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Original Article

Vagus nerve stimulation causes wake-promotion by affecting neurotransmitters via orexins pathway in traumatic brain injury induced comatose rats

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Abstract: Previous studies have demonstrated that vagus nerve stimulation (VNS) can decrease the amounts of daytime sleep and rapid eye movement in epilepsy patients with traumatic brain injury (TBI). Orexins, synthesized in lateral hypothalamus, are important neurotransmitters with sleep/wakefulness cycle, which have great connection with the projection area of vagus nerve in the brain. The aim of this study was to investigate the wake-promoting effects of VNS in comatose rats caused by TBI and its mechanisms with neurotransmitters. Additionally, we attempted to clarify the function of orexins on the awakening effects of VNS. One hundred and twenty Sprague-Dawley rats were randomly assigned to four groups: control group, TBI group, stimulated (TBI+VNS) group, and an antagonist (TBI+SB334867+VNS) group. We established TBI models by free fall drop. In the stimulated group, the TBI induced comatose rats were stimulated by the vagus nerve. In the antagonist group, comatose rats were given SB334867, an orexin receptor 1 antagonist, by intracerebroventricular injection and VNS. Then, the behavior changes were evaluated and the receptors of neurotransmitters in the prefrontal cortex were detected by the western-blot and immunohistochemistry. Results have shown that 8 of 30 rats were awakened in the TBI group, 20 of 30 awakened in the stimulated group and 12 of 30 rats were awakened in the antagonist group. Between the TBI group and the stimulation group, the expression of orexin receptor-1 (OX1R), N Methyl D Aspartate receptor-1 (NMDAR1), 5-hydroxytryptamine 2A receptor (5-HT_{2A}R), histamine 1 receptor (H1R), norepinephrine α1 receptor (α1-AR) were higher and gamma aminobutyric acid b receptor (GABAbR) was lower in stimulated groups ($P < 0.05$). However, the expression of OX1R, NMDAR1, 5-HT_{2A}R, H1R, α1-AR were lower and GABAbR was higher in antagonist group than the stimulated group ($P < 0.05$). Taken together, these data indicate that VNS has wake-promoting effects. One possible mechanism is that VNS upregulates the expression of excitatory neurotransmitters and decreases the expression of inhibitory neurotransmitters. More importantly, orexins may play a key role in wake-promoting effects of VNS.

Keywords: Vagus nerve stimulation, orexins, traumatic brain injury, wake-promotion

Introduction

Coma is often caused by Traumatic brain injury (TBI), cerebrovascular accident, or alcoholism [1]. TBI occurs in 200 million people per year in the world, leading to heterogeneous neurological outcomes with high morbidity and mortality [2]. Recent progress in intensive care and first-aid potentially decrease mortality for severe TBI, but survivors may be left with impaired consciousness resulting in a comatose state, which also brings huge economic burdens on society and families. At present, we believe that the pathogenesis of coma could be resultant from damage to the Ascending Reticular Activating System (ARAS) in brainstem and neurotransmitter imbalances between wakefulness/sleep [3-5]. There are two kinds of neurotransmitters, excitatory neurotransmitters and inhibitory neurotransmitters, which control the state transition of consciousness. Excitatory neurotransmitters such as orexins, glutamate, dopamine (DA), 5-hydroxytryptamine (5-HT), histamine (His) and noradrenaline (NA) help maintain awareness [3, 6-8]. Inhibitory neurotransmitters such as γ-aminobutyric acid

(GABA) and glycine, on the contrary, could lead to sleeping or somnolent states [9]. Our previous investigation suggested that orexins is the most important neurotransmitter in sleeping and wakefulness [3-5]. Orexins, which including orexin-A and orexin-B, produced in the lateral hypothalamus (LHA) are associated with growth hormones, metabolic rate, autonomic function, food intake and sleep/wakefulness cycle [10]. They have two receptors in the central nervous system, orexin receptor 1 and receptor 2 (OX1R and OX2R), which are important for wake-promotion [10].

Clinical treatment methods for coma include drugs, hyperbaric oxygen, music therapy, sensory stimulation, cell transplantation, and nerve stimulation [11, 12]. Nerve stimulation comprises various mechanisms including deep brain stimulation (DBS), cervical spinal cord stimulation (cSCS), transcranial direct current stimulation (tDCS), transcranial magnetic stimulation (TMS), median nerve electrical stimulation (MNES) and vagus nerve stimulation (VNS). VNS, approved by the U.S. Food and Drug Administration since 1997, has been widely used in treating cognitive disorders, refractory epilepsy, and resistant depression [13]. To our knowledge there is no direct connection between wake-promotion and VNS. But numerous studies have shown that VNS can reduce the amount of daytime sleep and rapid eye movement, and thereby extend the amount of awaken time in patients with epilepsy caused by TBI [14-18]. A prospective pilot clinical trial was also intend to examine the effectiveness of VNS in severe TBI impeding consciousness patients, which is promising [19]. However the mechanism of above effects is still unclear, some mechanisms such as influenced of related neurotransmitters may lay the foundation of wake-promoting of VNS. The purpose of this investigation was to study the wake-promoting effects and mechanism of VNS in comatose rats caused by TBI, as well as the function of orexins in awakening effects of VNS.

Material and methods

Establishment of coma model caused by TBI

Pathogen-free adult male Sprague-Dawley rats, weighing 250-300 g, were used in this study. Rats were obtained from the Institute of Laboratory Animals at Nanchang University (Nanchang, Jiangxi Province, China). All rats were housed in the Laboratory Animal center of the First Affiliated Hospital of Nanchang University (Nanchang, Jiangxi Province, China), and were maintained under controlled temperature and light conditions with food and water continuously available. Animals were divided into four groups: control group, TBI group, stimulated (TBI+VNS) and antagonist groups (TBI+SB-334867+VNS). Each group comprised 30 rats. Rats in the TBI, stimulated, and antagonist groups were anesthetized using diethyl ether inhalation and allowed to breathe air spontaneously. After anesthesia, the skull was exposed via a 5 mm vertical incision using conventional surgical techniques. A cross hit point was marked by a syringe needle at 2 mm left of the midline and 1 mm anterior to the coronal suture. A cylindrical impact hammer weighing 400 g was dropped from a vertical height of 40-44 cm along a previously marked space, resulting in a concave fracture of skull [20]. After injury, the incision was closed, and the animal was disinfected and moved to a cage. One hour later, they were classified into degrees of I-VI consciousness according to sensory and motor functions [21]. Degree I: Normal activity freely engaged in cages; degree II: decreased activity; degree III: decreased activity with motor in coordination; degree IV: righting reflex could be elicited and animals could stand up; degree V: righting reflex disappeared and animals could react to pain; degree VI: animals have no reaction to pain. We defined rats in degrees V and VI, which lasted at least 30 minutes, as rats in a comatose state and suitable for the following procedures.

Intracerebroventricular injection of the OX1R antagonist, SB334867

Under sterile conditions, an injection catheter was inserted into the left cerebral ventricle of rats in the antagonist group. Each rat was pretreated with gentamicin (0.1 mL/100 g, intramuscular injection) and anesthetized with 10% chloral hydrate (0.3 mL/100 g, intraperitoneal injection) before surgery. The rats were positioned on a stereotaxic frame (ZS-B/S, Beijing ZhongshiDichuang Science and Technology Development Co., Ltd., Beijing, China), with the coordinates to map the guide cannula as follows: 1.0 mm posterior to bregma, 1.5 mm lateral to the midline, and 4.5 mm ventral to the skull surface, with the incisor bar 3.2 mm below the interauricular line. An injection catheter was inserted into the cerebral ventricle of rats

in the antagonist group under sterile conditions. The OXR1 inhibitors SB334867 (Tocris Bioscience, Ellisville, MO, USA) 10 mg/kg was dissolved in 60:40 dimethyl sulfoxide solution and administrated at a dose of 5 μL. After rats awoke from anesthesia, they were prepared for VNS.

Vagus nerve stimulation

In the stimulated and antagonist groups, rats were treated with VNS, using a low frequency electrical stimulator (ES-420, ITO Physiotherapy & Rehabilitation, Tokyo, Japan). Before stimulation, rats were anesthetized with 10% chloral hydrate (0.3 mL/100 g, administrated intraperitoneal injection). The head and neck were disinfected with betadine and then shaved. A small incision was made on the left ventral side of the neck adjacent to the midline to approach the left vagus nerve at the cervical level. Blunt dissection of the subcutaneous fat, salivary glands, sternohyoid and sternocleidomastoid was made to expose the carotid sheath that wrapped the vagus nerve and carotid artery, separating the left vagus nerve by 5 mm. A self-VNS electrode surrounded the vagus nerve and an ohmmeter ensured electrode contact with the nerve. VNS parameters were as follows: frequency, 30 Hz; electrical current, 1.0 mA; pulse width, 0.5 ms; total stimulation time, 15 minutes [22]. Following surgery, each animal received gentamicin (0.1 mL/100 g, im). According to the assessment criteria for degrees of consciousness specified above, we observed and evaluated animals' behavior and consciousness again one hour after suoperation. The TBI group underwent a procedure identical to the stimulated groups, but without any electric current output. And the same manner such as sham operation and anesthesia were also done in the control group.

Histological processing

Rats were euthanized with excess diethyl ether at 6 hours after VNS in the stimulated and antagonist groups, respectively, along with a corresponding control and TBI-induced comatose rat simultaneously. Then prefrontal cortex tissues (within the frontal lobe) were extracted, and immunohistochemistry and western blot analysis were used to detect the expression of OX1R, NMDAR1, 5-HT_{2A}R, H1R, α1-AR and GABAbR levels.

Western blot analysis

The tissue samples obtained were homogenized using a Tissue Protein Extraction Kit (CW0891, Beijing Kangwei Biotechnology Co., Ltd, Beijing, China). Homogenates were off centered at 12,000 × g for 10 minutes at 4°C. Supernatants were separated into small aliquots and stored at -80°C. The amount of total protein in each sample was detected by the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA). The amount of proteins in each homogenated sample was equal and loading buffers was boiled in water for 5 minutes and run on a 10% sodium dodecyl sulfate/polyacrylamidegel. The proteins were transferred to poly vinyli denedi fluoride membranes (PVDF) followed by electrophoresis. Membranes were infiltrated for 2 to 3 hours at room temperature with TBS-T buffer (150 mM NaCl, 20 mM TrisHCl, pH 7.4, 0.1% Tween 20 mL) containing 5% milk. The blots were incubated overnight with rabbit anti-OX1R polyclonal antibody (1:200; ab68718, Abcam, New Territories, HK Co., Ltd., China), rabbit anti-NMDAR1 polyclonal antibody (1:200; BA0612, Wuhan Boster Biotechology Co., Ltd., China), rabbit anti-GABAbR polyclonal antibody (1:500; ab131417, Abcam, HK Co., Ltd., China) and rabbit anti-rat β-actin monoclonal antibody (1:400; CW0096, Beijing Kangwei, Beijing, China) at 4°C overnight. Following the incubation, the membranes were washed four times with TBS-T for one hour, and incubated at room temperature for 1 hour with horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) (ZB-2301, Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) diluted 1:2,000 in TBS-T containing 5% milk. The concentration of proteins was detected using the bicinchoninic acid protein assay. The blots were stripped by incubation for 30 minutes at 70°C with a solution containing 2% sodium dodecyl sulfate, 100 mM β-mercaptoethanol in 62.5 mM Tris-HCl, pH 6.8, and re-probed using rabbit anti-β-actin monoclonal antibody (CW0096, Beijing Kangwei) to monitor loading of gel lanes. Then, blots were incubated with a chemiluminescence substrate (32109, ECL Plus, Amersham Biosciences, NJ, USA) and quantified using Image Lab (Bio-Rad). Western blot analysis of OX1R in rat prefrontal cortex tissues was performed at 6 hours. The data generated represent optical density measurements of individual bands from western blot normalized to β-actin.

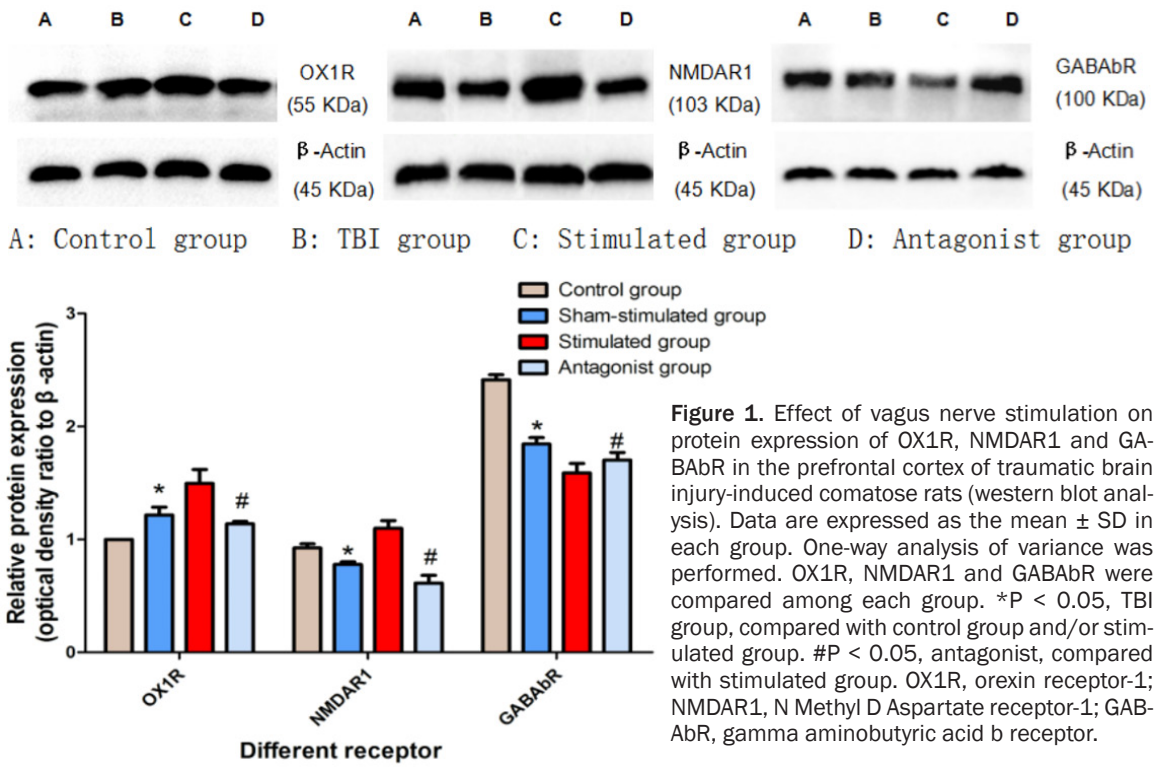

Figure 1. Effect of vagus nerve stimulation on protein expression of OX1R, NMDAR1 and GABAbR in the prefrontal cortex of traumatic brain injury-induced comatose rats (western blot analysis). Data are expressed as the mean ± SD in each group. One-way analysis of variance was performed. OX1R, NMDAR1 and GABAbR were compared among each group. *P < 0.05, TBI group, compared with control group and/or stimulated group. #P < 0.05, antagonist, compared with stimulated group. OX1R, orexin receptor-1; NMDAR1, N Methyl D Aspartate receptor-1; GABAbR, gamma aminobutyric acid b receptor.

Immunohistochemistry

The animals were decapitated at 6 hours after VNS. The rats were anesthetized and perfused through the heart with 4% paraformaldehyde. Then, the brains were carefully removed, and 40-μm thick coronal sections were cut. The sections were rinsed by phosphate buffered saline (PBS) and treated with 0.3% hydrogen-peroxide (H_2O_2) for 30 min. The sections were then rinsed three times for 5 min and incubated with normal goat serum for 20 min. The sections were then incubated overnight (4°C) with diluted rabbit anti-OX1R (1:200; ab68718, Abcam, New Territories, HK Co., Ltd., China), rabbit anti-NMDAR1 polyclonal antibody (1:200; BA0612, Wuhan Boster Biotechnology Co., Ltd., China) and rabbit anti-5-HT2A R polyclonal antibody (1:400; BA2048, Wuhan Boster Biotechnology Co., Ltd., China), rabbit anti-H1R polyclonal antibody (1:200; BA1633-1, Wuhan Boster Biotechnology Co., Ltd., China) and rabbit anti-GABAbR polyclonal antibody (1:500; ab131417, Abcam, HK Co., Ltd., China). Following extensive rinsing in PBS, the sections were subsequently incubated with abiotinylated goat anti-rabbit antibody. Then, the sections were visualized using diaminobenzidine as a chromogen in a peroxidase reaction.

Statistics analysis

Western blot data were processed with mean ± standard deviation and immunohistochemistry data as the mean rank. One-way analyses of variance with Turkey's test were performed for comparison of results from western-blot. Kruskal Wallis-Test was used for comparison of results from Immunohistochemistry. All data were analyzed with SPSS 17.0 software (SPSS, Chicago, IL, USA). P < 0.05 was accepted as statistically significant.

Results

VNS improves the consciousness grades of comatose rats caused by TBI

In the present study, evaluation of consciousness was conducted through a double blind method 1 hour after completion of the experiment. A total of 20 rats expired and were excluded from this study in TBI (3), stimulated (8) and antagonist (9) groups. Results showed that only 8 of 30 rats awakened from the coma in the TBI group (IV degree: 8, V degree: 10, VI degree: 12). In the stimulated group, 20 rats reawakened (II degree: 4, III degree: 6, IV degree: 10, V degree: 8, VI degree: 2), and 10 rats were still in comatose state. Twelve rats were revived

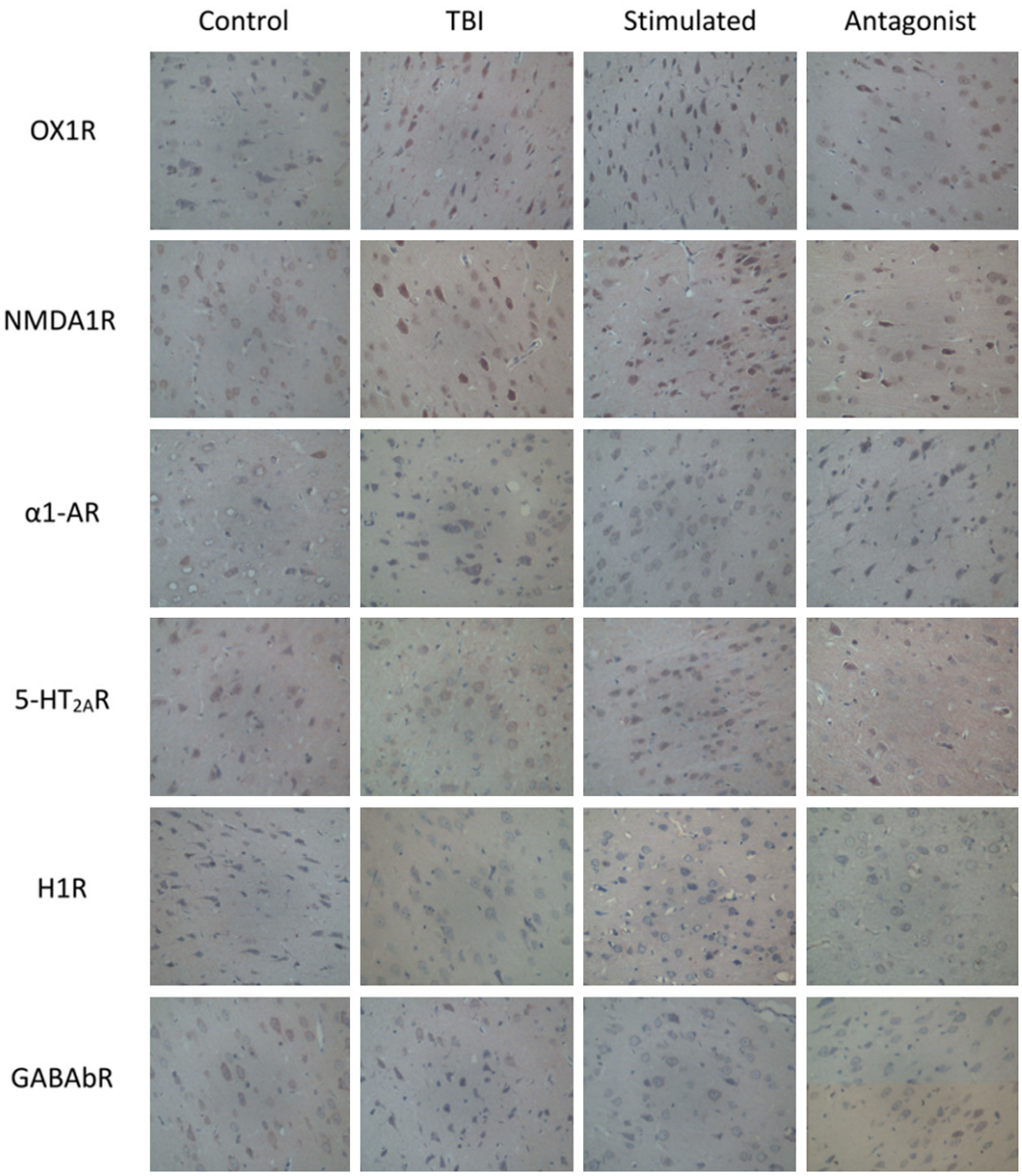

Figure 2. Representative photomicrographs of OX1R, NMDAR1, 5-HT_{2A}R, H1R, α1-AR and GABAbR immunoreactivity localization in the prefrontal cortex. OX1R, NMDAR1, 5-HT_{2A}R, H1R, α1-AR and GABAbR immunostaining was detected within the cytoplasm of neurons in the prefrontal cortex. 400 times magnification. OX1R, orexin receptor-1; NMDAR1, N Methyl D Aspartate receptor-1; 5-HT_{2A}R, 5-hydroxytryptamine 2A receptor; H1R, histamine 1 receptor; α1-AR, norepinephrineα1 receptor; GABAbR, gamma aminobutyric acid b receptor.

from comas in the antagonist group (III degree: 5, IV degree: 7, V degree: 10, VI degree: 8). A comparison of the number of rats in degrees of consciousness I-IV revealed the following order: TBI group < antagonist group < stimulated group < control group. These data indicate that VNS could promote recovery of consciousness in comatose rats.

VNS upregulates the expression of excitatory neurotransmitters but downregulates inhibitory neurotransmitters in the prefrontal cortex

The levels of excitatory neurotransmitter receptors such as OX1R, NMDAR1, 5-HT_{2A}R, H1R, α1-AR and inhibitory neurotransmitter receptors such as GABAbR expression in the prefron-

Table 1. The rank mean of OX1R, NMDAR1, 5-HT_{2A}R, H1R, α1-AR and GABAbR by Kruskal Wallis-Test

	N	Control	TBI	Stimulation	Antagonist	X^2	P
OX1R	15	23.70	30.03	41.05	26.77	12.141	0.007
NMDA1R	15	35.00	25.00	39.00	23.00	12.070	0.007
5-HT_{2A}R	15	16.17	32.33	42.33	31.17	21.241	0.000
H1R	15	34.43	23.40	39.30	24.87	10.558	0.014
α1-AR	15	19.30	32.70	40.90	29.10	19.677	0.000
GABAbR	15	35.73	31.87	29.93	34.47	8.442	0.038

Data are expressed as the rank mean in each group. P < 0.05 indicates statistical significance. OX1R, orexin receptor-1; NMDAR1, N Methyl D Aspartate receptor-1; 5-HT_{2A}R, 5-hydroxytryptamine 2A receptor; H1R, histamine 1 receptor; α1-AR, norepinephrine α1 receptor; GABAbR, gamma aminobutyric acid b receptor.

tal cortex were measured by western blot and immunohistochemistry 6 hours after VNS. The relative levels of OX1R expressions were: control group < TBI group < stimulated group (P < 0.05) (**Figures 1**, **2** and **Table 1**); the NMDAR1 expressions were: TBI gr-oup < control group < stimulated group (P < 0.05) (**Figures 1**, **2** and **Table 1**); the 5-HT_{2A}R expressions were: control group < TBI group < stimulated group (P < 0.05) (**Figure 2** and **Table 1**); the H1R expressions were: TBI group < control group < stimulated group (P < 0.05) (**Figure 2** and **Table 1**); the α1-AR expressions were: control group < TBI group < stimulated group (P < 0.05) (**Figure 2** and **Table 1**); the GABAbR expressions were: stimulated group < TBI group < control group (P < 0.05) (**Figures 1**, **2** and **Table 1**). These data also demonstrate that excitatory neurotransmitter receptors such as the expression of OX1R, NMDAR1, 5-HT_{2A}R, H1R, α1-AR in the stimulated groups are higher than the TBI group, and inhibitory receptors such as GABAbR expression in the stimulated group are lower than the TBI group. From the above analysis, we concluded that VNS may upregulate the expression of excitatory neurotransmitters but downregulates inhibitory neurotransmitters to promote wakefulness.

Orexins play a key role in regulating other neurotransmitters in VNS induced wake-promotion

Figure 1 and **Table 1** also demonstrate that NMDAR1, 5-HT_{2A}R, H1R and α1-AR expression in the antagonist group were lower than the stimulated group (P < 0.05). In addition, GABAbR expression in the antagonist group was higher than the stimulated group (P < 0.05). These indicate that orexins have an important role in regulating excitatory and inhibitory neurotransmitters for wake-promotion with VNS.

Discussion

The purpose of this experiment was to investigate the wake-promoting effects of VNS in comatose rats caused by TBI and its mechanisms with neurotransmitters. In addition, we attempted to clarify the function of orexins on the awakening effects of VNS. The present study indicates that 1) VNS could promote the consciousness grades of comatose rats caused by TBI; 2) VNS upregulates the expression of excitatory neurotransmitters and decreases the expression of inhibitory neurotransmitters may one of mechanism for wake-promotion of VNS; 3) and more importantly, orexins may play a key role in wake-promoting effects of VNS.

At present, based on previously published literature and present study, one of explanations for VNS-induced wake-promotion might be due to the influenced of related neurotransmitters [23]. It is known that excitatory neurotransmitters such as orexins, NA, Glutamate, 5-HT, DA, His could promote wakefulness, on the contrary, inhibitory neurotransmitter such as GABA could promote sleeping [4, 6-8]. Our previous studies have shown that MNES could increase the expression of orexins and NMDA, and decrease the expression of GABAbR in the prefrontal cortex to promote the wakefulness in comatose rats caused by TBI [3-5, 24]. Therefore, VNS induced wake promotion may have relationship to some neurotransmitters of sleep/wakefulness. Recent evidence suggests that some effects of VNS may be due to activation of the locus coeruleus nucleus to release norepinephrine (NE) throughout the neuraxis in the central nervous system [25]. The NE system plays a significant role in recovery from TBI, particularly in wake promotion and sleep inhibition [25]. There are also reported that VNS induces phrenic long-term facilitation by activating receptors of 5-TH [26]. As surowka et al., found that peripheral vagus nerve stimulation significantly affects lipid composition and protein secondary structure with dopamine-related brain regions in rats [27]. Other studies also indicated that VNS have great connection with

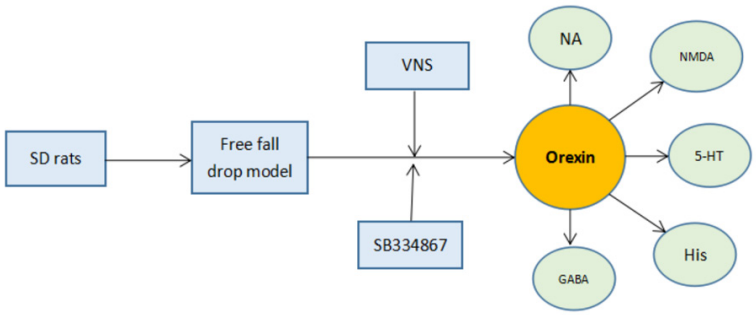

Figure 3. Schematic overview of the wake-promotion of VNS in comatose rats caused by TBI. VNS, Vagus nerve stimulation; NA, noradrenaline; NMDA, N Methyl D Aspartate; 5-HT, 5-hydroxytryptamine; His, histamine; GABA, gamma aminobutyric acid.

dopamine system [28-30]. On the other side, electrical stimulation of vagus nerve could also modulate the histamine content of mast cells in the rat jejunal mucosa [31]. Furthermore, It's reported that VNS might modulate the cortical excitability of brain areas associated with epileptogenesis and that GABA receptor contributes to above effects [32]. And from the results of this experiment, we can also know VNS could upregulate the expression of NA, Glutamate, 5-HT, His, orexins and reduction of GABA in the prefrontal cortex to promote wakefulness.

Previous studies have shown that there are three systems in control of the sleep/wakefulness cycle, including the orexinergic system, monoaminergic system and cholinergic system [33, 34]. The orexinergic system could also play a key role in controlling the monoaminergic system and cholinergic system and have widely projecting fibers in the central nervous system. What's more, orexins could directly activate the cerebral cortex or regulate other wakefulness systems to promote awakening [34-36]. For example, orexins could excite the monoaminergic system and cholinergic system, and suppress the ventrolateral preoptic nucleus (VLPO) to strengthen wakefulness [35]. It was also reported that orexins send out fibers to the tuberomammillary nucleus (TMN) to activate the neurons of TMN and release histamine, and improve the expression levels of histamine to excite the cortex and maintain arousal [37]. In addition, injection of orexin-A to the basal forebrain can promote the release of acetylcholine in the somatosensory cortex to improve the state of wakefulness, and reduced the above effects by injection of OX1R antagonist [38]. Moreover, it has been found that orexin neurons of the hypothalamus could activate the 5-HT neurons of the LC and dorsal raphe nucleus (DRN) to promote awakening [39, 40]. For instance, injection of orexins in the LC and DRN can prolong the time of wakefulness and injection of OX1R antagonist could cutoff these effects [39, 40]. It has also been found that orexins neurons of the LHA have connections with the medial prefrontal cortex (MPFC) through the laterodorsal tegmental nucleus (LTD) by visual tracking technology, and injection of orexin-A in the LTD could improve the arousal reaction and decrease REM time [41]. In addition, it was reported that Orexin-A can increase glutamatergic neurotransmission in the prefrontal cortex to modulate neuronal activity, which play a role in cognitive arousal [42]. It was also reported that pharmacological or genetic OX1R inhibition significantly attenuated the cortical glutamate release elicited by MK-801 in mouse cortex [43]. What's more, prior studies have indicated that orexingeric neurons of LHA could inhibit GABAgeric neurons of VLPO to promote awakening from sleep [33, 44]. The data of our experiment also showed that orexins have a positive effect with upregulation of NMDAR1, 5-HT_{2A}R, H1R and α1-AR, and reduction of GABAbR in wake-promotion of VNS, this conclusion is consistent with previous studies. All in all, orexins plays an important role in regulating other neurotransmitters to promote wakefulness by VNS.

In conclusion, the findings of this study provide preliminary evidence that VNS could promote arousal and improved consciousness in TBI-induced comatose rats. In addition, we have demonstrated that the upregulated expression of OX1R, NMDAR1, 5-HT2AR, H1R and α1-AR and decreased expression of GABAbR in the prefrontal cortex may be one of the mechanisms involved in consciousness-promoting effects. Importantly, orexins may play a key role in wake-promotion using VNS (**Figure 3**). With this understanding VNS could be a potential treatment for comatosed individuals affected by TBI and more comatose patients will be benefit from VNS in the future. Additional studies are needed however, to test the clinical effects

and mechanisms of VNS and its complications in humans.

There are several limitations to this study including: 1) We could have examined the wake-promoting effects of VNS by a more sensitive method, such as electroencephalogram, Glascow coma scale or evoked potential; 2) A larger sample size and a more accurate method of TBI models should have been utilized; 3) Direct clinical application is hindered by the fact that VNS requires open-operation procedures. Future studies should explore a non-invasive method of VNS.

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Disclosure of conflict of interest

None.

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