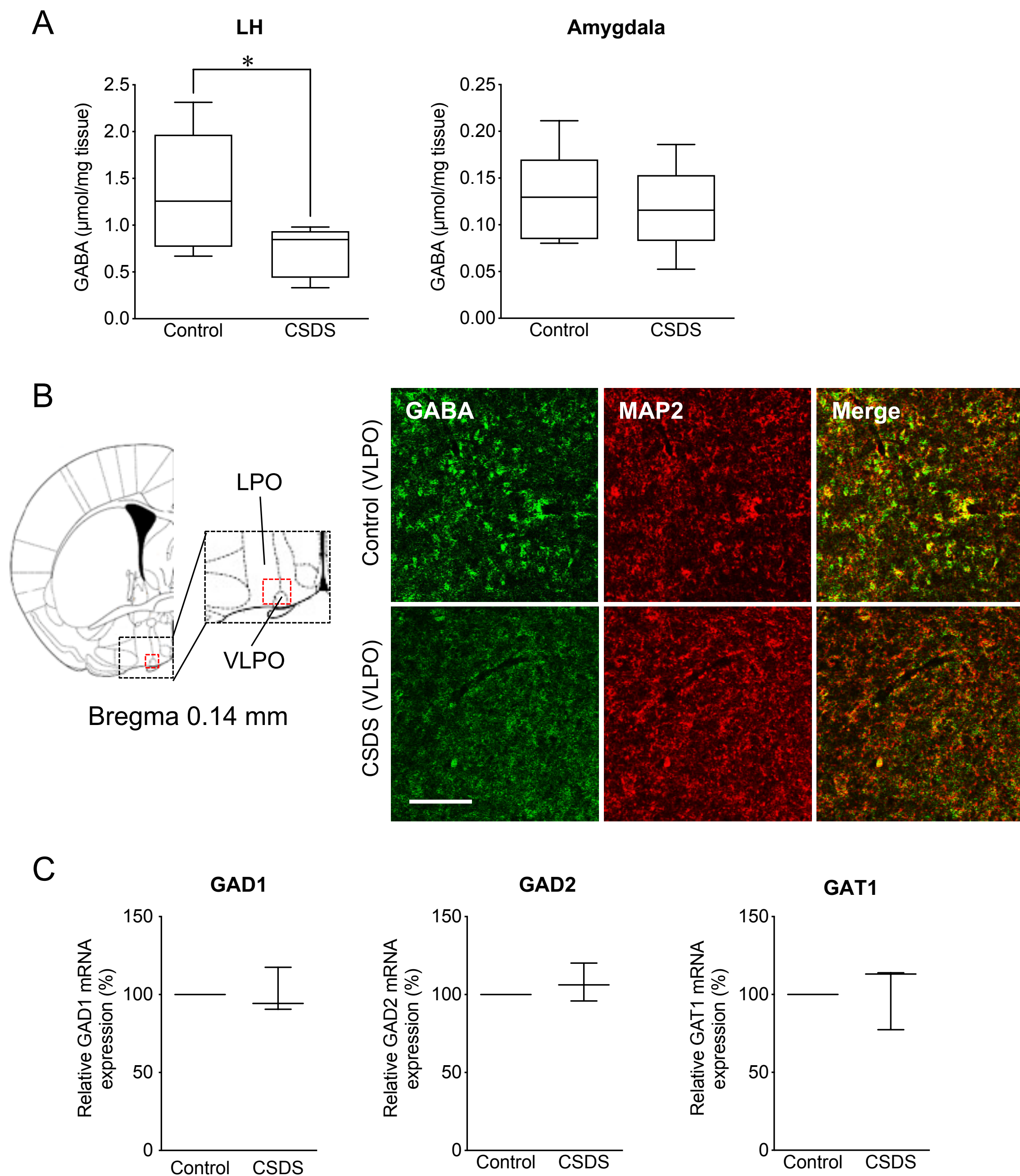

Fig. 1-5. CSDS reduces GABA levels in the ventrolateral preoptic nucleus (VLPO). (A) Changes in GABA content in the lateral hypothalamus (LH) and amygdala of control and CSDS mice, measured by HPLC. Data are expressed as means ± SEMs (n = 6–7). *p < 0.05 (unpaired t test). (B) (*left*) Position of mouse VLPO imaging. Stippled red lines represent imaging area. (*Right*) Representative images of GABA-IR (green) and MAP2-IR (red) in the VLPO of control and CSDS mice. Scale bar = 50 μm. (C) qRT-PCR analysis of glutamic acid decarboxylase 1 and 2 (GAD1 and GAD2, respectively) and GABA transporter 1 (GAT1) mRNAs in the VLPO of control and CSDS mice. (n = 3). The lower and upper edges of each box show the 25th–75th percentiles, respectively, whereas the horizontal line within the box shows the median. The whiskers span all data points included within each point.

surrounding regions due to the small size of this brain region), I performed immunostaining with an anti-GABA antibody in the VLPO. A number of GABA-immunoreactivity (IR) puncta were detected on MAP2-IR neurons in the VLPO and surrounding regions in control mice, whereas very few were detected in CSDS mice (Fig. 1-5B). However, the expression of mRNA for GAD1, GAD2, and GABA transporter 1 (GAT1) in the VLPO did not differ between control and CSDS mice (Fig. 1-5C).

Reduced inhibitory effect of brotizolam on hyperactivated orexinergic neurons in CSDS mice

I examined the effects of the benzodiazepine analog brotizolam on the spontaneous firing of orexinergic neurons in the LH. Whole-cell patch-clamp recordings showed that application of brotizolam (10 μM for 150 s) significantly decreased the spontaneous activity of orexinergic neurons in LH slices from control mice (Fig. 1-6A–B; $t_3 = 7.479$, $p = 0.005$). By contrast, brotizolam had no effect on the increased spontaneous activity of LH orexinergic neurons derived from CSDS mice (Fig. 1-6 A–B). The rate of spontaneous firing after brotizolam application relative to baseline firing in the CSDS group was significantly higher than in the control group (Fig. 1-6C; $t_4 = 7.050$, $p = 0.002$).

Reduced hypnotic effect of brotizolam in CSDS mice

I next investigated whether the failure of brotizolam to affect the firing of orexigenic neurons in mice exposed to CSDS also influenced the hypnotic effect. I first determined the hypnotic effect of a barbiturate pentobarbital, which directly activates $GABA_A$ receptors (34), in CSDS mice. The onset time and duration of sleep induced by an i.p. injection of pentobarbital (40 mg/kg) in CSDS mice were not different from those in control mice (Fig. 1-7A). Single i.p. injections of brotizolam (0.03, 0.1, 0.17, 0.3, and 1.0 mg/kg) dose dependently extended the duration of sleep

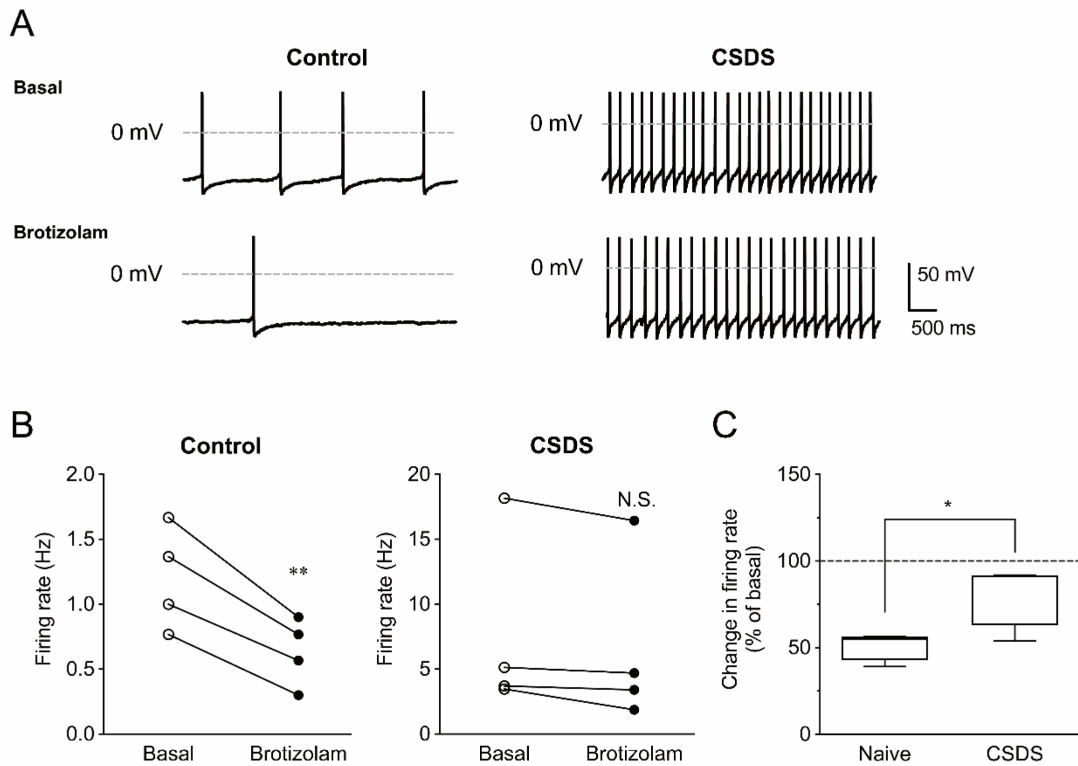

Fig. 1-6. Reduced inhibition of spontaneous firing of hyperactivated LH orexinergic neurons by brotizolam in CSDS mice. (A) Representative traces and (B) individual spontaneous firing rates (Hz) of orexinergic neurons in LH slices of control and CSDS mice before (basal) and after brotizolam (10 μM) perfusion. Spontaneous firing was recorded for 30 s, and the mean firing rate during this period was considered the basal firing rate. **$p < 0.01$ (unpaired t test). N.S. = not significant. (C) Rate of spontaneous firing after brotizolam application relative to baseline firing activity. The lower and upper edges of each box show the 25th–75th percentiles, respectively, whereas the horizontal line within the box shows the median. The whiskers span all data points included within each point. (n = 4 cells/3 mice). *$p < 0.05$ (unpaired t test).

after pentobarbital administration in control mice, with effective sleep rates of 16.7%, 33.3%, 50%, 55.6%, and 87.5%, respectively (Fig. 1-7B *left panel*, $R^2 = 0.97780$). Thus, the ED_{50} of brotizolam required to prolong pentobarbital-induced sleep in control mice was calculated as 0.185 mg/kg. This dose was then used to compare the hypnotic effect of brotizolam between control and CSDS mice. Brotizolam shortened the onset and increased the duration of sleep induced by pentobarbital in control mice, but these effects were significantly attenuated in CSDS mice (Fig. 1-7B; onset time: $t_6 = 3.205$, $p = 0.019$, duration time: $t_6 = 3.026$, $p = 0.023$). I further

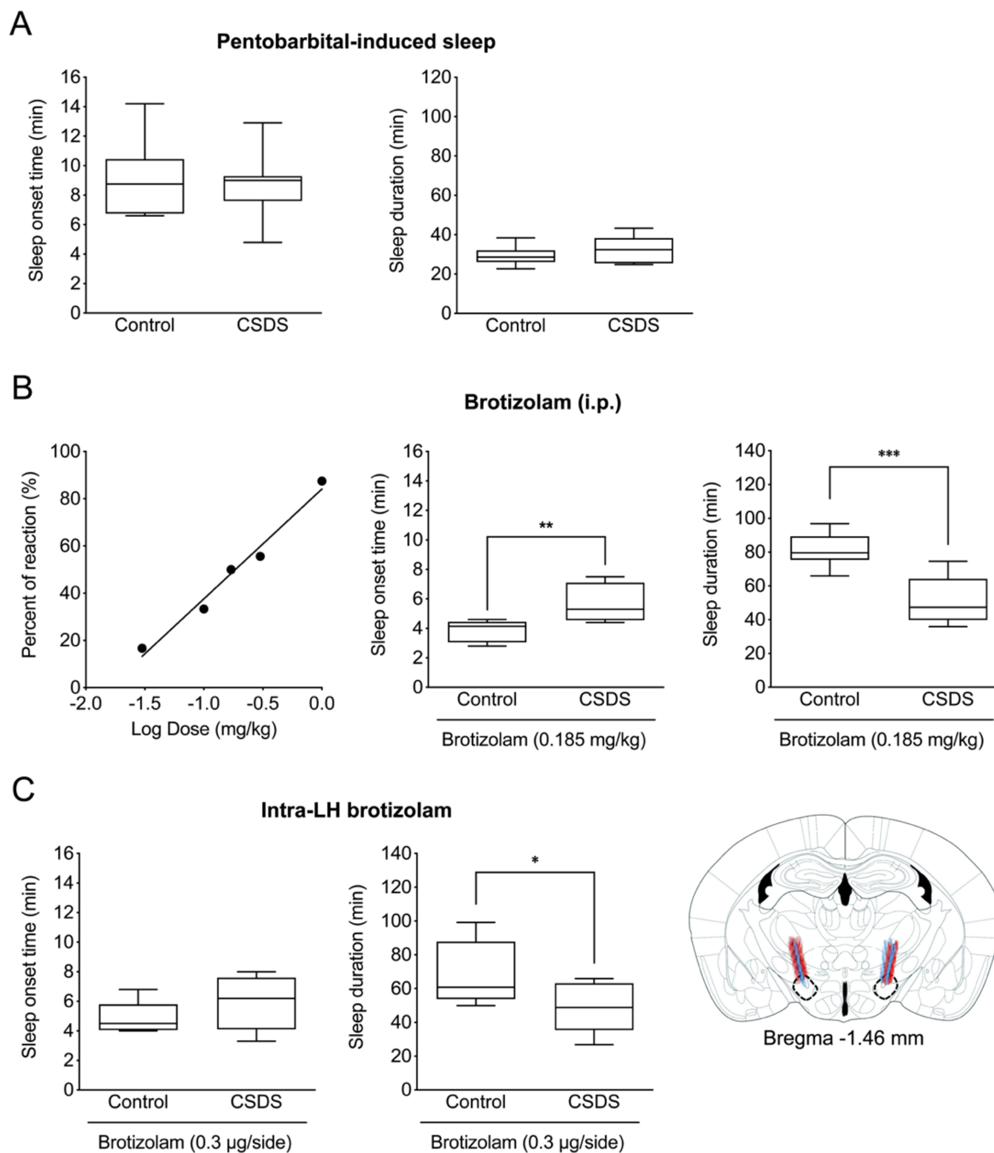

Fig. 1-7. Reduced potent hypnotic effect of brotizolam in CSDS mice. (A) No changes in the hypnotic effect of pentobarbital in CSDS mice. Pentobarbital (40 mg/kg) was injected intraperitoneally into control (n = 6) and CSDS mice (n = 7), and then the onset time (*left panel*) and duration of sleep (*right panel*) were measured as described in the Materials and Methods. (B) Comparison of hypnotic effect (potentiation of pentobarbital-induced sleep) of brotizolam in control (n = 8) and CSDS mice (n = 9). Brotizolam at a dose of calculated ED_{50} on pentobarbital-induced sleep in control mice (0.185 mg/kg; see Results section) was injected intraperitoneally 30 min before the i.p. injection of pentobarbital (40 mg/kg). Then, the onset time (*center panel*) and duration of sleep (*right panel*) were measured as described in the Materials and Methods. $^{**}p < 0.01$ and $^{***}p < 0.001$ (unpaired t-test). (D) Comparison of hypnotic effect induced by bilateral microinjection of brotizolam (0.3 μg/μL/side) into the lateral hypothalamus (LH) of control (n = 5) and CSDS mice (n = 7). At 30 min after microinjection of brotizolam into the bilateral LH, mice were injected i.p. with pentobarbital (40 mg/kg), and subsequently subjected to pentobarbital-induced sleeping test as described above. $^{*}p < 0.05$ (unpaired t-test). The lower and upper edges of each box show the 25th–75th percentiles, respectively, whereas the horizontal line within the box shows the median. The whiskers span all data points included within each point. (*Right*) Localization of microinjection sites in the mouse LH region. Stippled lines represent the LH region; colored solid lines show cannula placement (blue, control mice; red, CSDS mice).

verified the impact of CSDS on brotizolam-induced hypnotic effect by bilateral microinjection of brotizolam into the mouse LHs. Fig. 1-7*C* shows the placement of microinjection probes within the mouse LHs. Bilateral microinjections of brotizolam (0.3 μg/side) into the LH prolonged pentobarbital-induced sleep in control mice but to a significantly lesser extent in CSDS mice (Fig. 1-7C; $t_{10} = 2.256, p = 0.048$). The intra-LH brotizolam microinjection attenuated the reduced sleep onset in CSDS mice, but the difference from controls was not significant.

Hypnotic effects of suvorexant and valproate were not altered in CSDS mice

As the CSDS mice showed altered responsivity to the hypnotic effect of brotizolam, I tested the effects of two other clinically used drugs, the nonselective orexin receptor antagonist suvorexant, and the antiepileptic valproate, which increases the level of GABA at the synaptic cleft. Single i.p. injections of suvorexant (10, 13.3, 17.8, and 23.7 mg/kg) administrated 30 min before pentobarbital dose dependently extended the duration of sleep in control mice, with effective sleep rates of 23.1%, 26.3%, 37.5%, and 62.5%, respectively (Fig. 1-8A *left panel*, $R^2 = 0.87442$), resulting in an ED_{50} of 20.4 mg/kg. Suvorexant (20.4 mg/kg, i.p.) similarly reduced the time to onset and extended the duration of pentobarbital-induced sleep in control and CSDS mice (Fig. 1-8A *center and right panels*). Valproate (30, 100, 150, and 200 mg/kg) also prolonged pentobarbital-induced sleep in control mice in a dose-dependent manner, with effective sleep rates of 7.1%, 31.6%, 44.4%, and 70.0%, respectively (Fig. 1-8B *left panel*, $R^2 = 0.89216$), for an ED_{50} of 144.7 mg/kg. There were no significant differences in the onset time and duration of pentobarbital-induced sleep after i.p. injection of valproate (144.7 mg/kg, i.p.) between control and CSDS mice (Fig. 1-8B *center and right panels*).

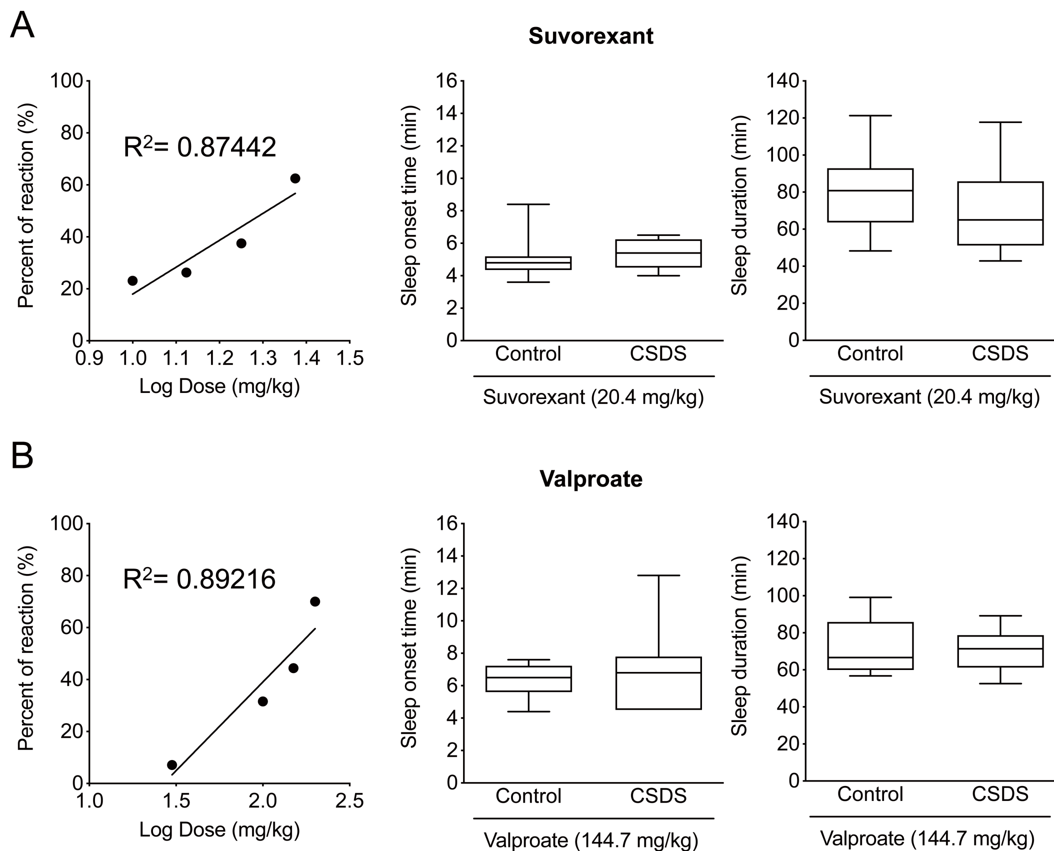

Fig. 1-8. No changes in the hypnotic effect of suvorexant and valproate in CSDS mice. The hypnotic effect of suvorexant (A) and valproate (B) in control and CSDS mice were evaluated based on the degree to their potentiating effect of pentobarbital-induced sleeping. Suvorexant (20.4 mg/kg) or valproate (144.7 mg/kg) was injected intraperitoneally at 30 min before the i.p. injection of pentobarbital (40 mg/kg). Then, the onset time (*center panel*) and duration of sleep (*right panel*) were measured as described in the Materials and Methods. The doses of suvorexant and valproate were calculated as ED_{50} on pentobarbital-induced sleep in control mice, as described for Fig. 1-7. The lower and upper edges of each box show the 25th–75th percentiles, respectively, whereas the horizontal line within the box shows the median. The whiskers span all data points included within each point. (n = 8–12).

DISCUSSION

The CSDS paradigm utilized in this study, which induces social avoidance and anxiety-like behaviors in mice, also altered sleep characteristics resembling insomnia in susceptible mice. These were observed as decreases in the total time and bout duration of immobility-defined sleep, which correlate with EEG and EMG analyses of sleep disturbance (25) and are consistent with results of previous studies showing that CSDS disrupts sleep characteristics assessed by EEG and

EMG as well as circadian body temperature fluctuations and locomotor activity (11,35). I not only provide evidence that LH orexinergic neurons are hyperactivated in these mice, but also show that these neurons were less responsive to a benzodiazepine, resulting in a reduced hypnotic effect that was likely caused by a decrease in GABA levels in the VLPO. By contrast, the hypnotic effects of an orexin receptor antagonist and valproate were not altered in these mice.

Whether there is a functional change in orexinergic neurons in depression has been controversial (36). Previous preclinical studies demonstrated that the expression of orexin in the hypothalamus is decreased in mice exposed to CSDS (37,38), and orexin A concentrations in CSF are lower in suicidal patients with MDD (39). However, other studies reported elevated concentrations of orexin A in CSF (40) and blood (41) samples from patients with depression. Grafe et al. (42) found that the inhibition of orexinergic neurons reduces depression-like behaviors and promotes resilience to social defeat in CSDS mice. Furthermore, a recent study demonstrated that stimulation of these neurons via designer receptors exclusively activated by designer drugs decreases social behaviors and induces anxiety-like behaviors in mice (43). The number of orexin-positive neurons in the LH was also found to be increased in a mouse depression model produced by corticosterone injections (44). However, our findings suggest that CSDS increases the spontaneous activity of orexinergic neurons in the LH, which may contribute to sleep disturbances associated with the observed depression-like behaviors in these mice. The hyperactivation of these neurons and sleep disturbances are unlikely directly related to the expression of orexin-related molecules, as the expression of PPO in the LH and orexin receptors in the TMN was not affected. Further investigations are needed to elucidate the underlying mechanisms.

I also found a decrease in GABA-IR in the VLPO, and a reduced GABA content in the LH including nerve endings of GABAergic neurons projected from VLPO after CSDS. GABA is an important component of the sleep regulatory system, as GABAergic neurons are activated during

sleep and send inhibitory output to $GABA_A$ receptors on orexinergic neurons in the LH (14,45). Hence, the increased spontaneous firing of LH orexinergic neurons observed in CSDS mice may be a consequence of relaxed inhibitory GABAergic transmission from the VLPO. As the transcription of rate-limiting enzymes in GABA synthesis (e.g., GAD1/GAD2 and GAT1) was not altered in CSDS mice, the mechanism underlying the observed decrease in GABA content in the VLPO is unclear at the present time. However, our results are consistent with reduced GABA in the hippocampus and cortex in patients with depression (14).

The major finding of this study is that the inhibitory effect of the benzodiazepine brotizolam on the spontaneous firing of LH orexinergic neurons was suppressed in CSDS mice, which led to lower hypnotic potency of this drug. Benzodiazepines produce a leftward shift of the concentration-response curve for GABA, due to an increase in the affinity for GABA at its binding site, that is, these drugs cannot open ligand-activated Cl^- channels of in the absence of GABA (21). By contrast, the hypnotic effect of pentobarbital was unaffected. These findings may reflect the different modes of actions of these drugs. Barbiturates such as pentobarbital can also drive Cl^- currents, even in the absence of GABA (34). The reduced effect observed even by direct microinjection of brotizolam into the LH further indicates that the sleep regulatory GABAergic system in the LH is disturbed in CSDS mice. Although the changes in the expression and activity of $GABA_A$ receptors need to be investigated in the future, I surmise that the reduced potency of brotizolam on LH orexinergic neurons and pentobarbital-induced sleep in CSDS mice resulted from reduced inhibitory drive in the sleep regulatory system, since lower amounts of GABA were detected in the VLPO. Further evidence for this is the observation that the hypnotic effect of valproate was unaffected, as this drug increases the level of endogenous GABA at the synaptic cleft by inhibiting GABA transaminase (46).

The dual orexin receptor antagonist suvorexant, which inhibits the binding of orexin to its

receptors and suppresses arousal regulatory systems, is approved for the treatment of insomnia in the United States, Europe, Japan, and Australia (47). The data presented here indicate that suvorexant at ED_{50} induces a similar hypnotic effect in CSDS and control mice and suggest the possible utility of this orexin receptor antagonist for treating insomnia in patients with depression. Consistent with this, a prospective clinical study found that treatment with suvorexant improved the quality of sleep and reduced the severity of anxiety and depression in 40 psychiatric inpatients (48). Furthermore, the selective orexin-2 receptor antagonist seltorexant improved sleep in antidepressant-treated MDD patients with persistent insomnia (49,50).

I showed for the first time that CSDS impacts the balance between arousal and sleep regulatory systems, resulting in reduced GABA levels in the VLPO and increased spontaneous firing of orexinergic neurons in the LH. Furthermore, the decreased responsiveness of these hyperactivated neurons reduced the hypnotic potency of brotizolam but not of the orexin receptor antagonist suvorexant. These results provide new insights into the mechanisms underlying the reduced potency of benzodiazepine hypnotics in a mouse model of depression and suggest that orexin receptor antagonists may be effective hypnotics for the treatment of insomnia complicated with depression.

Chapter 2: Assessment of suvorexant and eszopiclone as alternatives to benzodiazepines for treating insomnia in patients with major depressive disorder

INTRODUCTION

Benzodiazepines are widely prescribed hypnotics used for treating insomnia in patients with depression (5-7). An early double-blind, placebo-controlled clinical study showed that the addition of a benzodiazepine to tricyclic antidepressant treatment not only reduced the increased total rapid eye movement sleep often observed in patients with MDD but also improved depressive symptoms (51). However, MDD patients can still experience residual insomnia despite being treated with benzodiazepines (52,53), and rare cases of benzodiazepine treatment-resistant insomnia in MDD patients are observed (8). Furthermore, the chronic use of benzodiazepines is associated with worsening symptoms of depression and the development of dependence or tolerance to benzodiazepines (54-56). Taken together, these observations indicate the important need for a drug that can be used as an alternative to a benzodiazepine for treating insomnia that is highly comorbid with depression. However, treatment guidelines do not state a defined strategy to treat insomnia in patients with MDD (5).

Recent commonly prescribed hypnotics for insomnia treatment include suvorexant, a dual orexin receptor antagonist, and non-benzodiazepine GABA receptor agonists (i.e., z-drugs), such as eszopiclone and zolpidem (57,58). Suvorexant is used to effectively treat insomnia characterized by difficulty achieving and/or maintaining sleep, and is associated with a low risk of developing tolerance and dependence (59,60). Eszopiclone is approved for the long-term treatment of sleep onset and sleep maintenance insomnia (61), and there is no evidence suggesting a risk of tolerance or dependence during chronic use (62). Previous clinical studies demonstrate

that these hypnotic drugs are also beneficial for treating insomnia in patients with MDD (63,64). Although the number of studies is limited, a clinical study showed that treatment with suvorexant for 1 week improves total sleep time and time to sleep onset in patients with MDD (63). An open-label randomized clinical trial demonstrated that the addition of zolpidem to the selective serotonin reuptake inhibitor (SSRI) escitalopram improved total sleep time, wake time after sleep onset, and sleep-onset latency in patients with MDD (64). However, it is not known if suvorexant or z-drugs can be used as alternatives to benzodiazepine treatment in MDD patients who have residual insomnia despite receiving treatment with benzodiazepines.

To address this question, in this Chapter, I conducted a prospective, two-arm, open-label randomized study to evaluate the efficacy and safety of switching from benzodiazepines to suvorexant or eszopiclone for treating insomnia in patients with MDD.

METHODS

Patients and screening

This study was approved by the Kyoto University Graduate School and Faculty of Medicine, Ethics Committee (No. C1353). I conducted an open-label randomized study from April 2018 to March 2020 at Kyoto University Hospital. All patients were required to be 16–89 years old and meet the Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-5) criteria for MDD and insomnia associated with MDD. In addition, patients were required to have insomnia symptoms with a score of > 7 on the Insomnia Severity Index Japanese version (ISI-J) (65), despite receiving treatment with benzodiazepines for more than 2 weeks of treatment.

Major exclusion criteria included 1) Patients taking other hypnotics; 2) Patients judged not to be accurately taking the study medication; 3) Patients with severe physical disease; 4) Patients

with a history of severe drug hypersensitivity or drug allergy; 5) Women who were pregnant or wished to become pregnant during the study period, and lactating mothers; 6) Patients taking potent inhibitors of cytochrome P450 3A; 7) Patients taking agents that cause insomnia, such as steroids, dopamine receptor agonists, and β-blockers; 8) Patients judged to be high-risk for substance abuse; 9) Patients judged to be high-risk for suicide; and 10) Patients judged to be inappropriate for inclusion in this study by a psychiatrist. Patients were withdrawn after the run-in period if medication compliance was inadequate or non-compliant, or if they had an onset of suicidal ideation assessed by the Beck Depression Inventory-II (BDI-II) (66). Patients were allowed to voluntarily withdraw after the start of the study. As shown in Fig. 1-1, the run-in period was defined as 2 weeks of treatment with benzodiazepines prior to the start of the study. Written informed consent was obtained from all patients during the run-in period.

Randomization

The screening of potential participants was conducted by a pharmacist and a psychiatrist. A stratified block randomization scheme was used to assign eligible participants at baseline to eszopiclone or suvorexant in a 1:1 ratio using a computer-generated randomization scheme based on age (< 65 and ≥ 65 years old) and gender in randomly assigned block sizes of 2, 4, or 6. All investigators (i.e., pharmacists and doctors involved in this study) remained blinded to treatment allocation during the run-in period and were unblinded after randomization.

Study design and procedure

After the 2 week run-in period, patients were randomized to receive eszopiclone or suvorexant for 4 weeks instead of benzodiazepines (Fig. 2-1). The oral dose of each drug was determined in accordance with the package insert; namely, by age (< 65 years: eszopiclone 3 mg/day, suvorexant

20 mg/day; ≥ 65 years: eszopiclone 2 mg/day, suvorexant 15 mg/day). Patients took eszopiclone or suvorexant each night immediately before bed. At the beginning of the study, the dose of benzodiazepine was reduced to 1/2 of the dose taken during the run-in period, and discontinued 2 weeks after the start of the study. The use of other hypnotic medicines was not permitted. However, antidepressant, antipsychotic, and antianxiety agents were permitted if patients had been taking these for 2 weeks or more before the run-in period. However, a change of dose was not permitted; patients were excluded from the study if the dose of antidepressant, antipsychotic, and antianxiety agents was changed.

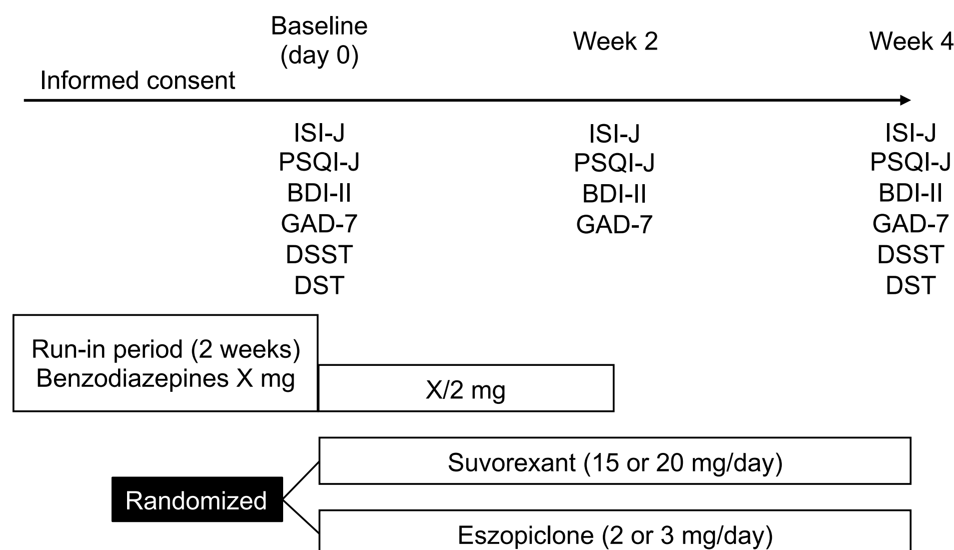

Fig. 2-1. A schematic of the study from screening to study completion. The 2 weeks of treatment with benzodiazepines prior to the beginning of the study was defined as the run-in period. Written informed consent was obtained from all patients during the run-in period. The assessment of each symptom was conducted on day 0 (baseline), and 2 and 4 weeks after switching to the study drugs. ISI-J, Insomnia Severity Index Japanese version; PSQI-J, Pittsburgh Sleep Quality Index Japanese version; BDI-II, Beck Depression Inventory-II; GAD-7, Generalized Anxiety Disorder-7; DSST, digit symbol substitution test; DST, digit span test.

Efficacy assessment

The efficacy of eszopiclone and suvorexant was assessed at the beginning of the study (baseline), and 2 and 4 weeks after the start of the study (Fig. 2-1), by several self-administered

questionnaires: the ISI-J for insomnia severity (65), the Pittsburgh Sleep Quality Index Japanese version (PSQI-J) for subjective sleep quality (67), the BDI-II for depressive symptoms (66,68), and the Generalized Anxiety Disorder-7 (GAD-7) score for anxiety symptoms (69). The cognitive function and the next-day residual effects of the drugs were assessed using the digit span test (DST) and digit symbol substitution test (DSST) at the beginning of the study (baseline) and 4 weeks after the start of the study (70,71). Safety was assessed using open-ended questioning for adverse events throughout the study.

Primary efficacy measure (assessment of insomnia severity)

The primary endpoint of the study was a change in the severity of insomnia from baseline as measured by the ISI-J 2 and 4 weeks after switching from benzodiazepines to eszopiclone or suvorexant (65). The ISI-J is a 7-item self-report questionnaire used to assess the severity of insomnia and consists of questions related to sleep onset, sleep maintenance, early-morning awakening problems, sleep dissatisfaction, the interference of sleep difficulties with daytime functioning, the noticeability of sleep problems by others, and the distress caused by sleep difficulties. Each item is rated on a 5-point Likert scale (a score of 0 indicates no problems; 4 indicates very severe problems), and the total score ranges from 0–28 (a score of 0 ≤ to ≤ 7 indicates an absence of insomnia; 7 < to ≤ 14 indicates subthreshold insomnia; 14 < to ≤ 21 indicates moderate insomnia; 21 < to ≤ 28 indicates severe insomnia).

Secondary efficacy measure

The secondary endpoint was a change from baseline in the subjective quality of sleep, depressive symptoms, anxiety symptoms at 2 and 4 weeks after switching medication, and cognitive function at 4 weeks after switching medication.

The PSQI-J assesses seven factors (sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbance, the use of sleep medicine, and daytime dysfunction) (67). Each item

is rated from 0–3 (a score of 0 indicates no insomnia; 3 indicates severe insomnia), and the total score ranges from 0–21. A PSQI-J total score of > 5.5 suggests clinical insomnia (a higher score indicates more severe insomnia).

The severity of depressive symptoms was measured by the BDI-II, which consists of 12 self-assessed questions related to somatic/affective function (loss of pleasure, crying, loss of interest, indecisiveness, worthlessness, loss of energy, sleep problems, irritability, appetite problems, concentration, fatigue, and loss of interest in sex) and nine self-assessed questions related to cognitive function (sadness, pessimism, past failure, guilty feelings, punishment feelings, self-dislike, self-criticalness, suicidal ideation, and agitation) (66,68). Each item is rated on 4-point Likert scale (a score of 0 indicates no depression; 3 indicates severe depression), and the total score ranges from 0–63 (a score of 0 ≤ to ≤ 13 indicates no depression; 13 < to ≤ 19 indicates mild depression; 19 < to ≤ 28 indicates moderate depression; 28 < indicates severe depression).

Anxiety symptoms were measured by GAD-7, which consists of a 7-item self-questionnaire (69). Each item is rated on a 4-point Likert scale (a score of 0 indicates no problems and a score of 3 indicates the patient has a problem nearly every day), and the total score ranges from 0–21 (a score of 0 ≤ to < 5 indicates minimal anxiety, 5 ≤ to < 10 indicates mild anxiety, 10 ≤ to < 15 indicates moderate anxiety, 15 ≤ to ≤ 21 indicates severe anxiety).

Cognitive function was assessed by the DSST and DST contained in the Wechsler Adult Intelligence Scale (70,71). The DSST is a paper-based tool designed to measure visual–motor coordination, short-term memory, concentration, and processing speed, and is rated on a score from 0–19 (a lower score indicates worse cognitive function). Patients are provided with a set of symbols with a matching key and scored on the total number of symbols that are drawn correctly in 120 seconds. The DST was used to evaluate attention span and short-term memory, and verbal working memory, and is rated from 0–19 (a lower score indicates poorer short-term memory).

The DST contains Digit Span Forward (DSF) and Digit Span Backward (DSB). DSF requires the participant to repeat back increasingly lengthy strings of numbers, and DSB requires the participant to repeat series of numbers backward. DST score was scored on the total score of DSF and DSB score.

Safety was assessed by evaluating adverse events and the onset of suicidal ideation throughout the study period. Any new or worsening signs and symptoms of illness, regardless of whether or not they were related to the study drugs, were recorded as adverse events. The occurrence of rebound insomnia was assessed by changes in the ISI-J total score relative to baseline at each assessment point.

Statistical Analysis

Sample size determination

I used the average of ISI score and standard deviation values reported in previous studies as reference for sample size determination (72). In the suvorexant group, it was estimated that 20 patients per treatment group would be needed to achieve a mean change in ISI-J score from baseline to week 4 of 4.0 points (SD = 4.5) with 80% power, using a 2-sided α level of 0.05.

Patient demography and clinical characteristics

Differences in patient demographics and characteristics at baseline between the suvorexant and eszopiclone groups were examined statistically. Comparisons of gender, the using benzodiazepines before switching to the study drugs, and the using antidepressants were performed with Fisher's exact test because the frequency count for these characteristics was expected to be < 5. Comparisons of age, the baseline ISI-J total score, the PSQI-J total score and seven component scores, the BDI-II total score and two factor scores, the GAD-7 total score, the DSST score, and the DST score were performed with the Mann–Whitney test due to the low

number of enrolled patients.

Primary efficiency analysis

Changes in insomnia severity mediated by treatment with suvorexant or eszopiclone were examined statistically. Insomnia severity (the mean ISI-J total score) within each group at baseline, week 2, and week 4 were compared with Friedman non-parametric analysis and Dunn's multiple comparison tests. To analyze the change after each treatment, changes in the ISI-J score from baseline between the suvorexant and eszopiclone groups at weeks 2 and 4 were examined statistically using the Mann–Whitney test.

Secondary efficiency analysis

Changes in sleep quality (the PSQI-J total score and the seven component scores), depressive symptoms (the BDI-II total score and two factor scores), and anxiety symptoms (the GAD-7 total score) within each group at baseline, week 2, and week 4 were compared statistically using Friedman non-parametric analysis and Dunn's multiple comparison tests. To analyze the change after each treatment, changes in the PSQI-J score, the BDI-II score, and the GAD-7 total score from baseline between the suvorexant and eszopiclone groups at weeks 2 and 4 were examined statistically using the Mann–Whitney test.

Changes in cognitive function and next-day residual effects (mean DSST and DST scores) within each treatment group at baseline, week 2, and week 4 were compared statistically using a paired *t*-test.

Safety analysis

The safety of suvorexant or eszopiclone treatment was assessed by examining statistically the frequency of the adverse events. The incidence of adverse events within each group was compared at baseline and during the study using Fisher's exact test.

General overview

Missing data values were assigned using last observation carried forward (LOCF) for the primary efficiency analysis and most secondary analyses, except for the DSST and DST data. Because the DSST and DST scores were assessed only twice, the DSST and DST scores were analyzed using observed case data.

All data were analyzed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) or JMP Pro 14.0.0 (SAS Institute, Inc., Cary, NC, USA). A two-sided p-value of < 0.05 was considered statistically significant.

RESULTS

Patients

In the present study, doctors and pharmacists selected participants based on strict screening criteria after careful observation of the MDD patients' condition due to concerns about suicidal ideation in patients receiving suvorexant (52,59). As shown in Fig. 2-2, 279 patients were screened for study inclusion; 18 of the screened patients were randomized and received treatment, and 16 (89%) of the randomized patients completed the study. Two patients in the eszopiclone group discontinued the study due to adverse events (unpleasant taste). Initially, it was planned to enroll 20 patients per treatment group. However, the analysis was conducted with nine patients per treatment group due to the low number of patients who met the selection criteria during the study period. The baseline clinical characteristics of the study participants are summarized in Tables 2-1 and 2. There were no significant differences between treatment groups in demographic parameters (gender and age) at baseline. Before switching to study medications, 77.8% of patients in the suvorexant group (seven patients) and 66.7% of patients in the eszopiclone group (six patients) were taking brotizolam. In addition, 55.6% of patients in the suvorexant group (five

patients) and 55.6% of patients in the eszopiclone group (five patients) were taking an SSRI (paroxetine, escitalopram, or sertraline). There was no statistically significant difference in the type of benzodiazepines or antidepressants used between the treatment groups (Table 2-1). There were no significant differences in insomnia severity (ISI-J total score), sleep quality (PSQI-J subscale), severity of depressive symptoms (BDI-II total score), anxiety symptoms (GAD-7 total score), or cognitive function (DSST and DST scores) between the treatment groups at baseline (Table 2-2).

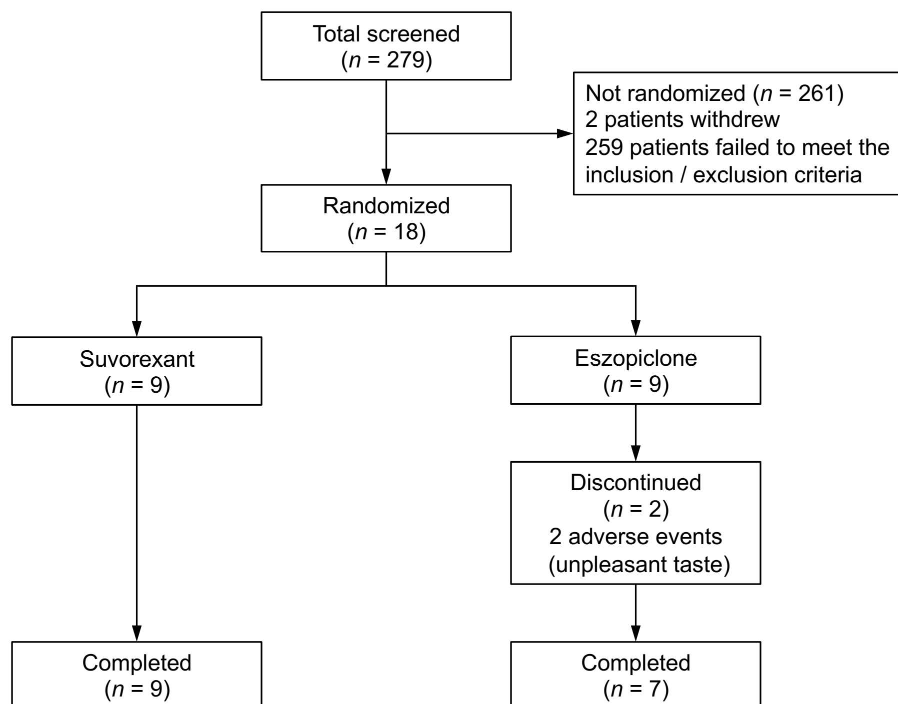

Fig. 2-2. Flow-chart of patient selection

Primary efficacy analysis (assessment of insomnia severity)

As shown in Table 2-2, the mean ISI-J total score at baseline was 13.3 in the suvorexant group (classed as subthreshold insomnia) and 16.1 in the eszopiclone group (classed as moderate insomnia). Treatment with eszopiclone for 4 weeks significantly reduced the ISI-J total score

compared with the score at baseline (Table 2-2; Friedman ANOVA test / Dunn's test, $p = 0.040$), and improved insomnia severity to subthreshold insomnia. Incidentally, two discontinued patients treating with eszopiclone showed a numerical improvement ISI-J total score at the week 2 from baseline. Suvorexant treatment for 4 weeks also reduced the ISI-J total score, but this effect was not significant. The changes in the ISI-J total scores from the baseline were -3.3 (suvorexant) and -4.5 (eszopiclone) at 2 weeks, and -4.3 (suvorexant) and -4.1 (eszopiclone) at 4 weeks after the switch from benzodiazepines (Table 2-3). The difference between the two groups was not statistically significant at each assessment

Table 2-1. Patient Demographics and Baseline Characteristics.

	Suvorexant n = 9	Eszopiclone n = 9	*p*
Gender, n (%)			
Female	6 (66.7)	5 (55.6)	>0.99
Male	3 (33.3)	4 (44.4)	
Age, mean (SD)	57.7 (13.8)	59.7 (10.5)	0.618
Benzodiazepine, n (%)			
Brotizolam	7 (77.8)	6 (66.7)	>0.99
Nitrazepam	1 (11.1)	2 (22.2)	
Rilmazafone	0 (0)	1 (11.1)	
Lormetazepam	1 (11.1)	0 (0)	
Antidepressant, n (%)			
Paroxetine	3 (33.3)	1 (11.1)	0.100
Escitalopram	1 (11.1)	1 (11.1)	
Sertraline	1 (11.1)	3 (33.3)	
Duloxetine	1 (11.1)	0 (0)	
Venlafaxine	1 (11.1)	0 (0)	
Milnacipran	1 (11.1)	0 (0)	
Trazodone	2 (22.2)	3 (33.3)	
Mirtazapine	0 (0)	1 (11.1)	
Clomipramine	0 (0)	1 (11.1)	
No antidepressant	0 (0)	1 (11.1)	

Baseline parameters were collected on day 0. *p*-values reflect the difference between the suvorexant group and the eszopiclone group at baseline (Mann-Whitney *U* test).

point. Rebound insomnia was not observed in either group throughout the study period. In the subgroup analysis, the ISI-J total scores were classified according to the presence (GAD-7 score ≥ 10) or absence (GAD-7 score < 10) of moderate anxiety, and changes in scores were compared within the suvorexant and eszopiclone groups (Fig. 2-3). Treatment with suvorexant tended to improve the ISI-J total score in MDD patients with moderate anxiety over time (ISI-J -8.4 at week 4), whereas it did not affect the score in MDD patients who did not have moderate anxiety (ISI-J +0.3 at week 4). Treatment with eszopiclone tended to improve the ISI-J total score in MDD patients who had (ISI-J -2.8 at week 4) and did not have moderate anxiety (ISI-J -6.6 at week 4), whereas more improvement was noted in the patients who did not have moderate anxiety. There was no statistically significant difference in the change of the ISI-J total score in patients who had and did not have anxiety between the two treatment groups at each assessment point.

Secondary efficacy analysis

Assessment of subjective sleep quality

The mean PSQI-J total score at baseline was 10.2 in the suvorexant group and 13.0 in the eszopiclone group, and both scores exceeded the cut-off score (> 5.5) of PSQI-J for insomnia (Table 2-2). Treatment with suvorexant or eszopiclone did not affect the PSQI-J total score during the study period. The change in the PSQI-J total score from baseline to 4 weeks after the switch in medication was -0.4 for suvorexant and -0.1 for eszopiclone at 2 weeks, and 0.3 for suvorexant and -1.0 for eszopiclone (Table 2-3). The change in the PSQI-J total score between the two groups was not significantly different at each assessment point. Treatment with suvorexant or eszopiclone did not significantly affect the seven component scores of the PSQI-J, but tended to reduce the score for daytime disfunction and sleep duration.

Table 2-2. Summary of Primary and Secondary Efficacy Endpoints.

Endpoint	Suvorexant					Eszopiclone					*p*
				p					*p*		
Rating scale score, mean (SD)	Baseline	Week 2	Week 4	Baseline versus week 2	Baseline versus week 4	Baseline	Week 2	Week 4	Baseline versus week 2	Baseline versus week 4	Suvorexant versus eszopiclone at baseline
Primary Efficacy Endpoint											
ISI-J											
Total score	13.3 (5.1)	10.0 (3.8)	9.0 (4.7)	>0.99	>0.99	16.1 (4.1)	11.6 (4.4)	12.0 (4.2)	0.040	0.040	0.247
Secondary Efficacy Endpoint											
PSQI-J											
Total score	10.2 (3.9)	9.8 (3.1)	10.6 (4.0)	>0.99	>0.99	13.0 (3.5)	12.9 (3.7)	12.0 (3.7)	>0.99	>0.99	0.209
PSQI-J 7 component score											
Sleep quality	1.4 (0.7)	1.2 (0.6)	1.6 (0.7)	>0.99	>0.99	1.8 (0.4)	1.8 (0.4)	1.6 (0.5)	>0.99	>0.99	0.153
Sleep latency	1.8 (0.8)	1.8 (0.9)	2.1 (1.1)	>0.99	>0.99	2.1 (0.7)	2.2 (0.8)	2.2 (0.8)	>0.99	>0.99	0.473
Sleep duration	0.8 (1.1)	0.7 (0.9)	0.9 (1.1)	>0.99	>0.99	1.9 (1.1)	1.7 (0.8)	1.3 (0.9)	>0.99	0.377	0.107
Habitual sleep efficacy	0.4 (1.0)	0.4 (1.0)	0.6 (1.0)	>0.99	>0.99	1.3 (1.3)	1.4 (1.4)	1.3 (1.3)	>0.99	>0.99	0.199
Sleep disturbance	1.2 (0.4)	1.1 (0.6)	1.4 (0.7)	>0.99	>0.99	1.2 (0.6)	1.0 (0.5)	1.0 (0.5)	>0.99	>0.99	>0.99
Use of sleep medication	3.0 (0)	3.0 (0)	3.0 (0)	>0.99	>0.99	3.0 (0)	3.0 (0)	3.0 (0)	>0.99	>0.99	–
Daytime disfunction	1.6 (0.7)	1.6 (1.0)	1.0 (0.8)	>0.99	0.580	1.7 (0.9)	1.8 (1.1)	1.6 (1.2)	>0.99	>0.99	0.674
BDI-II											
Total score	19.0 (11.5)	16.0 (13.1)	16.1 (13.9)	>0.99	>0.99	30.2 (13.8)	28.8 (14.9)	26.8 (15.1)	>0.99	0.297	0.118
BDI-II two factor score											
Somatic / affective score	12.6 (7.0)	9.9 (6.6)	9.7 (7.3)	0.867	0.472	18.6 (8.4)	17.4 (8.0)	16.4 (8.3)	0.716	0.135	0.098
Cognitive score	6.4 (4.7)	6.1 (6.6)	6.4 (6.9)	>0.99	>0.99	11.7 (6.8)	11.3 (7.5)	10.4 (7.4)	>0.99	>0.99	0.097
GAD-7											
Total score	8.2 (5.1)	7.1 (5.6)	6.4 (5.0)	>0.99	0.867	11.8 (4.6)	8.8 (5.0)	9.1 (4.7)	0.055	0.029	0.141
DSST score	9.1 (3.8)	–	9.8 (4.1)	–	0.132	6.7 (3.8)	–	8.1 (3.6)	–	0.058	0.213
DST score	11.1 (2.6)	–	12.2 (3.9)	–	0.282	9.0 (2.2)	–	9.4 (2.3)	–	0.078	0.130

The data presented are mean scores in the suvorexant group and the eszopiclone group on day 0 (baseline), and at weeks 2 and 4. ISI-J, Insomnia Severity Index Japanese version; PSQI-J, Pittsburgh Sleep Quality Index Japanese version; BDI-II, Beck Depression Inventory-II; GAD-7, Generalized Anxiety Disorder-7; DSST, digit symbol substitution test; DST, digit span test. *p*-values reflect the change from baseline at week 4.

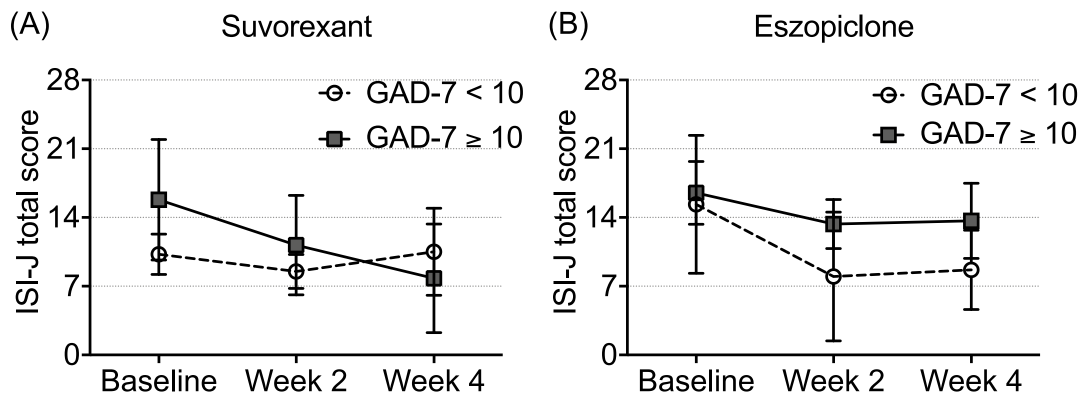

Fig. 2-3. Changes in the ISI-J total score classified according to the presence (GAD-7 score ≥ 10) or absence (GAD-7 score < 10) of moderate anxiety in suvorexant- (A) or eszopiclone-treated (B) groups. (A) In the suvorexant group, five patients had moderate anxiety and four patients did not have moderate anxiety. (B) In the eszopiclone group, six subjects had moderate anxiety and three patients did not have moderate anxiety. *p*-values reflect the change from baseline analyses with one-way ANOVA and Dunn's multiple comparison tests. (A) Baseline versus week 2: $p > 0.99$ (GAD-7 score < 10), $p > 0.99$ (GAD-7 score ≥ 10); baseline versus week 4: $p > 0.99$ (GAD-7 score < 10), $p = 0.173$ (GAD-7 score ≥ 10). (B) Baseline versus week 2: $p = 0.31$ (GAD-7 score < 10), $p = 0.182$ (GAD-7 score ≥ 10); baseline versus week 4: $p = 0.124$ (GAD-7 score < 10), $p = 0.337$ (GAD-7 score ≥ 10) (Friedman test and Dunn's multiple comparison test).

Assessment of depressive symptom severity

The mean BDI-II total score at baseline was 19.0 in the suvorexant group (classed as mild depression) and 30.2 in the eszopiclone group (classed as severe depression) (Table 2-2). Neither suvorexant nor eszopiclone had a significant effect on the BDI-II total score, but the score tended to decrease in both treatment groups. Eszopiclone improved the severity of depression symptoms to moderate depression. The change in the BDI-II total score from baseline was -3.0 for suvorexant and -1.4 for eszopiclone at 2 weeks, and -2.9 for suvorexant and -3.3 for eszopiclone 4 weeks after switching treatment (Table 2-3). There was no significant difference in change of the mean BDI-II total score between the two treatment groups at each assessment point. Of the 21 items in the BDI-II, suvorexant and eszopiclone tended to decrease the somatic/affective score, although these decreases were not significant.

Assessment of anxiety symptom severity

The mean GAD-7 total score at baseline was 8.2 in the suvorexant group (classed as mild

anxiety) and 11.8 in the eszopiclone group (classed as moderate anxiety) (Table 2-2). Treatment with eszopiclone for 4 weeks significantly reduced the GAD-7 total score compared with the score at baseline (Table 2-2; Friedman ANOVA test / Dunn's test, p = 0.029), and improved anxiety severity to mild anxiety. There was a tendency for the total GAD-7 score to decrease over time during the 4 weeks of treatment with suvorexant, but this effect was not significant. The change in the GAD-7 total score from baseline was -1.1 for suvorexant and -3.0 for eszopiclone at 2 weeks, and -1.8 for suvorexant and -2.7 for eszopiclone 4 weeks after switching treatment (Table 2-3). There was no significant difference in the change of the total GAD-7 score between the two treatment groups at each assessment point.

Assessment of cognitive function

The mean of the DSST total scores at baseline was 9.1 in the suvorexant group and 6.7 in the eszopiclone group, and the mean of the DST total scores at baseline was 11.1 in the suvorexant group and 9.0 in the eszopiclone group. Treatment with suvorexant or eszopiclone did not significantly alter the DSST and DST total scores at week 4 (Tables 2-2 and 3).

Table 2-3. Summary of the Change from Baseline Score at Weeks 2 and 4.

	Week 2			Week 4		
Endpoint	Suvorexant	Eszopiclone	*p*	Suvorexant	Eszopiclone	*p*
Change score from baseline, mean (SD)						
ISI-J total score	-3.3 (5.0)	-4.5 (3.5)	0.397	-4.3 (8.0)	-4.1 (3.7)	0.746
PSQI-J total score	-0.4 (1.5)	-0.1 (2.3)	0.498	0.3 (1.3)	-1.0 (1.9)	0.690
BDI-II total score	-3.0 (7.1)	-1.4 (7.2)	0.444	-2.9 (9.0)	-3.3 (8.5)	0.473
GAD-7 total score	-1.1 (4.3)	-3.0 (3.3)	0.303	-1.8 (3.8)	-2.7 (1.8)	0.383
DSST score	–	–	–	+0.8 (1.3)	+1.4 (1.5)	0.432
DST score	–	–	–	+1.1 (2.7)	+0.4 (0.5)	0.835

The data presented are changes in mean scores in the suvorexant group and the eszopiclone group from baseline (day 0) at weeks 2 and 4. ISI-J, Insomnia Severity Index Japanese version; PSQI-J, Pittsburgh Sleep Quality Index Japanese version; BDI-II, Beck Depression Inventory-II; GAD-7, Generalized Anxiety Disorder-7; DSST, digit symbol substitution test; DST, digit span test. p-values reflect the difference between the suvorexant group and the eszopiclone group at each timepoint (Mann-Whitney U test).

Safety

Adverse events with an incidence rate of > 10% over 4 weeks in each treatment group are summarized in Table 2-4. Common adverse events seen in either group during the study period were somnolence, headache, fatigue, fall, irritation, abnormal dreams, and dizziness. However, the overall incidence of these adverse events did not significantly change during the study period compared with the corresponding baseline in each treatment group. In the eszopiclone group, four patients experienced an unpleasant taste 4 weeks after switching from benzodiazepines (44.4%). Throughout the study, the overall adverse events seen in either group were mild in intensity. In addition, no patients experienced suicidal ideation during the study period.

Table 2-4. Summary of Adverse Events.

	Baseline (using benzodiazepines only)	During study period (using suvorexant or eszopiclone)	*p* versus baseline
Suvorexant			
Somnolence	7 (77.8)	4 (44.4)	0.170
Headache	4 (44.4)	5 (55.6)	>0.99
Fatigue	8 (88.9)	8 (88.9)	>0.99
Fall	3 (33.3)	2 (22.2)	>0.99
Irritation	4 (44.4)	3 (33.3)	>0.99
Abnormal dreams	4 (44.4)	6 (66.7)	0.637
Dizziness	2 (22.2)	2 (22.2)	>0.99
Unpleasant taste	0 (0)	0 (0)	>0.99
Eszopiclone			
Somnolence	5 (55.6)	6 (66.7)	>0.99
Headache	5 (55.6)	4 (44.4)	>0.99
Fatigue	6 (66.7)	5 (55.6)	>0.99
Fall	4 (44.4)	2 (22.2)	0.620
Irritation	5 (55.6)	5 (55.6)	>0.99
Abnormal dreams	6 (66.7)	3 (33.3)	0.347
Dizziness	4 (44.4)	5 (55.6)	>0.99
Unpleasant taste	0 (0)	4 (44.4)	0.082

The adverse events presented are any that occurred at a rate ≥ 10% in either group. *p*-values reflect the change in the incidence of adverse events throughout the study (Fisher's exact tst).

DISCUSSION

In this randomized open-label study, it was shown that switching from benzodiazepines to suvorexant or eszopiclone was well tolerated and improved insomnia severity in MDD patients who had insomnia despite receiving more than 2 weeks of benzodiazepine treatment. Although the sample size used in our assessment was small due to low enrollment, the findings suggest that suvorexant and eszopiclone may be useful alternatives to benzodiazepines for treating insomnia in patients with MDD.

The present study in MDD patients showed that treatment with either suvorexant or eszopiclone for 4 weeks decreased the ISI-J total score to a similar level. The effect of suvorexant on insomnia severity was not statistically significant, but comparable to that described in a Phase 3 clinical trial of suvorexant in patients with primary insomnia (the ISI score was -4.0 after 4 weeks of treatment) (72). Eszopiclone significantly improved insomnia, the primary efficacy endpoint, although the level of improvement was less than that reported in a previous clinical trial in patients with schizophrenia (the ISI score was -10.7 after 8 weeks of treatment) (73). By contrast, suvorexant or eszopiclone did not improve sleep quality assessed by the PSQI-J. This result is inconsistent with a previous report showing that suvorexant significantly improves the PSQI score in psychiatric patients with depression and anxiety (74). It is unclear why suvorexant and eszopiclone did not affect sleep quality in this study. The long-term use of benzodiazepines may affect self-rated sleep quality after medication is switched; nevertheless, no patients experienced rebound insomnia after switching from benzodiazepines. Further additional and expanded studies are needed to address this issue.

Switching from benzodiazepines to suvorexant or eszopiclone tended to improve secondary outcomes such as depression severity and anxiety in MDD patients, as judged by the reduction in total BDI-II and GAD-7 scores from baseline. These results are consistent with previous clinical

studies (61,74,75), and are supported theoretically by several preclinical studies. In mice, the facilitation of orexin neurotransmission is closely related to the development of depression and panic anxiety, suggesting that orexin1/2 receptor antagonists have antidepressant and anxiolytic effects (76-78). In addition, eszopiclone has a greater affinity for α2 and α3 subunits of the $GABA_A$ receptor than benzodiazepines; these subunits mediate the anxiolytic and antidepressant effects of ligands that act on the $GABA_A$ receptor (79,80). During the study period, no patients experienced a new onset of suicidal ideation, which is an infrequent adverse event seen in depressed patients after suvorexant treatment (36,52). Taken together, the present findings support the benefit of suvorexant or eszopiclone as an alternative to benzodiazepine treatment for MDD patients with insomnia.

Besides complaints of insomnia, MDD patients often suffer from cognitive impairments such as reduced concentration and memory difficulties (81). In this study, treatment with suvorexant or eszopiclone failed to affect the DSST and DST total scores in patients with MDD, but encouragingly no patients experienced further cognitive impairment or had increased next-day residual effects after switching from benzodiazepines. Furthermore, although the incidence of adverse effects seen in the suvorexant or eszopiclone groups was greater than that observed in previous studies (61,63,72,82), switching from benzodiazepines to suvorexant or eszopiclone did not increase the incidence of adverse effects. An unpleasant taste, which is the most frequently reported adverse effect of eszopiclone (62), was observed in four patients after switching from benzodiazepines to eszopiclone. However, the incidence of this adverse effect was comparable to that observed in previous studies (82,83). Overall, our results suggest that switching from benzodiazepines to suvorexant or eszopiclone to treat insomnia in MDD patients poses no significant clinical risk or inconvenience for patients.

More than 50% of MDD patients have a comorbid anxiety disorder (84,85). A previous

prospective clinical study showed that suvorexant improves the quality of sleep in psychiatric patients with depression and moderate anxiety symptoms (74). Considering this finding, I classified patients with MDD according to the presence or absence of moderate anxiety, as judged by the GAD-7 total score, and re-analyzed the effect of suvorexant and eszopiclone on insomnia severity. Although a significant difference was not observed, suvorexant is likely to be more effective in improving the insomnia severity in MDD patients who have moderate anxiety than in MDD patients who do not have moderate anxiety. By contrast, eszopiclone was more effective in improving insomnia severity in patients who did not have moderate anxiety than in patients who had moderate anxiety. Thus, the presence or absence of anxiety symptoms may be a useful criterion for selecting patients likely to benefit from switching from benzodiazepines to suvorexant or eszopiclone, although this needs to be further investigated in additional studies.

In conclusion, this study suggests that suvorexant or eszopiclone are beneficial alternatives to benzodiazepines for treating residual insomnia in MDD patients. Switching to suvorexant or eszopiclone is unlikely to increase the risk of adverse events or worsen rebound insomnia. Thus, the present findings will support reconsideration of the management of insomnia in MDD patients, and more specifically, indicate that comorbid insomnia could be treated with suvorexant or eszopiclone.

LIMITATION

The primary limitation of this study was the small sample size in each treatment group. Furthermore, missing data values were assigned using LOCF in the small sample size. This may lower the reliability of the findings. In addition, the study included patients receiving different medications such as benzodiazepines and antidepressants. Regarding the baseline clinical characteristics of study participants, the BDI-II score for depression severity was higher in the

eszopiclone group than in the suvorexant group, although this difference was not significant. These differences in baseline clinical characteristics cannot be ruled out as a possible confounding factor. A second limitation of this assessment was the use of patient self-administered questionnaires, rather than the objective measurement of sleep parameters and activity using polysomnography. This limitation is problematic and may result in the effect of the treatment being overestimated. The open-label design of this study is also a limitation, since the possibility of introducing bias by patients or investigators during assessment cannot be excluded.

Summary

In this study, based on a previous retrospective study suggesting less potent hypnotic efficacy of benzodiazepines in patients with MDD, I investigated 1) the brain mechanism of insomnia under depression, 2) the mechanism of decreased efficacy of benzodiazepines under depression, and 3) the efficacy of suvorexant and eszopiclone as alternatives to benzodiazepines for treating insomnia in patients with MDD. I made the following findings.

In Chapter 1, I demonstrated the hyperactivity of orexinergic neurons in the LH (arousal regulatory systems) and decreased GABA level in the VLPO (sleep regulatory systems) in the CSDS mouse model of depression. These perturbed balance between arousal and sleep regulatory systems may be responsible for the sleep disturbance and the decreased hypnotic potency of benzodiazepine brotizolam, but not of the orexin receptor antagonist suvorexant, in CSDS mice.

In Chapter 2, I found that suvorexant or eszopiclone are beneficial alternatives to benzodiazepines in MDD patients who have residual insomnia despite receiving treatment with benzodiazepines. Furthermore, switching to suvorexant or eszopiclone is unlikely to increase the risk of adverse events or worsen rebound insomnia.

Overall, this study provides new insights into the mechanisms underlying the sleep disturbance and reduced hypnotic potency of benzodiazepines in a mouse model of depression; i.e., the hyperactivity of orexinergic neurons and decreased GABA level. Furthermore, present prospective study on MDD patients who have benzodiazepine-resistant insomnia showed the utility of suvorexant or eszopiclone as alternatives to benzodiazepines. Thus, the present findings will support reconsideration of the management of insomnia in MDD patients using benzodiazepines, and more specifically, indicate that comorbid insomnia could be treated with suvorexant or eszopiclone.

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List of Publications

1. Decreased inhibitory effect of brotizolam on hyperactivated orexinergic neurons in the lateral hypothalamus is responsible for its reduces hypnotic potency in a mouse chronic social defeat stress model of depression.

Masaru Miyayama, Yuumi Shimizu, Yuki Shigetsura, Koki Tsuji, Madoka Koyanagi, Nozomi Asaoka, Takashi Ogihara, Naoya Nishitani, Atsushi Yonezawa, Tomohiro Omura, Shunsaku Nakagawa, Shuji Kaneko, Tomohiro Terada, Takayuki Nakagawa, Satoshi Imai, Kazuo Matsubara

Under submission

2. Assessment of suvorexant and eszopiclone as alternatives to benzodiazepines for treating insomnia in patients with major depressive disorder.

Yuki Shigetsura, Satoshi Imai, Hiroki Endo, Yuumi Shimizu, Keita Ueda, Toshiya Murai, Kotaro Itohara, Shunsaku Nakagawa, Atsushi Yonezawa, Yasuaki Ikemi, Noriaki Kitada, Tomohiro Terada, Takayuki Nakagawa, Kazuo Matsubara

Clinical Neuropharmacology. ***In press.***

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