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D_1/D_2 receptor-targeting L-stepholidine, an active ingredient of the Chinese herb *Stephonia*, induces non-rapid eye movement sleep in mice

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ABSTRACT

L-stepholidine, an active ingredient of the Chinese herb *Stephonia*, is the first compound known to have mixed dopamine D_1 receptor agonist/D_2 antagonist properties and to be a potential treatment medication for schizophrenia. In schizophrenic patients insomnia is a common symptom and could be partly related to the presumed over-activity of the dopaminergic system. To elucidate whether stepholidine modulates sleep behaviors, we observed its effects on sleep–wake profiles in mice. The results showed that stepholidine administered i.p. at doses of 20, 40 or 80 mg/kg significantly shortened the sleep latency to non-rapid eye movement (non-REM, NREM) sleep, increased the amount of NREM sleep, and prolonged the duration of NREM sleep episodes, with a concomitant reduction in the amount of wakefulness. Stepholidine at doses of 40 and 80 mg/kg increased the number of state transitions from wakefulness to NREM sleep and subsequently from NREM sleep to wakefulness. However, stepholidine had no effect on either the amount of REM sleep or electroencephalogram power density of either NREM or REM sleep. Immunohistochemistry study showed that stepholidine dose-dependently increased c-Fos expression in neurons of the ventrolateral preoptic area, a sleep center in the anterior hypothalamus, as compared with the vehicle control. These results indicate that stepholidine initiates and maintains NREM sleep with activation of the sleep center in mice, suggesting its potential application for the treatment of insomnia.

Dopamine is critically involved in sleep–wake regulation (Dzirasa et al., 2006; Lu et al., 2006), based on the findings from basic to clinical research. Dopaminergic neurons in the ventral tegmental area fire tonically during quiet wakefulness and non-rapid eye movement (non-REM, NREM) sleep, and show bursts during appetitive waking behavior and REM sleep (Dahan et al., 2007). Arousal behaviors are associated with increased forebrain dopamine secretion and can be enhanced by blockade of dopamine reuptake. Extracellular dopamine levels are lower during the light period, when rats typically sleep, than during the dark period, in the medial prefrontal cortex and the nucleus accumbens (Feenstra et al., 2000; Lena et al., 2005). Anatomical localization of wake-active dopaminergic neurons revealed that 50% of these neurons in the ventral periaqueductal gray matter expressed c-Fos protein during wakefulness, whereas none expressed c-Fos during sleep (Lu et al., 2006). The dopamine D_1 and D_2 receptors (R) are the most widely and abundantly expressed receptors for dopamine in the brain (Kobayashi et al., 2004). Activation of postsynaptic D_1R or D_2R promotes a waking state that largely resembles normal spontaneous waking (Isaac and Berridge, 2003), whereas their blockade induces sleep (Monti and Monti, 2007). In humans, altered central dopaminergic synaptic transmission has been implicated in Parkinson's disease, schizophrenia, and attention deficit hyperactivity disorder (Lewis and Gonzalez-Burgos, 2006; Mazei-Robison et al., 2005; Schultz, 2007). Patients with these diseases exhibit dramatic sleep disturbances and altered sleep architecture (Abbott, 2005; Keshavan et al., 2004; Krystal et al., 2008). These findings suggest that the dopaminergic system containing D_1R/D_2R plays a role in regulating the sleep–wake cycle.

L-stepholidine (SPD) was isolated from the Chinese herb *Stephania* and characterized to possess dual properties toward dopamine receptors, acting as a partial D_1R agonist and as a full D_2R antagonist (Dong et al., 1997; Jin et al., 2002). Clinical trials have indicated that SPD is effective in the treatment of both positive and negative syndromes in schizophrenia (Cai, 1988). The pathogenesis of schizophrenia is suggested to involve dysfunction of dopamine D_1R in the medial prefrontal cortex, which is accompanied by secondary dopamine D_2R hyperactivity in subcortical regions such as the ventral tegmental area and the nucleus accumbens. D_1R dysfunction is suggested to be responsible for the negative symptoms of schizophrenia whereas the D_2R hyperactivity might result in the positive symptoms of this disorder (Davis et al., 1991; Okubo et al., 1997). Above that, Schizophrenic patients experience disturbed sleep, including long sleep-onset latencies, poor sleep efficiency, NREM deficits, and short REM latencies (Benson, 2006; Monti and Monti, 2005). If SPD would

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improve the disturbed sleep of schizophrenic patients, it would be helpful for providing a positive clinical outcome in the treatment of schizophrenia. Although we tested the sleep induction of SPD at one dose in 4 mice (Qiu et al., 2008), the effects of SPD on the sleep–wake cycle and mechanisms remain to be elucidated.

In this study, we administered SPD to mice by giving them an i.p. bolus injection during their wakefulness period and found that SPD shortened sleep latency and increased NREM sleep, but did not alter the sleep electroencephalogram (EEG) power density. These effects differed from those of diazepam, a common benzodiazepine derivative drug for insomnia (Tobler et al., 2001). Immunostaining showed that SPD increased c-Fos expression in neurons of the ventrolateral preoptic area (VLPO), a sleep center, in the hypothalamus. These findings suggest the potential application of SPD for the treatment of insomnia.

1. Methods and materials

1.1. Animals

Male inbred C57BL/6J mice, weighing 22–26 g (11–13 weeks old), were obtained from the Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). The animals were housed individually under ambient temperature of 22 ± 0.5 °C with a relative humidity of 60 ± 2% and an automatically controlled 12-h light/12-h dark cycle (lights on at 07:00, illumination intensity ≈ 100 lux), and they had free access to food and water. Experimental protocols were approved by the Medical Experimental Animal Administrative Committee of Shanghai. Every effort was made to minimize the number of animals used and any pain and discomfort experienced by the subjects.

1.2. Chemicals

SPD powder (purity > 99.5%) was obtained from Shanghai Institute of Materia Medica, Chinese Academy of Science (Jin et al., 2002), and dissolved in 0.1 mM H_2SO_4 (10 mg/150 μl) to be sulfated. The solution was adjusted to pH 5 with 0.1 mM NaOH and brought up with saline to the final desired concentration (Mo et al., 2005; Wang et al., 2007). Diazepam was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). Rabbit polyclonal anti-c-Fos antibody was purchased from Abcam (Cambridge, MA). Biotinylated donkey anti-rabbit IgG and avidin–biotin–peroxidase came from Vector Laboratories (CA); and 3, 3′-diamino-benzidine-tetrahydrochloride (DAB) from Sigma (Saint Louis, MO).

1.3. Polygraphic recordings and vigilance state analysis

Under pentobarbital anesthesia (50 mg/kg, i.p.), mice were chronically implanted with electrodes for polysomnographic recordings of EEG and electromyogram (EMG). Two stainless steel screws (1 mm in diameter) were inserted through the skull into the cortex (antero-posterior, + 1.0 mm; left–right, − 1.5 mm from bregma or lambda) according to the atlas of Franklin and Paxinos (1997) and served as EEG electrodes. Two insulated stainless steel, Teflon-coated wires were bilaterally placed into both trapezius muscles and served as EMG electrodes. All electrodes were attached to a microconnector and fixed onto the skull with dental cement.

The EEG and EMG recordings were carried out by means of a slip ring designed so that the behavioral movement of the mice would not be restricted. After a 10-day recovery period, the mice were housed individually in transparent barrels and habituated to the recording cable for 3–4 days before polygraphic recording. For the study of spontaneous sleep–wakefulness cycles, each animal was recorded for 24 h beginning at 19:00, the end of the light period. The animals then entered the pharmacological phase of the study, in which sleep–wakefulness parameters were recorded for 48 h. The data collected during the first 24 h also served as baseline comparison data for the second experimental day.

Cortical EEG and EMG signals were amplified, filtered (EEG, 0.5–30 Hz; EMG, 20–200 Hz), digitized at a sampling rate of 128 Hz, and recorded by using SleepSign (Kissei Comtec, Nagano, Japan) as described before (Huang et al., 2001; Qu et al., 2006). When complete, polygraphic recordings were automatically scored offline by 10-s epochs as wake, REM, and NREM sleep by SleepSign according to standard criteria (Huang et al., 2005; Kohtoh et al., 2008; Qu et al., 2008). As a final step, defined sleep–wake stages were examined visually and corrected, if necessary.

1.4. Pharmacological treatments

SPD was prepared as described above immediately before use and administered i.p. at 21:00 on the experimental day at a dose of 20, 40 or 80 mg/kg ($n = 4, 6$, and 4 in each group, respectively). Diazepam dissolved in 5% DMSO and suspended in 0.03% Tween-80, was administered i.p. at a dose of 6 mg/kg ($n = 6$). For baseline data, mice were injected i.p. with vehicle (20 ml/kg). We used separate groups of mice for each dose.

1.5. c-Fos immunohistochemistry

Four groups of mice were used. One group was treated with vehicle; and the others were injected i.p. with SPD at doses of 20, 40, and 80 mg/kg, respectively. At 90 min after the SPD administration, the animals were anesthetized with 10% chloral hydrate and perfused via the heart with saline solution followed by ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Their brains were then removed, post-fixed in 4% PFA for 6 h, and immersed in 30% sucrose overnight. Thereafter, frozen sections were cut at 30 μm in coronal planes by use of a freezing microtome (Jung Histocut, model 820-II, Leica, Germany). The sections were stored in a cryoprotectant solution (Tao et al., 2001; Yin and Sun, 1999) at − 20 °C for histological analysis.

Immunohistochemistry was performed in accordance with the free-floating method described earlier (Qiu et al., 2003). Sections were fixed in 4% PFA for 10 min and incubated with 0.3% H_2O_2 for 15 min to quench the endogenous peroxidase activity. The sections were next placed for 30 min at 37 °C in blocking solution containing 10% normal rabbit serum with 0.3% Triton X-100 in 0.01 M phosphate-buffered saline (PBS, pH 7.2) and then incubated at 4 °C for 24 h with a rabbit polyclonal antibody against c-Fos at a 1:1000 dilution in PBS containing 1% normal rabbit serum and 0.3% Triton X-100. On the second day, the sections were incubated with a 1:200 dilution of biotinylated donkey anti-rabbit secondary antibodies for 30 min followed by a 1:200 dilution of avidin–biotin–peroxidase for 45 min at 37 °C. The peroxidase reaction was visualized with 0.05% DAB in 0.1 M phosphate buffer and 0.01% H_2O_2. Sections were mounted, dehydrated, and cover slipped. As controls, adjacent sections were incubated without the primary antibody to confirm that no non-specific staining had occurred.

The sections were examined under bright-field illumination using a Leica DMLB2 microscope (Germany). Images were captured with a CoolSNAP-Procf digital camera (RT_{KE} DIAGNOSTIC, SPOT instrument).

1.6. Statistical analysis

All results were expressed as means ± SEM ($n = 4$–6). Time-course of the hourly amounts of each stage, histograms of sleep/wake amounts, sleep/wake stage transition number, number and duration of sleep/wake bouts were analyzed by the paired t-test, with each animal serving as its own control. The comparison between drug-treated groups as well as number of c-Fos immunoreactive neuron was analyzed by ANOVA followed by Fisher's probable least-squares difference (PLSD) test. In all cases, $P < 0.05$ was taken as the level of significance.

2. Results

2.1. SPD increased NREM sleep and shortened sleep latency

We injected SPD into mice at 21:00 intraperitoneally at a dose of 20, 40 or 80 mg/kg and examined the sleep–wake pattern of the animals by recording their EEG and EMG. Diazepam was given at 6 mg/kg as a positive control. Fig. 1 shows typical examples of polygraphic recordings and corresponding hypnograms from an individual mouse given SPD at a dose of 80 mg/kg. During the period of 21:00 to 02:00, this mouse spent more time in wakefulness than in other sleep states under the vehicle control (Fig. 1A). When SPD was injected on the experimental day, however, the animal started sleep within several minutes and spent more time in sleep than the controls (Fig. 1B). Similar changes were

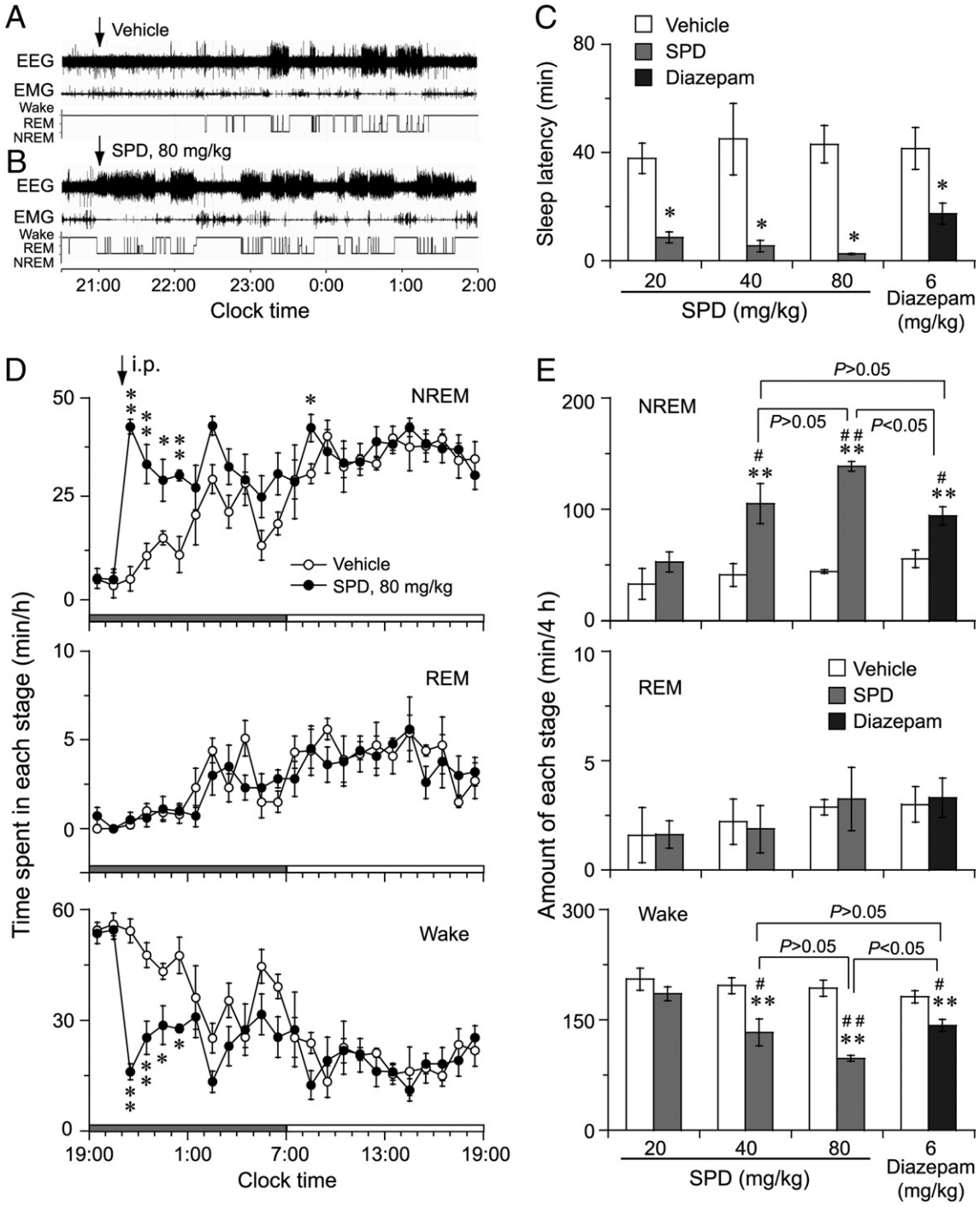

Fig. 1. Sleep-stage distribution produced by an i.p. administration of SPD to mice. Typical examples of polygraphic recordings and corresponding hypnograms in a mouse treated with vehicle (A) or SPD at a dose of 80 mg/kg (B). (C) Effect of SPD and diazepam on NREM sleep latency. Open and filled bars show the profiles for the respective baseline day (vehicle injection) and experimental day (SPD and diazepam injection). *, $P<0.05$, two-tailed paired t-test. (D) Time-course changes produced by the i.p. administration of SPD at 80 mg/kg. Each circle represents the hourly mean ± SEM of NREM, REM sleep, and wakefulness. Open and filled circles indicate the baseline and experimental day profiles, respectively. SPD was given at 21:00. The horizontal filled and open bars on the χ-axes indicate the 12-h dark and 12-h light periods, respectively. *, $P<0.05$, **, $P<0.01$, compared with vehicle control as assessed by two-tailed paired t-test. (E) Total time spent in NREM and REM sleep and wakefulness for 4 h after the SPD and diazepam administration. Open and filled bars show the profiles for the respective baseline day (vehicle injection) and experimental day (SPD and diazepam injection). Values are means ± SEM ($n=4$–6). *, $P<0.05$, **, $P<0.01$, compared with vehicle control as assessed by two-tailed paired t-test. #, $P<0.05$, ##, $P<0.01$, compared with SPD at 20 mg/kg as assessed by ANOVA followed by Fisher's PLSD test.

observed with the lower concentrations (20 and 40 mg/kg) of SPD (data not shown).

As shown in Fig. 1C, the i.p. injection of SPD shortened remarkably the NREM sleep latency, which is defined as the time from the saline or SPD injection to the appearance of the first NREM sleep episode lasting for at least 20 s. The latency to NREM sleep in mice treated with SPD (20, 40, and 80 mg/kg) and diazepam (6 mg/kg) was 8.6, 5.4, 2.5, and 17 min, which were markedly and significantly shorter than the latency of 38, 45, 43, and 42 min after the vehicle injection ($P<0.05$, paired t-test). The short sleep latency of the SPD-injected mice clearly indicates that SPD accelerated the initiation of NREM sleep.

Figs. 1D and E summarize time-courses of the hourly amounts of NREM and REM sleep and wakefulness at 80 mg/kg and their cumulative amounts for 4 h after the SPD injection, respectively. As compared with the vehicle control, SPD at 80 mg/kg markedly increased the amount of NREM sleep, commencing the first hour after the SPD injection. This augmentation in sleep time was accompanied by a reduction in wakefulness. The increase in NREM sleep and decrease in wakefulness lasted more than 4 h after the injection. There was no further disruption of the sleep architecture during the subsequent period (Fig. 1D). Similar time-course profiles were observed with lower doses of 20 and 40 mg/kg, but the effect on sleep was small and lasted only about 1–2 h after the injection (data not shown).

We calculated the total time spent in NREM and REM sleep and wakefulness for 4 h after SPD and diazepam injection (Fig. 1E). SPD given at 40 and 80 mg/kg significantly increased the total amounts of NREM sleep by 156% and 214%, and decreased the total amount of wakefulness by 32.3% and 49.2% ($P<0.01$, paired t-test), respectively, during that 4-h period compared with the vehicle control. However, SPD at 20 mg/kg did not affect the cumulative amount of NREM sleep or that of wakefulness for 4 h after injection. There was no essentially significant difference in REM sleep before and after the administration of SPD at these 3 doses ($P>0.05$, paired t-test). On the other hand, diazepam at 6 mg/kg increased the total amount of NREM sleep by 69.6%, and decreased that of wakefulness by 21.5%, respectively, during that 4 h period compared with the vehicle control ($P<0.01$, paired t-test). Although there was no significant difference in REM sleep for 4 h after injection between diazepam and its vehicle control, diazepam did reduce REM sleep from 1.4 min to 0.6 min in the first 2 h. ANOVA analysis revealed that SPD increased NREM sleep ($F_{3,19}=6.052$, $P<0.01$) and the effect of SPD (80 mg/kg) was stronger than that of SPD at 20 mg/kg and diazepam at 6 mg/kg ($P<0.05$, PLSD) but not significantly different from that of SPD at 40 mg/kg ($P>0.05$, PLSD). These results clearly indicate that SPD increased NREM sleep.

2.2. Effects of SPD on characteristics of sleep–wake episodes

To better understand the sleep–wake profile caused by SPD, we determined the NREM sleep and wake bout distribution as a function of bout or episode duration (Fig. 2). Compared with its own control after the vehicle injection, SPD at 20 mg/kg had no effect on NREM sleep or duration of wake bouts in dark and subsequent light periods; whereas SPD at 40 and 80 mg/kg increased the number of NREM sleep bouts with duration ranges of 60–120, 240–480, 480–960, and 960–1920 s (Fig. 2A). At these 2 dosages, SPD increased the number of wake bouts with duration ranges of 10–30 and 30–60 s, whereas it decreased that in the range of 1920–3840 s (Fig. 2B). Similar to the results of SPD 80 mg/kg, compared with vehicle control, diazepam increased the number of NREM sleep bouts with duration ranges of 30–60, 60–120, and 120–240 s, and that of wake bouts with duration ranges of 10–30, 30–60, 60–120, 120–240 and 240–480 s, whereas it decreased that in the range of 480–960 s (Fig. 2). In addition, as shown in Figs. 3A and B, SPD also changed the total number and mean duration of NREM and wake episodes. SPD (40 and 80 mg/kg) and diazepam (6 mg/kg) increased the number of NREM bouts by 1.9-, 2.2- and 1.8-fold, and those of wake episodes by 2.0-, 2.3-, and 1.8-fold, respectively; but the mean duration of NREM and REM sleep was not altered during a 4-h period after the injection. Meanwhile the mean duration of wake episodes was significantly decreased by 78% after the

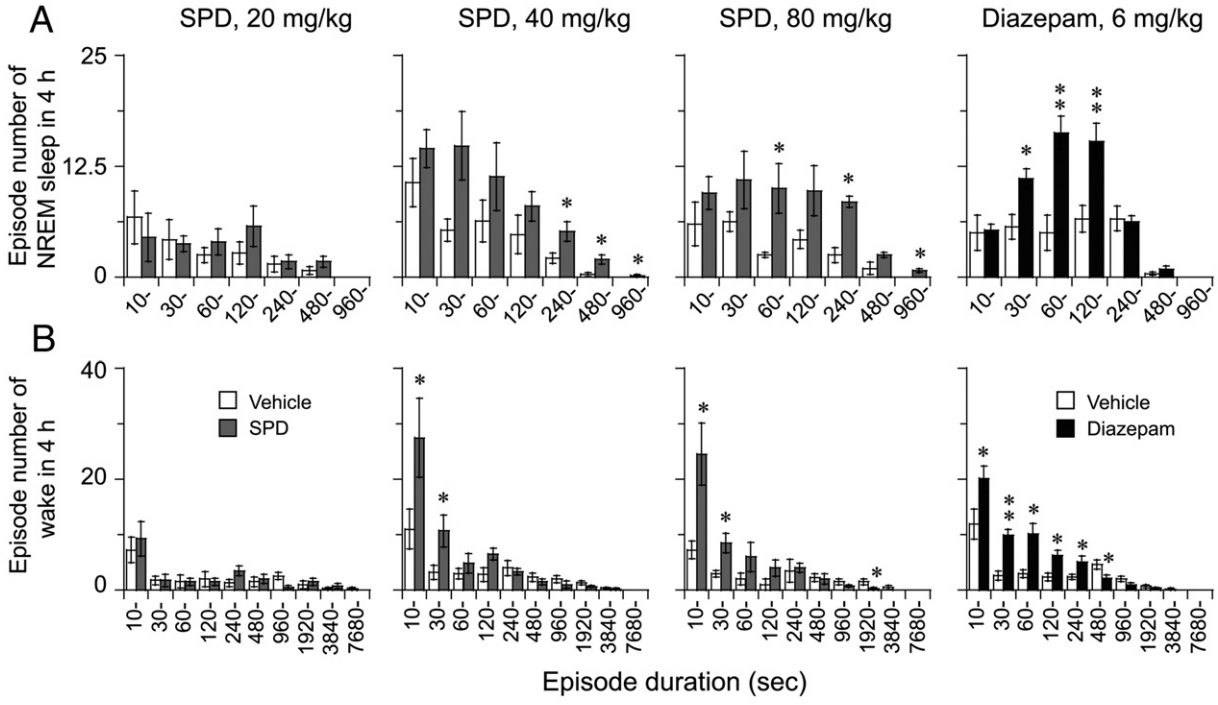

Fig. 2. Changes in number of NREM (A) and wake (B) bouts at different ranges of episode duration in 4 h after the administration of diazepam (6 mg/kg) and SPD at 20, 40 or 80 mg/kg. Open and filled bars show the profiles for the respective baseline day (vehicle) and experimental day (SPD or diazepam). Values are means ± SEM ($n=4$–6). *, $P<0.05$, **, $P<0.01$, compared with vehicle control as assessed by two-tailed paired t-test.

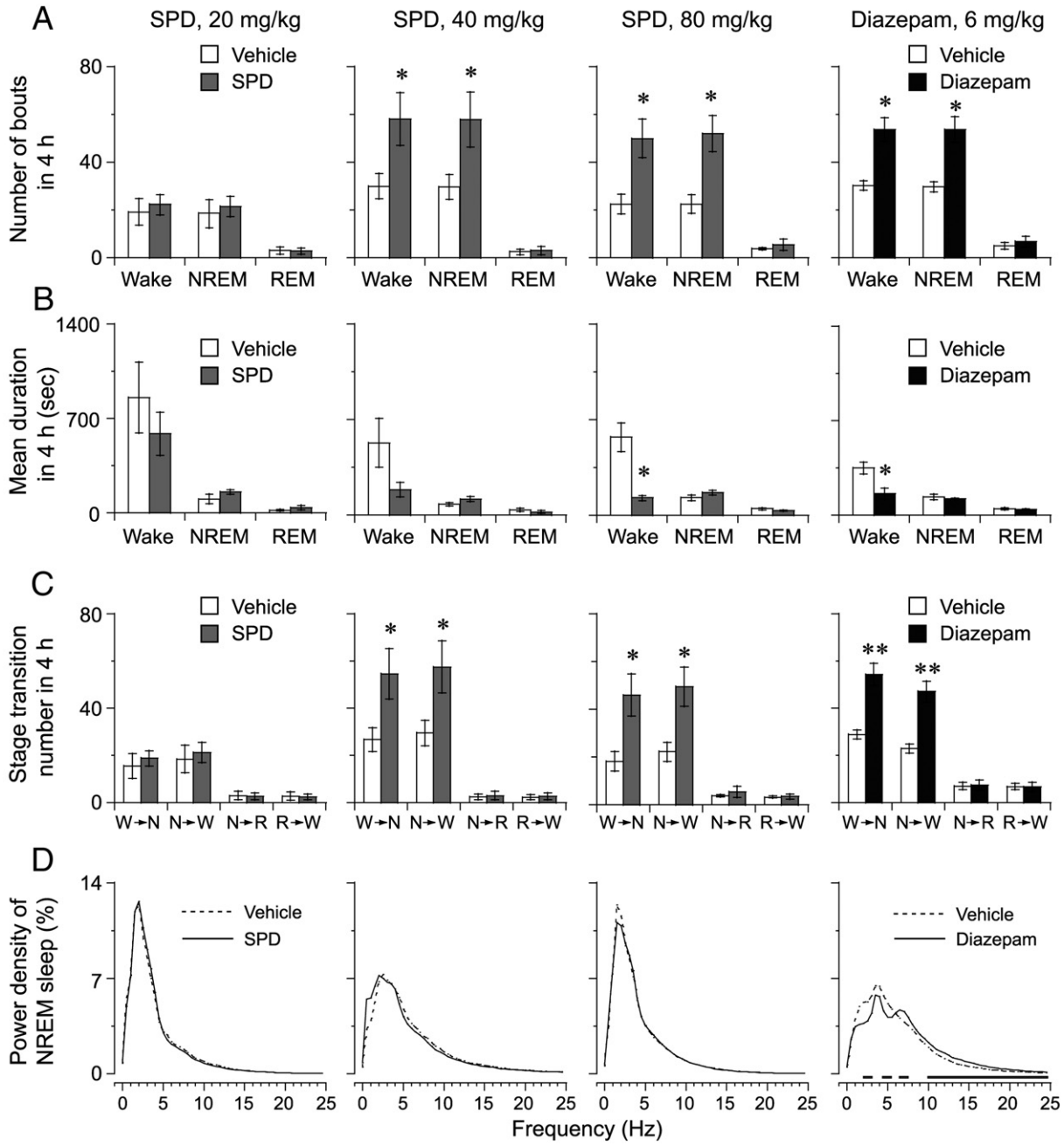

Fig. 3. Characteristics of sleep–wake episodes produced by the administration of diazepam (6 mg/kg) and SPD at 20, 40 or 80 mg/kg. Total number (A) and mean duration (B) of wake, NREM, and REM bouts in a 4-h period after the administration of SPD and diazepam. Open and filled bars show the profiles for the respective baseline day (vehicle) and experimental day (SPD or diazepam). (C) Sleep–wake state transitions for 4 h after the administration of SPD or diazepam. Open and filled bars show the profiles for the respective baseline day (vehicle) and experimental day (SPD or diazepam). W, N, and R represent the stage for wakefulness, NREM, and REM sleep, respectively. Values are means ± SEM ($n = 4$–6). *, $P < 0.05$, **, $P < 0.01$, compared with vehicle control as assessed by two-tailed paired t-test. (D) EEG power density of NREM sleep after the administration of SPD or diazepam. The horizontal bars indicate where there is a statistical difference ($P < 0.05$, paired t-test) between diazepam and vehicle control. There was no essential difference in EEG power density during NREM sleep between SPD treatment and the vehicle control ($P > 0.05$, paired t-test).

SPD injection of 80 mg/kg, and by 54% after diazepam treatment. On the other hand, the administration of SPD at 20 mg/kg did not affect either the bout number or mean duration of NREM sleep or wakefulness (Figs. 3A and B). During the subsequent period, there were no changes in episode counts or mean duration between SPD treatment and the control (data not shown).

As a result, SPD at 40 mg and 80 mg/kg and diazepam at 6 mg/kg increased the number of state transitions from wakefulness to NREM sleep and from NREM sleep to wakefulness (Fig. 3C). Neither a change in the number of transitions from NREM to REM nor in that from REM to wakefulness was found.

We then determined the EEG power spectra during NREM sleep of the mice. The power of each 0.5 Hz bin was first averaged across the sleep stages individually and then normalized as a group by calculating the percentage of each bin from the total power (0–24.5 Hz) of the individual animal. As shown in Fig. 3D, there was no significant difference in EEG power density of NREM sleep between the SPD treatment and the vehicle control. However, diazepam (6 mg/kg) decreased EEG power density of NREM sleep in the frequency range of 2–3.5 Hz and 4.5–5.5 Hz, but increased that in the frequency range of 6.5–7.5 Hz and 9.5–24.5 Hz. These findings indicated that SPD did not affect the EEG power density of NREM sleep.

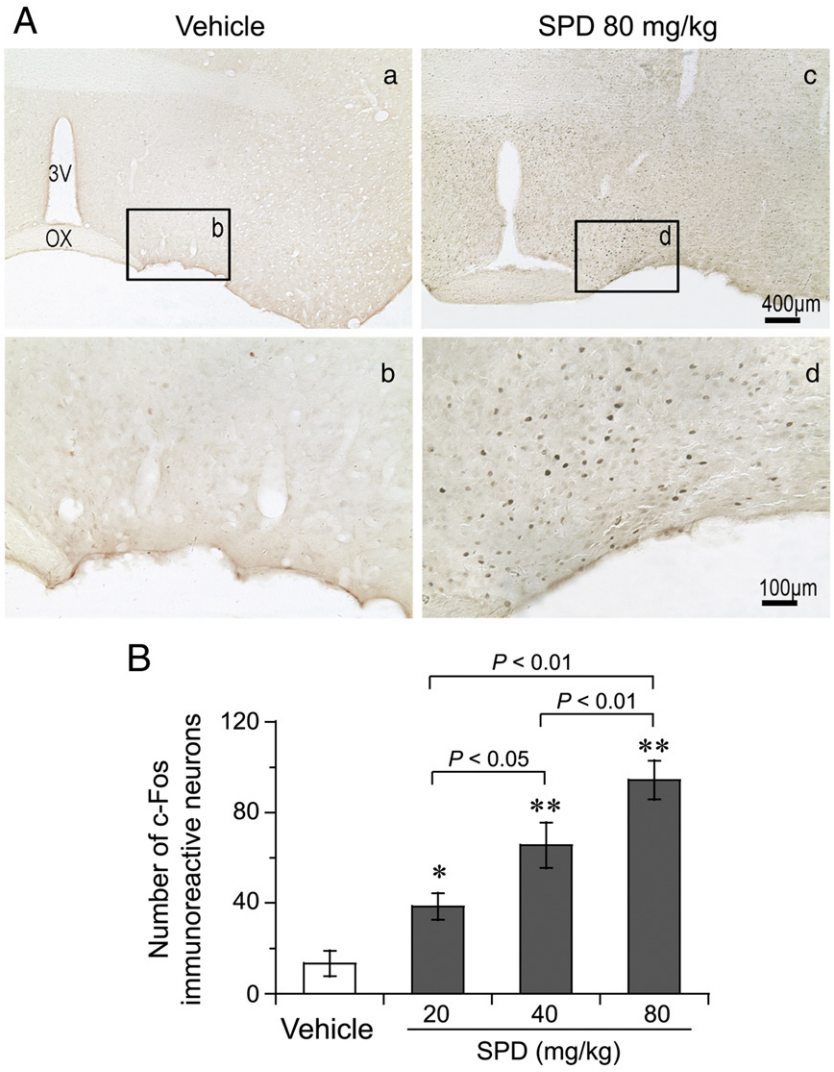

Fig. 4. Effect of SPD on c-Fos-immunoreactivity in the VLPO. (A) Representative photomicrographs of c-Fos immunostaining in the VLPO of vehicle- (a and b) and 80 mg/kg SPD- (c and d) administered mice (b, d: high-magnification views of the rectangular areas marked in "a" and "c," respectively). Scale bars: a and c, 400 μm; b and d, 100 μm. (B) The Number of c-Fos-immunoreactive neurons in VLPO after the SPD treatment. Values are means ± SEM ($n = 4–6$). *, $P<0.05$, **, $P<0.01$ by ANOVA followed by Fisher's PLSD test.

2.3. SPD increased c-Fos expression in the VLPO

To study the effect of SPD on the VLPO sleep center, we accounted the number of c-Fos-immunoreactive neurons in the VLPO. Fig. 4A shows representative photomicrographs of the VLPO of mice treated with vehicle or SPD 80 mg/kg and then immunostained for c-Fos. Analysis for the number of c-Fos-immunoreactive nuclei showed that SPD at 20, 40, and 80 mg/kg increased the expression of c-Fos in the VLPO by 1.9-, 3.9- and 6.1-fold, respectively, as compared with the vehicle control (Fig. 4B). These findings indicate that SPD activated the VLPO sleep center and increased NREM sleep.

3. Discussion

The present study clearly showed that SPD shortened NREM sleep latency and increased NREM sleep. Since SPD enhanced the expression of c-Fos-immunoreactivity in the VLPO but did not change the EEG power density of NREM sleep, SPD may be considered to induce sleep very similar to physiological sleep, suggesting its potential use for the treatment of insomnia.

The dopaminergic system containing D_1R/D_2R is proposed to play important roles in regulating the sleep–wake cycle (Dzirasa et al., 2006). Activation of postsynaptic dopamine D_1R or D_2R or blockade of presynaptic D_2R reduces sleep and increases wakefulness. Opposite effects were observed after blockade of the postsynaptic receptors or activation of the D_2 autoreceptors (Isaac and Berridge, 2003; Python et al., 1996). SPD, belonging to the tetrahydroprotoberberines, has dual actions, being a partial D_1R agonist and a D_2R antagonist (Jin et al., 2002). In this study, we found that SPD promoted NREM sleep, suggesting that blockade of D_2R plays a major role in the hypnotic action of SPD.

D_1R is a postsynaptic receptor coupled to adenylate cyclase, and its stimulation facilitates the activity of the enzyme. D_2R is the predominant D_2-like subtype in the brain and has been characterized also on neuron cell bodies and dendrites in the substantia nigra pars compacta and the ventral tegmental area, where it serves an autoreceptor function. D_2R mRNA is also present in the cerebral cortex. The areas of highest expression of the D_1R and D_2R in the rat brain include the striatum (caudate-putamen), the nucleus accumbens, and the olfactory tubercle (Monti and Monti, 2007). By examining c-Fos expression as a marker of neural activation, Mo et al. (2005) and Guo et al. (1998) found that SPD increases c-Fos expression in the forebrain areas, such as the medial prefrontal cortex, the nucleus accumbens, and the lateral septal nucleus, areas enriched in both D_1Rs and D_2Rs. In the present study, we found that SPD significantly enhanced the c-Fos-immunoreactivity in the VLPO, although the location of the SPD-targeting dopamine receptors is unclear. The VLPO contains one essential population of sleep-promoting

neurons, which are more active during sleep as indicated by the expression of c-Fos (Gong et al., 2000; Sherin et al., 1996) and has been demonstrated to play a critical role in the promotion of NREM sleep (Lu et al., 2000; Saper et al., 2005). The VLPO cluster more heavily innervates the histaminergic neurons (Sherin et al., 1998), and these histaminergic ones are closely linked to the transition between arousal and NREM sleep (Huang et al., 2006, 2007). SPD increased c-Fos protein expression in the VLPO, indicating that the hypnotic effects of SPD may be mediated by activation of the sleep center VLPO.

Here we provided data showing that SPD increased the amount of NREM sleep, but did not alter the EEG power density of NREM sleep. It is well known that delta activity is an indicator of the depth of NREM sleep (Faulhaber et al., 1997; Lancel, 1997; Neckelmann and Ursin, 1993). Nitrazepam, widely and commonly used to treat insomnia in humans, significantly decreases the total waking time and increases the total NREM sleep time but causes a remarkable decrease in the delta activity during NREM sleep (Tokunaga et al., 2007). This phenomenon has been also reported for other benzodiazepines, such as triazolam, flunitrazepam, and midazolam (Kopp et al., 2003, 2004; Lancel et al., 1996; Shinomiya et al., 2005; Tan et al., 1998; Tobler et al., 2001). The discrepancy between the increase in sleep continuity, the reduction of power in the lower EEG frequencies, caused by benzodiazepines in humans is consistent with the findings in mice (Tobler et al., 2001). Differing from those benzodiazepines, SPD induced NREM sleep very similar to physiological sleep. Activation of sleep–active neurons in the VLPO by the SPD administration further supports our conclusion.

Schizophrenic patients always have difficulty in initiating sleep and sleep less during the night, subsequently exhibiting a tendency to sleep more during the day. Our findings showed that SPD significantly shortened the onset time to enter the NREM sleep stage. Earlier pharmacokinetics studies revealed that SPD could be easily absorbed and quickly penetrate into the brain (Zhang et al., 1990). Compared with diazepam (Fig. 1B), other nitrazepam and histamine H_1-antagonists, such as diphenhydramine, chlorpheniramine, and cyproheptadine (Saitou et al., 1999; Tokunaga et al., 2007), SPD exhibited the most potent effects in shortening the sleep latency to NREM sleep. Therefore, it is reasonable to presume that SPD would be an effective sleep inducer for the treatment of insomnia, especially helping with difficulty in falling asleep.

In conclusion, SPD activated the VLPO neurons, increased the amount of NREM sleep, and shortened sleep latency in mice. Unlike often-used sleep pills such as benzodiazepines, SPD did not alter the EEG power density, indicating that SPD induces NREM sleep similar to that seen in physiological sleep and may be potentially used for the treatment of insomnia.

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