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Diltiazem potentiates pentobarbital-induced hypnosis *via* 5-HT_{1A} and 5-$HT_{2A/2C}$ receptors: Role for dorsal raphe nucleus

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ABSTRACT

It has been reported that the sedative component of pentobarbital is mediated by GABA receptors in an endogenous sleep pathway and the ventrolateral preoptic area (VLPO)-tuberomammillary nucleus (TMN) or VLPO-dorsal raphe nucleus (DRN) neural circuit is important in the sedative response to pentobarbital. Our previous findings indicated that the VLPO-TMN neuronal circuit may play crucial part in the augmentative effect of diltiazem on pentobarbital sleep and the serotonergic system may be involved. This study was designed to investigate the role of DRN and the serotonergic receptors 5-HT_{1A} and 5-$HT_{2A/2C}$ in the augmentative effect of diltiazem on pentobarbital-induced hypnosis in rats. The results showed that diltiazem (5 mg/kg, i.g.) significantly reversed pentobarbital-induced (35 mg/kg, i.p.) reduction of c-Fos expression in 5-HT neurons of DRNV (at −7.5 mm Bregma), DRND, DRNVL and MRN (at −8.0 mm Bregma). However it did not influence this reducing effect of pentobarbital on non-5-HT neurons either in DRN or in MRN. Moreover, the effect of diltiazem (1 or 2 mg/kg, i.g.) on pentobarbital-induced (35 mg/kg, i.p.) hypnosis was significantly inhibited by 5-HT_{1A} agonist 8-OH-DPAT (0.5 mg/kg, i.p.) and 5-$HT_{2A/2C}$ agonist DOI (0.5 mg/kg, i.p.), and potentiated by 5-HT_{1A} antagonist *p*-MPPI (2 mg/kg, i.p.) and 5-$HT_{2A/2C}$ antagonist ritanserin (2 mg/kg, i.p.), respectively. From these results, it should be presumed that the augmentative effect of diltiazem on pentobarbital-induced sleep may be related to 5-HT_{1A} and 5-$HT_{2A/2C}$ receptors, and DRN may be involved. In addition, it also suggested that the DRN may play a multi-modulating role in sleep–wake regulation rather than being recognized simply as arousal nuclei.

1. Introduction

To date, most of the researches on the actions of GABAergic general anesthetics have been directed at the molecular level (Lin et al., 1993; Tanelian et al., 1993). The barbiturate anesthetic pentobarbital enhances $GABA_A$ receptor function or GABAergic neurotransmission in a variety of systems (Akaike et al., 1990; Collingridge et al., 1984). The connection between the activation of $GABA_A$ receptors and the hypnotic response is still, however, obscure. Moreover, it is not known at which anatomical sites the $GABA_A$ receptors that mediate the hypnotic response are located.

Recent pharmacological studies have also uncovered relationships between receptor-based effects of anesthetics and brain regions known to be involved in sleep (Tung and Mendelson, 2004). A hypothesis regarding sleep generation is that the activation of the GABAergic ventrolateral preoptic nucleus (VLPO) neurons during sleep inhibits the activity of nuclei involved in arousal such as the histaminergic neurons of tuberomammillary nucleus (TMN) or serotonergic neurons of the dorsal raphe nucleus (DRN) (Saper et al., 2001). Such pathways appear to be involved in GABA-based anesthetic action as well (Lu et al., 2008; Nelson et al., 2002). Our previous results of immunohistochemistry analysis also demonstrated that the potentiating effect of L-type Ca^{2+} channel blocker diltiazem on pentobarbital-induced hypnosis may be related to the activation of GABAergic neurons in the VLPO and the simultaneous depression of histaminergic neurons in the TMN (Zhao et al., 2009). However, the role of DRN in the potentiating mechanism of diltiazem on pentobarbital-induced hypnosis has not been investigated yet.

Serotonin has long been implicated in the regulation of sleep–wake states (Jouvet, 1999; Ursin, 2002), although it is still controversial where and how serotonin exerts its principal effect on such regulation. Our previous reports indicated that pentobarbital-induced hypnosis was significantly potentiated by 5-HTP, and attenuated by *p*-chlorophenylalanine (*p*-CPA), a specific, potent and irreversible inhibitor of tryptophan 5-monooxygenase, the rate-limiting enzyme of biosynthesis of 5-HT (Zhao et al., 2004, 2006, 2009). These data indicated that serotonergic system may be involved in the pentobarbital-induced hypnosis. In addition, our findings also suggested that the serotonergic pathway may be involved in the augmentative activity of diltiazem on pentobarbital-induced hypnosis (Zhao et al., 2006, 2009).

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The serotonin receptors can be classified into seven classes, designated 5-HT_{1-7}. Most of the work on this aspect of the serotonin–sleep–waking problem so far has been done on the 5-HT_{1A} and 5-HT_2 receptors (Ursin, 2002). Systemic administration of 5-HT_{1A} agonist 8-OH-DPAT (Bjorvatn et al., 1997; Monti and Jantos, 1992) and 5-$HT_{2A/2C}$ agonist DOI (Dugovic, 1992) increased wakefulness and reduced slow wave sleep (SWS) and rapid eye movement (REM) sleep in freely moving rats. These effects were reversed by each corresponding antagonist, respectively (Dugovic and Wauquier, 1987; Silhol et al., 1992; Sorensen et al., 2001). Based on these findings, here we address the question of whether the augmentative effect of diltiazem on pentobarbital-induced hypnosis involves DRN pathway and also examine the roles of 5-HT_{1A} and 5-$HT_{2A/2C}$ receptors in the augmentative activity of diltiazem on pentobarbital-induced hypnosis using *in vivo* polysomnogram analysis technique.

2. Material and methods

2.1. Animals

Male Sprague–Dawley rats (220–240 g, Grade I, purchased from the Animal Center of Peking University, Beijing) were used. All experiments were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) for the use of experimental animals and approved by the Peking University Committee on Animal Care and Use. The rats were housed in acrylfiber cages individually and had *ad libitum* access to food and water. They were exposed to a 12:12 h light/dark schedule with lights on at 8:00 AM. The ambient temperature averaged 23 ± 1 °C and the relative humidity was 50 ± 10%. The experiments were carried out from 9:00 AM to 3:00 PM in a quiet room maintained the same condition mentioned above.

2.2. Drugs and drug administration

The following drugs were used in this study: pentobarbital, diltiazem, (+)-8-hydroxy-2-(di-*n*-propylamino)tetralin HBr (8-OH-DPAT), 4-(2′-methoxy- phenyl)-1-[2′-(*n*-2″-pyridinyl)-*p*-iodobenzamino-]ethyl-piperazine HCL (*p*-MPPI), R(−)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl (DOI), and 6-[2-[4-[bis(4-fluorophenyl)methylidene] piperidin-1-yl]ethyl]-7-methyl-[1,3]thiazolo[3,2-a]pyrimidin-5-one (ritanserin) (All from Sigma-Aldrich, St. Louis, USA).

Diltiazem was dissolved in distilled water with different concentrations (0.2, 0.4 or 1 mg/ml) for intragastrical administration (i.g.). Pentobarbital (7 mg/ml), 8-OH-DPAT (0.1 mg/ml), *p*-MPPI (0.4 mg/ml) and DOI (0.1 mg/ml) was dissolved in physiological saline for intraperitoneal injection (i.p.) respectively. Ritanserin (0.4 mg/ml) was dissolved in saline with 1% Tween 80 for intraperitoneal injection. All of these drugs were administrated in a constant volume of 5 ml/kg body weight. Diltiazem was administered 90 min prior to pentobarbital administration. 8-OH-DPAT, *p*-MPPI, DOI and ritanserin were respectively injected intraperitoneally to the right abdomen 20 min before pentobarbital administration.

2.3. Surgery

At least 1 week prior to any sleep recordings the animals were instrumented by standard procedures as reported (Shinomiya et al., 2004). Briefly, under chloral hydrate (300 mg/kg) anesthesia, two stainless steel screws attached to insulated wire were implanted in the skull over the frontal-parietal cortex to record the EEG. One was placed approximately 2 mm anterior and 2 mm to the right of Bregma, another was placed approximately 3 mm posterior and 2 mm to the left of Bregma and a ground electrode was placed between the two 3 mm lateral to midline. For the EMG, a pair of wire electrodes was threaded through the nuchal muscles. These electrodes were attached to a miniature connector which was affixed to the skull with dental acrylic.

After the surgery, rats were injected with antibiotics for 3 days and allowed to recover for 7 days prior to the initiation of experiments. For purposes of habituation they were connected to the recording apparatus at least 1 day before the experiments.

2.4. EEG and EMG recordings and analysis

For electrophysiological recording, a lightweight shielded cable was plugged into the connector on the rat's head and attached to a counterbalanced swivel that permitted free movement. All rats were studied in an electrically shielded box and noise-attenuated environment free from interruptions. The signals were routed to an electroencephalograph (Model MP 150, BIOPAC Systems, Goleta, CA, USA). Recordings were performed 5 min before pentobarbital injections. The signals were amplified and filtered (EEG, 0.5–30 Hz; EMG, 16–128 Hz), then digitized at a sampling rate of 128 Hz and recorded using AcqKnowledge software (BIOPAC Systems). Sleep stage was analyzed since pentobarbital injection till the rat was first awaken. The EEG/EMG recordings were analyzed in 4 s epochs by standard criteria embedded in SleepSign v.2.0 (BIOPAC Systems) and sleep–wake states were automatically classified wakefulness, REM sleep, light sleep (LS) and SWS. As a final step, the defined sleep–wake stages were examined and corrected according to the visual observation of the animal which was recorded by video camera.

Each epoch was assigned to one of the following categories: wakefulness (low-amplitude EEG activity, high-voltage EMG activity), REM sleep [Fast fourier transform (FFT) theta ratio of EEG ≥ 50%, desynchronized EEG, absence of tonic EMG, occasional body twitches while maintaining a recumbent sleep posture], SWS (FFT delta ratio of EEG ≥ 70%, large-amplitude, synchronous EEG with sleep spindles present, greatly diminished tonic EMG, eyes closed, small eye movement potentials present recumbent posture, usually lying on the animal's side or curled up with head down), LS (FFT delta ratio of EEG < 70%, high-amplitude slow or spindle EEG activity, low-amplitude EMG activity). Non rapid eye movement (NREM) sleep time equals SWS time plus LS time. Total sleep (TS) time equals NREM sleep time plus REM sleep time.

2.5. Perfusion and fixation

Animals were perfused through the ascending aorta with 0.9% NaCl solution 45 min after administration of pentobarbital (35 mg/kg, i.p.), followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Whole brains were immediately removed and post-fixed in the same fixative at 4 °C for 24 h, and then immersed in 30% sucrose in phosphate-buffered saline (PBS, 0.01 M, pH 7.4) at 4 °C for cryoprotection. Prior to sectioning of tissue, brains were blocked at approximately −6.0 mm with reference to Bregma, rapidly frozen in liquid *n*-hexane cooled by the mixture of solid carbon dioxide and ethanol, and stored at −80 °C.

2.6. Immunohistochemistry

Six alternate sets of 20 μm thick brain sections were prepared using a cryostat (Leica CM1850, Leica Microsystems UK Ltd., Milton Keynes, UK). One set of sections (i.e. every 6th section) was used for double-immunostaining for tryptophan hydroxylase (TrpOH) and c-Fos. Staining for TrpOH was performed as follows: sections were washed in 0.01 M PBS then incubated in 3% H_2O_2 followed by washing in 0.01 M PBS (3 × 2 min). Sections were immersed in PBS containing 0.3% Triton X-100 (PBST) for 20 min, followed by incubation with an affinity-purified sheep anti-rabbit TrpOH polyclonal antibody (Cat. No. 9260–2505, Biogenesis, Ltd., Poole, UK, generated using recombinant rabbit TrpOH as antigen, isolated as inclusion bodies from *E. coli* and purified by preparative SDS-PAGE) diluted 1:5000 in PBST at 4 °C for 16 h. Sections were washed using 0.01 M PBS (3 × 2 min), then incubated with Polymer Helper (reagent 1, Polink-2 plus® Polymer Horseradish

peroxidase Detection System For Goat Primary Antibody, Cat. No. PV-9003, GBI, USA) for 20 min. Sections were washed again using 0.01 M PBS (3×2 min) then incubated with poly-HRP anti-Goat IgG (reagent 2, Polink-2 plus® Polymer Horseradish peroxidase Detection System For Goat Primary Antibody, Cat. No. PV-9003, GBI, USA) for 40 min. After washing with 0.01 M PBS (3×2 min), sections were incubated with diaminobenzidine (Cat. No. ZLI-9017, Zhongshan, Ltd., Beijing, China) until brown reaction product was achieved.

Prior to immunohistochemical staining of the same sections for c-Fos, sections were rinsed in 0.01 M PBS (3×5 min). Sections were incubated with rabbit polyclonal anti-c-Fos (Ab-5) antiserum (Cat. No. PC38, Oncogene Research Products, Calbiochem, Nottingham, UK) diluted 1:2500 in PBST at 4 °C for 16 h. Sections were washed using 0.01 M PBS (3×2 min) then incubated with Anti-rabbit Polymer AP (alkaline phosphatase, Cat. No. DSD-0014, GBI, USA) for 120 min. Sections were washed again using 0.01 M PBS (3×2 min) then incubated with substrate (AP-Red, Cat. No. ZLI-9042, Zhongshan, LTD., Beijing, China) for 30 min until red reaction product was achieved.

2.7. Cell counting

All brain sections were analyzed by an observer blind to the treatment conditions of individual rats. Anatomical nomenclature is based on that used in Paxinos and Watson (1998). The DRN and the MRN were analyzed at rostral, middle, and caudal levels (−7.50, −8.00, and −8.50 mm posterior to Bregma respectively) (Paxinos and Watson, 1998). In order to identify subpopulations of serotonergic neurons within the DRN differentially affected by drug treatment, this area was divided into the following regions: the dorsal (DRND) and ventral (DRNV) parts (−7.50, −8.00, and −8.50 mm Bregma), the ventrolateral part (DRNVL, −8.00, and −8.50 mm Bregma), and the interfascicular part (DRNI, −8.50 mm Bregma) on the basis of the rat brain atlas by Paxinos and Watson (1998). From each subject two double-immunostained sections were selected for each rostrocaudal level counted and each section was assessed for the number of c-Fos-immunopositive/TrpOH-immunonegative [c-Fos(+)-Trp(−)] nuclei, the total number of TrpOH-immunopositive [c-Fos(±)-Trp(+)] cells (both c-Fos-immunopositive and c-Fos-immunonegative), and the number of double-immunostained [c-Fos(+)-Trp(+)] cells. Then the c-Fos positive ratio, the number of c-Fos(+)-Trp(+) cells divided by the number c-Fos(±)-Trp(+) cells and then multiply 100%, was compared in different treatment groups. Round or oval-shaped nuclei with pink-red immunostaining were counted as c-Fos-immunostained nuclei. Cells with light brown staining throughout the cytoplasm were counted as TrpOH-immunostained cells. Cells with light brown staining of the cytoplasm and pink-red staining of nuclei were counted as c-Fos/TrpOH double-immunostained cells.

2.8. Statistical analysis

All values are expressed as mean ± S.E.M. Sleep parameter results were analyzed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) test for multiple comparisons. The results from immunohistochemical experiment were analyzed by ANOVA followed by least significant difference (LSD). $P<0.05$ was considered statistically significant.

3. Results

3.1. The augmentative effect of diltiazem on pentobarbital-induced hypnosis

In consistence with previous report (Zhao et al., 2009), which observed in rats, the present result showed that L-type Ca^{2+} channel blocker diltiazem (2 or 5 mg/kg, i.g.) could potentiate the hypnotic effect of pentobarbital (35 mg/kg, i.p.) reflected by increasing total sleep (TS) time ($P<0.01$, Fig. 1). Sleep parameter analysis revealed that diltiazem (2 or 5 mg/kg, i.g.) led to a significant increase in NREM sleep time ($P<0.01$, Fig. 1) including SWS ($P<0.05$, Fig. 1), but had no effects on REM sleep (Fig. 1).

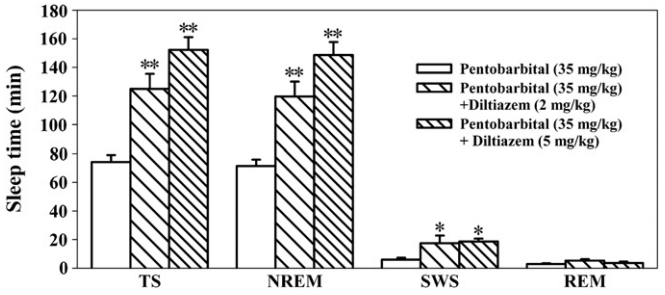

Fig. 1. The augmentative effects of diltiazem on pentobarbital-induced hypnosis in rats. TS [$F(2,29)=25.999$, $P<0.001$], NREM sleep [$F(2.29)=25.870$, $P<0.001$], SWS [$F(2,29)=5.095$, $P=0.013$], REM sleep [$F(2,29)=2.134$, $P=0.137$] were assessed (n = 10–11). Data are represented as mean ± S.E.M. * and ** $P<0.05$ and <0.01 *vs* Pentobarbital 35 mg/kg (SNK test).

3.2. Effect of diltiazem and pentobarbital on the c-Fos expression in DRN and MRN

3.2.1. The c-Fos positive ratio of the 5-HT neurons

The c-Fos and TrpOH immunostained neurons in DRN brain section are shown in Fig. 2. The c-Fos positive ratio of the 5-HT neurons in subdivisions of DRN including DRND, DRNV and DRNVL at rostral (−7.5 mm Bregma), middle (−8.0 mm Bregma) and caudal (−8.5 mm Bregma) levels was significantly decreased when the rats received pentobarbital (35 mg/kg, i.p.), respectively ($P<0.05$ or 0.01,

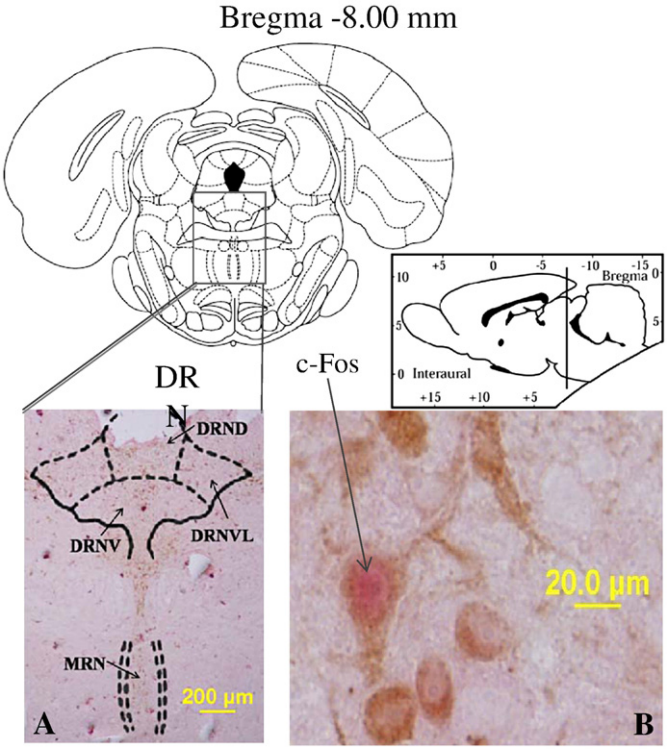

Fig. 2. (A) Illustrations of DRN brain sections are based on the atlas of Paxinos and Watson (1998). (B) Photomicrograph illustrating an example of c-Fos expression in TrpOH-immunostained neurons in DRN. Black arrow indicates a c-Fos-immunostained cell (pink nuclear staining).

Fig. 3). In MRN, pentobarbital (35 mg/kg, i.p.) showed the same trend toward decreasing c-Fos positive ratio of the 5-HT neurons especially at middle level with statistically significant difference ($P<0.01$, Fig. 3). These results demonstrated that pentobarbital (35 mg/kg, i.p.) inhibited the activity of the 5-HT neurons in DRN and MRN. However, the effect of pentobarbital (35 mg/kg, i.p.) on the c-Fos ratio was attenuated by diltiazem (5 mg/kg, i.g., Fig. 3). Pretreatment with diltiazem (2 or 5 mg/kg, i.g.) displayed the trend toward increasing the c-Fos positive ratio, as compared with the case of the sole pentobarbital (35 mg/kg, i.p.) administration (Fig. 3). And the statistically significant difference was showed at the dose of 5 mg/kg diltiazem in the following regions: DRNV at rostral level, DRND, DRNVL, MRN (both at the dose of 2 and 5 mg/kg) at middle level ($P<0.05$, Fig. 3).

3.2.2. The c-Fos expression of the non-5-HT neurons

As shown in Fig. 4, pentobarbital (35 mg/kg, i.p.) significantly inhibited the c-Fos expression in the Trp(−) non-5-HT neurons in DRND (at −8.0 mm Bregma, $P<0.01$), DRNV (at −8.0, 8.5 mm Bregma, $P<0.05$), DRNVL (at −8.0 mm Bregma, $P<0.05$). This inhibitory effect of pentobarbital was not affected by the pretreatment of diltiazem (2 or 5 mg/kg, i.g., Fig. 4). The difference results observed in 5-HT and non 5-HT neurons in DRN indicated that the 5-HT neurons may be involved in the augmentative effect of diltiazem on pentobarbital-induced hypnosis, but not the non-5-HT neurons in DRN.

3.3. The effect of diltiazem on pentobarbital-induced hypnosis is attenuated by 8-OH-DPAT and potentiated by p-MPPI

Pretreatment with diltiazem (2 mg/kg, i.g.) significantly prolonged the pentobarbital-induced (35 mg/kg, i.p.) sleep time in rats ($P<0.05$, Fig. 5A). In contrast, 8-OH-DPAT (0.5 mg/kg, i.p.), the 5-HT_{1A} receptor agonist, significantly decreased pentobarbital-induced (35 mg/kg, i.p.) sleep time in rats ($P<0.05$, Fig. 5A). Sleep parameter analysis revealed that diltiazem (2 mg/kg, i.g.) led to a significant increase in NREM sleep ($P<0.01$) and SWS ($P<0.05$, Fig. 5A). 8-OH-DPAT performed the opposite effect with significant reduction in NREM sleep ($P<0.05$, Fig. 5A). For evaluating the interaction between diltiazem and 8-OH-DPAT on the effects of pentobarbital-induced sleep, experiment with co-administration of these two drugs were conducted in rats. As a result, 8-OH-DPAT (0.5 mg/kg, i.p.) significantly inhibited the augmentative effects of diltiazem (2 mg/kg, i.g.) on TS ($P<0.05$, Fig. 5A) and NREM sleep ($P<0.01$, Fig. 5A) in pentobarbital-treated (35 mg/kg, i.p.) rats. Interestingly, diltiazem (2 mg/kg, i.g.) in combination with 8-OH-DPAT (0.5 mg/kg, i.p.) significantly enhanced REM sleep time ($P<0.05$, Fig. 5A).

Our previous studies showed that systemic administration of the 5-HT_{1A} receptor antagonist *p*-MPPI dose-dependently increased

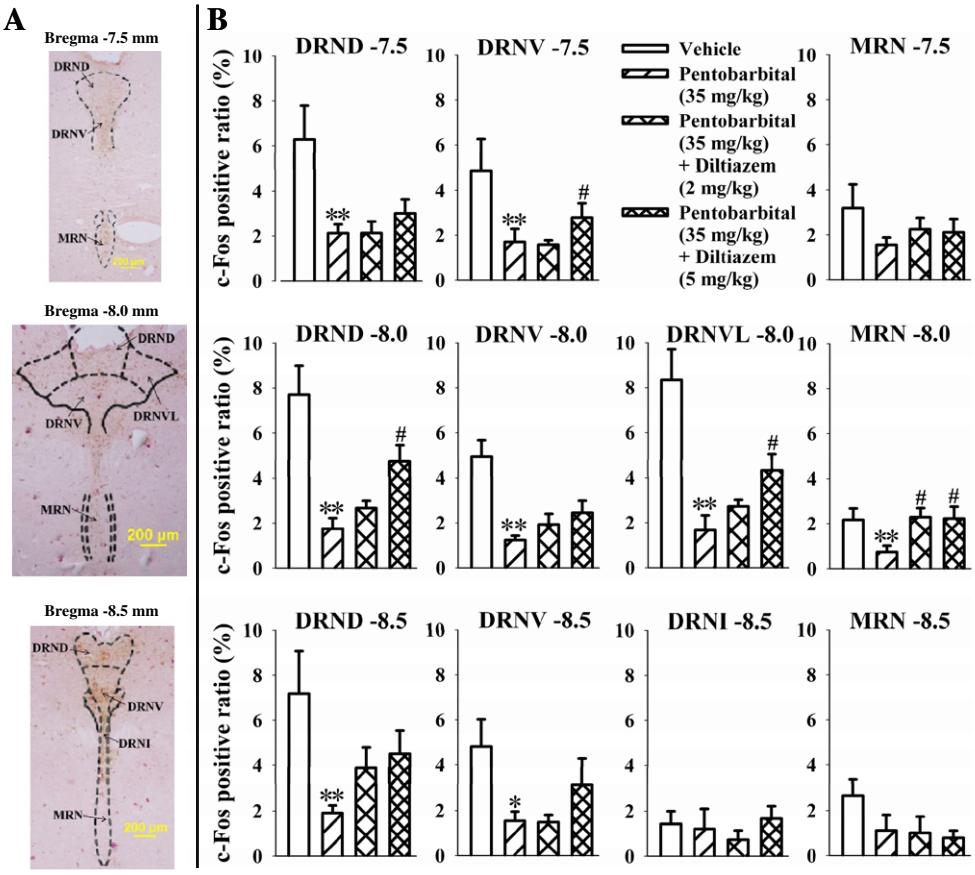

Fig. 3. (A) Photomicrographs of coronal rat brain sections stained for c-Fos (pink)/TrpOH (brown) to illustrate the distribution of 5-HT neurons within anatomical subdivisions of the brainstem raphe complex. Dotted lines delineate subdivisions of the midbrain raphe nuclei according to Paxinos and Watson (1998). Photographs are arranged in rostrocaudal order from top to bottom: −7.5 mm Bregma, −8.0 mm Bregma, −8.5 mm Bregma. (B) Effects of diltiazem, pentobarbital and their co-administration on the c-Fos positive ratio of the 5-HT neurons in subdivisions of DRN and MRN. The statistical results in each graph as follow: DRND −7.5 [$F(3,22)=5.904$, $P=0.004$]; DRNV −7.5 [$F(3,22)=4.049$, $P=0.020$]; MRN −7.5 [$F(3,20)=1.019$, $P=0.405$]; DRND −8.0 [$F(3,27)=10.463$, $P<0.001$]; DRNV −8.0 [$F(3,28)=9.393$, $P<0.001$]; DRNVL −8.0 [$F(3,27)=11.386$, $P<0.001$]; MRN −8.0 [$F(3,27)=3.023$, $P=0.047$]; DRND −8.5 [$F(3,21)=2.826$, $P=0.063$]; DRNV −8.5 [$F(3,21)=2.506$, $P=0.087$]; DRNI −8.5 [$F(3,21)=0.474$, $P=0.704$]; MRN −8.5 [$F(3,21)=2.155$, $P=0.124$]. Data are represented as mean ± S.E.M. * and ** $P<0.05$ and <0.01 *vs* Vehicle, # and ## $P<0.05$ and <0.01 *vs* Pentobarbital 35 mg/kg (n = 6–8) (LSD test).

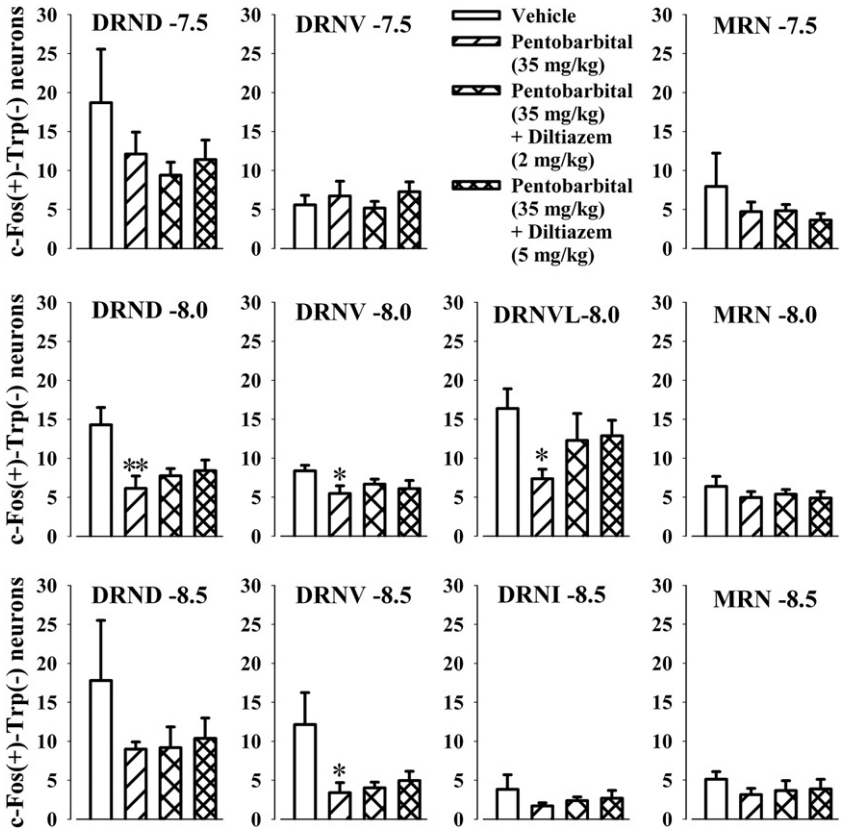

Fig. 4. Effects of diltiazem, pentobarbital and their co-administration on the c-Fos expression of the non-5-HT neurons of DRN and MRN. The statistical results in each graph as follow: DRND −7.5 [$F(3,22)=1.194, P=0.335$]; DRNV −7.5 [$F(3,22)=0.500, =0.686$]; MRN −7.5 [$F(3,20)=1.158, P=0.350$]; DRND −8.0 [$F(3,27)=4.466, P=0.011$]; DRNV −8.0 [$F(3,28)=2.089, P=0.124$]; DRNVL −8.0 [$F(3,27)=2.103, P=0.123$]; MRN −8.0 [$F(3,27)=0.576, P=0.636$]; DRND −8.5 [$F(3,21)=0.915, P=0.451$]; DRNV −8.5 [$F(3,21)=3.233, P=0.043$]; DRNI −8.5 [$F(3,21)=0.544, P=0.658$]; MRN −8.5 [$F(3,21)=0.489, P=0.694$]. Data are represented as mean ± S.E.M. * and ** $P<0.05$ and <0.01 *vs* Vehicle, # and ## $P<0.05$ and <0.01 *vs* Pentobarbital 35 mg/kg (n = 6-8) (LSD test).

pentobarbital-induced sleep time in rats (Wang et al., 2010). To investigate the interaction between diltiazem and *p*-MPPI, ineffective doses of diltiazem (1 mg/kg, i.g.) and *p*-MPPI (2 mg/kg, i.p.) which did not interfere with pentobarbital sleep were used for co-administration in this study. The results showed that co-administration of diltiazem (1 mg/kg, i.g.) and *p*-MPPI (2 mg/kg, i.p.) significantly increased TS ($P<0.05$, Fig. 5B), NREM sleep ($P<0.05$, Fig. 5B) and SWS ($P<0.01$, Fig. 5B) in pentobarbital-treated (35 mg/kg, i.p.) rats.

3.4. The effect of diltiazem on pentobarbital-induced hypnosis is attenuated by DOI and potentiated by ritanserin

DOI (0.5 mg/kg, i.p.), the 5-$HT_{2A/2C}$ receptor agonist, significantly inhibited not only the pentobarbital-induced (35 mg/kg, i.p.) TS time ($P<0.01$, Fig. 6A), NREM sleep ($P<0.01$, Fig. 6A) and SWS ($P<0.01$, Fig. 6A) in rats, but also the augmentative effects of diltiazem (2 mg/kg, i.g.) on pentobarbital-induced (35 mg/kg, i.p.) sleep reflected by inhibition of TS ($P<0.01$, Fig. 6A), NREM sleep ($P<0.01$, Fig. 6A) and SWS ($P<0.01$, Fig. 6A) in rats.

In our preliminary experiment, administration of 5-$HT_{2A/2C}$ receptor antagonist ritanserin dose-dependently increased pentobarbital-induced sleep time in rats (data not shown). To investigate the interaction between diltiazem and 5-$HT_{2A/2C}$ receptor antagonist ritanserin, ineffective doses of diltiazem (1 mg/kg, i.g.) and ritanserin (2 mg/kg, i.p.) which did not interfere with pentobarbital sleep were used for co-administration. The results of this experiment showed that the co-administration of diltiazem (1 mg/kg, i.g.) and ritanserin (2 mg/kg, i.p.) significantly increased TS ($P<0.05$, Fig. 6B), NREM sleep ($P<0.05$, Fig. 6B) and SWS ($P<0.05$, Fig. 6B) in pentobarbital-treated (35 mg/kg, i.p.) rats.

4. Discussion

In consistence with our previous report (Zhao et al., 2009), the present study also showed that L-type Ca^{2+} channel blocker diltiazem (2 or 5 mg/kg, i.g.) potentiated the hypnotic effect of pentobarbital (35 mg/kg, i.p.) reflected by increasing TS and SWS time (Figs. 1, 5A and 6A).

Lowry et al. (2008) have proposed six subdivisions of the DRN of the rat. The subdivisions comprise the rostral (DRNR), ventral (DRNV), dorsal (DRND), lateral including the ventrolateral part (DRNVL) and the adjacent ventrolateral periaqueductal gray (VLPAG), caudal (DRNC) and interfascicular (DRNI) parts of the DRN. Activation of the cellular immediate early gene *c-fos*, and subsequent accumulation of expressed c-Fos protein, is often used as surrogate marker of neuronal activity (Hoffman et al., 1993). C-Fos level has been shown to change in different regions of the brain during spontaneous sleep/wake episodes (Cirelli and Tononi, 2000). The present study showed that c-Fos positive ratio of the 5-HT neurons in DRN including DRND, DRNV and DRNVL at rostral (−7.5 mm Bregma), middle (−8.0 mm Bregma) and caudal (−8.5 mm Bregma) levels was significantly decreased when the rats received pentobarbital (35 mg/kg, i.p., Fig. 3). In MRN, pentobarbital (35 mg/kg, i.p.) showed the same trend toward decreasing c-Fos positive ratio of the 5-HT neurons especially at −8.0 mm relative to Bregma with statistically significant difference (Fig. 3). This pattern of pentobarbital action on serotonergic DRN and MRN neurons is consistent with the hypothesis regarding sleep generation is that the activation of VLPO neurons during sleep inhibits the activity of nuclei involved in arousal such as the DRN or TMN.

Previous research reported that systemic administration of pentobarbital increased the c-Fos expression in the VLPO, and

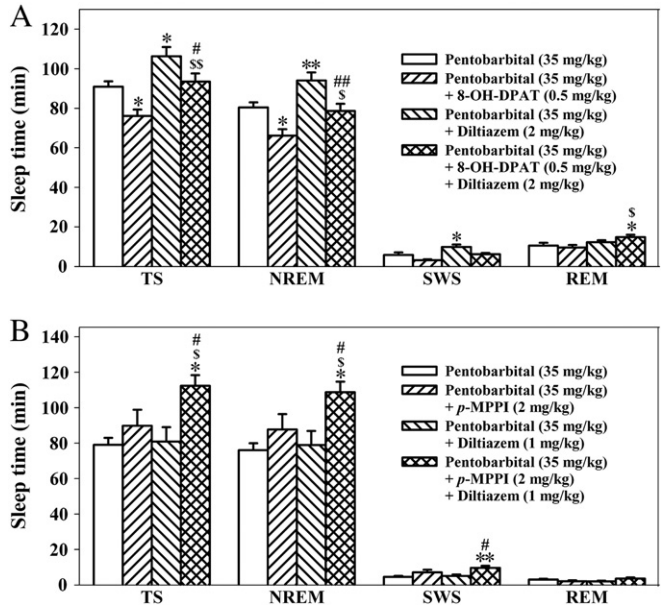

Fig. 5. The effect of diltiazem on pentobarbital-induced hypnosis is attenuated by 8-OH-DPAT and potentiated by *p*-MPPI. (A) Effects of 8-OH-DPAT, diltiazem and their co-administration on hypnosis in pentobarbital-treated rats. TS [$F(3,43)=11.277$, $P<0.001$], NREM sleep [$F(3,43)=11.843$, $P<0.001$], SWS [$F(3,43)=8.157$, $P<0.001$], REM sleep [$F(3,43)=3.134$, $P=0.035$] were assessed (n=11-12). (B) Effects of *p*-MPPI and diltiazem co-administration on hypnosis in pentobarbital-treated rats. TS [$F(3,21)=5.795$, $P=0.005$], NREM sleep [$F(3,21)=5.672$, $P=0.005$], SWS [$F(3,21)=5.773$, $P=0.005$], REM sleep [$F(3,21)=2.114$, $P=0.129$] were assessed (n=5-7). Data are represented as mean ± S.E.M. * and ** $P<0.05$ and <0.01 *vs* Pentobarbital 35 mg/kg, # and ## $P<0.05$ and <0.01 *vs* Pentobarbital 35 mg/kg + Diltiazem, $ and $$ $P<0.05$ and <0.01 *vs* Pentobarbital 35 mg/kg + agonist/antagonist (SNK test).

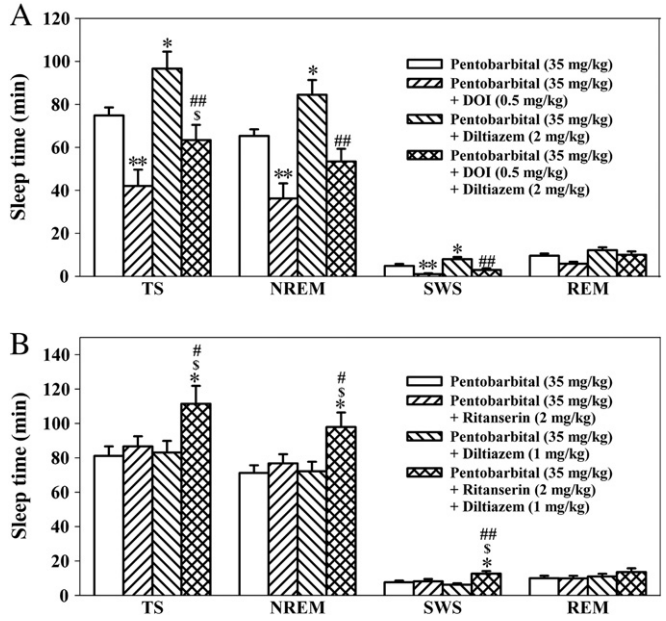

Fig. 6. The effect of diltiazem on pentobarbital-induced hypnosis is attenuated by DOI and potentiated by ritanserin. (A) Effects of DOI, diltiazem and their co-administration on hypnosis in pentobarbital-treated rats. TS [$F(3,38)=11.249$, $P<0.001$], NREM sleep [$F(3,38)=11.677$, $P<0.001$], SWS [$F(3,38)=13.002$, $P<0.001$], REM sleep [$F(3,38)=4.545$, $P=0.008$] were assessed (n=10–11). (B) Effects of ritanserin and diltiazem co-administration on hypnosis in pentobarbital-treated rats. TS [$F(3,39)=3.658$, $P=0.023$], NREM sleep [$F(3,39)=4.078$, $P=0.013$], SWS [$F(3,39)=5.208$, $P=0.004$], REM sleep [$F(3,39)=0.995$, $P=0.405$] were assessed (n=10–12). Data are represented as mean ± S.E.M. * and ** $P<0.05$ and <0.01 *vs* Pentobarbital 35 mg/kg, # and ## $P<0.05$ and <0.01 *vs* Pentobarbital 35 mg/kg + Diltiazem, $ and $$ $P<0.05$ and <0.01 *vs* Pentobarbital 35 mg/kg + agonist/antagonist (SNK test).

decreased the c-Fos expression in the TMN (Nelson et al., 2002; Zhao et al., 2009). Nelson et al. (2002) interpreted these data to indicate that pentobarbital activates VLPO neurons, which then release GABA into the TMN, or pentobarbital directly acts on $GABA_A$ receptors in TMN, thus inhibiting the activity of this arousal-producing nucleus and finally concluded that the sedative component of pentobarbital is mediated by $GABA_A$ receptors in an endogenous NREM sleep pathway. According to these ratiocinations, it is easy to presume that diltiazem could facilitate the inhibitory effect of pentobarbital on c-Fos positive ratio to the 5-HT neurons in DRN and MRN, because diltiazem can potentiate the hypnotic effect of pentobarbital. However, we observed surprisingly that the inhibitory effect of pentobarbital (35 mg/kg, i.p.) on the c-Fos ratio of the 5-HT neurons was reversed by diltiazem (5 mg/kg, i.g.) in some subdivisions of DRN. The significant differences were revealed in the following regions: DRNV (at −7.5 mm Bregma), DRND (at −8.0 mm Bregma), DRNVL (at −8.0 mm Bregma) and MRN (at −8.0 mm Bregma). These results indicated that the prolongation of pentobarbital-induced hypnosis by diltiazem may be related at least in part to the activation of some subdivisions of DRN and MRN. These findings also suggested that the role of DRN in sleep could not be characterized simply as arousal nuclei. Notwithstanding the unclear role of the subdivisions of DRN in sleep modulation, it could be presumed that DRN may participate in sleep modulation with multiple regulatory roles even as sleep promoting nuclei in some situations, but not just as arousal nuclei. The role characterizations of DRN subdivisions in sleep modulation need to be further evaluated.

DRN contains 5-HT and non-5-HT neurons. The latter express a variety of substances including dopamine, GABA and glutamate (Ochi and Shimizu, 1978; Wang et al., 2001). Moreover, numerous brain areas have neurons that project to the DRN and express monoamines, amino acids, acetylcholine or neuropeptides that directly or indirectly, through local circuits, regulate the activity of 5-HT cells (Steinbusch, 1981). In order to evaluate the roles of the non-5-HT neurons in the potentiating effect of diltiazem in pentobarbital treated rats, we also counted the number of c-Fos(+) expression in Trp(−) cells (non-5-HT neurons). As shown in Fig. 4, comparison of the numbers of c-Fos (+)-Trp(−) neurons in the DRN and MRN among all experimental groups revealed that pentobarbital-induced (35 mg/kg, i.p.) significant reduction of c-Fos number especially at DRND (−8.0 mm Bregma), DRNV (−8.0 and −8.5 mm Bregma) and DRNVL (−8.0 mm Bregma), respectively. And this reducing effect of pentobarbital was not affected by the pretreatment of diltiazem (2 or 5 mg/kg, i.g.). The results of these experiments indicated that diltiazem (5 mg/kg, i.g.) can selectively diminish the pentobarbital-induced (35 mg/kg, i.p.) reduction of c-Fos expression in TrpOH positive 5-HT neurons in DRN or MRN, but showed no effects on the non-5-HT neurons (Fig. 4).

Hjorth and Magnusson (1988) reported that systemic administration of 8-OH-DPAT, a selective 5-HT_{1A} receptor agonist in doses ranging from 0.1~2.0 mg/kg, consistently increases waking, and reduces SWS and REM sleep in rats (Bjorvatn et al., 1997; Dzoljic et al., 1992; Monti and Jantos, 1992, 1994). These vigilance effects of 8-OH-DPAT are hypothesized to reflect post-synaptic 5-HT_{1A} receptor stimulation. The present study showed that 8-OH-DPAT (0.5 mg/kg) administered intraperitoneally significantly cultivated total sleep and decreased NREM sleep without significant influence on SWS and REM sleep in pentobarbital-treated (35 mg/kg, i.p.) rats (Fig. 5A). In addition, 8-OH-DPAT (0.5 mg/kg, i.p.) significantly inhibited diltiazem (2 mg/kg, i.g.)-induced promotion of total sleep and NREM sleep in pentobarbital-treated (35 mg/kg, i.p.) rats (Fig. 5A). In contrast, 5-HT_{1A} receptor antagonist *p*-MPPI potentiated the effects of diltiazem on pentobarbital hypnosis significantly by increasing total sleep, NREM sleep and SWS (Fig. 5B). Moreover, co-administration of diltiazem (2 mg/kg, i.g.) and 8-OH-DPAT (0.5 mg/kg, i.p.) significantly increased REM sleep compared to both pentobarbital group and pentobarbital plus 8-OH-DPAT group in rats (Fig. 5A). This is a wander

result, because there is compelling evidence showing that systemic administration of a Ca^{2+} channel antagonist (Takahashi et al., 1999) or a 5-HT_{1A} receptor agonist (Hjorth and Magnusson, 1988) decreases REM sleep. Portas et al. (1996) provided direct evidence that suppression of DRN serotonergic activity increases REM sleep and Monti (2010) also proposed that the absence of a postsynaptic inhibitory influence on the mechanisms involved in the induction and maintenance of REM sleep. Although in this experiment, 8-OH-DPAT do not suppress REM sleep, may be due to the dose did not reach the effective level, but combined it with diltiazem significantly increased REM sleep, suggesting that diltiazem may diminish the suppression effects of 5-HT_{1A} receptor agonist 8-OH-DPAT on REM sleep.

Systemic administration of the serotonin 5-$HT_{2A/2C}$ receptor agonist DOI has been shown to reduce SWS and REM sleep and to increase waking in the rat. Pretreatment with the serotonin 5-$HT_{2A/2C}$ receptor antagonist ritanserin prevented the waking enhancement and the SWS deficit induced by DOI but not the REM sleep suppression (Dugovic, 1992; Dugovic et al., 1989). Present study showed that DOI (0.5 mg/kg) administered intraperitoneally significantly decreased total sleep time, NREM sleep and SWS in pentobarbital-treated (35 mg/kg, i.p.) rats (Fig. 6A). On the other hand, DOI (0.5 mg/kg, i.p.) also diminished the potentiating effect of diltiazem (2 mg/kg, i.g.) on pentobarbital (35 mg/kg, i.p.) sleep by suppression of total sleep time, NREM and SWS (Fig. 6A). Diltiazem (1 mg/kg, i.g.) also exhibited synergic effects with ritanserin (2 mg/kg, i.p.) on pentobarbital hypnosis by significant promotion of total sleep, NREM sleep and SWS (Fig. 6B). These results suggested that the 5-$HT_{2A/2C}$ receptors also involved in the augmentative effect of diltiazem on pentobarbital-induced hypnosis.

The results described here identify the DRN, like VLPO and TMN, also as a key element and the role of 5-HT_{1A} and 5-$HT_{2A/2C}$ receptors in the augmentative effect of diltiazem on pentobarbital-induced hypnosis, though other mechanisms remain possible. In addition, DRN cannot be simply characterized as arousal nuclei. The role characterizations of DRN subdivisions in sleep modulation need to be further evaluated.

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