Neuropharmacology 62 (2012) 775–783

Contents lists available at SciVerse ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Arousal effects of orexin A on acute alcohol intoxication-induced coma in rats

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ARTICLE INFO

Article history:
Received 6 January 2011
Received in revised form
24 August 2011
Accepted 28 August 2011

Keywords:
Orexins
Arousal
Alcohol intoxication
Coma
Prefrontal cortex
Histamine
Serotonin
Noradrenaline

ABSTRACT

The key role of the hypothalamic neuropeptides orexins in maintenance and promotion of arousal has been well established in normal mammalian animals, but whether orexins exert arousal effects under pathological condition such as coma was little studied. In this study, a model of unconscious rats induced by acute alcohol intoxication was used to examine the effects of orexins through intracerebroventricular injection. The results revealed that either orexin A or orexin B induced decrease of duration of loss of right reflex in alcohol-induced unconscious rats. In the presence of the selective orexin receptor 1 antagonist SB 334867 and orexin receptor 2 antagonist TCS OX2 29, the excitatory action of orexin A was completely blocked. Our data further presented that orexin A also induced reduction of delta power in EEG in these rats. Single-unit recording experiment *in vivo* demonstrated that orexin A could evoke increase of firing activity of prefrontal cortex neurons in unconscious rats. This excitation was completely inhibited by an H_1 receptor antagonist, pyrilamine, whereas application of α_1-adrenoreceptor antagonist prazosin or 5-HT_2 selective receptor antagonist ritanserin partially attenuated the excitatory effects of orexin A on these neurons. Consistently, the results of EEG recordings showed that microinjection of pyrilamine, prazosin, or ritanserin suppressed reduction of delta power in EEG induced by orexin A on unconscious rats. Thus, these data suggest that orexins exert arousal effects on alcohol-induced unconscious rats by the promotion of cortical activity through activation of histaminergic, noradrenergic and serotonergic systems.

This article is part of a Special Issue entitled 'Post-Traumatic Stress Disorder'.

1. Introduction

Orexin A and B (hypocretin-1 and -2, respectively) are hypothalamic neuropeptides cleaved from the same preprotein, prepro-hypocretin/preproorexin (de Lecea et al., 1998; Sakurai et al., 1998). Several lines of evidence have confirmed the key role of the orexin system in maintaining state of wakefulness (Saper et al., 2005; Sutcliffe and de Lecea, 2002), which mainly depends on excitatory actions on several subcortical arousal systems, including noradrenergic locus coeruleus (LC), histaminergic tuberomammillary nuclei (TMN), serotonergic dorsal raphe (DR), and the nonspecific thalamocortical projection system (Bayer et al., 2001; Bourgin et al., 2000; Brown et al., 2002; Burlet et al., 2002; Chen et al., 2008; Eriksson et al., 2001; Hagan et al., 1999; Huang et al., 2001; Jones, 2003; Trivedi et al., 1998), as well as the cerebral cortex (Bayer et al., 2004; Li et al., 2010; Song et al., 2006, 2005; Xia et al., 2005a, 2009). Nonetheless, little is known about whether orexins exert an arousal-promoting effect under pathological condition such as coma. Clinical investigations have shown that the level of orexin A in cerebrospinal fluid (CSF) is undetectable in "Hashimoto's encephalopathy" associated with coma (Castillo et al., 2004). Furthermore, Rejdak et al. describe a decreased level of orexin A in the CSF of patients after acute brain injury caused by haemorrhagic stroke (Rejdak et al., 2005). These results are in line with the previous observation in patients with traumatic brain injury (Ripley et al., 2001). Most importantly, orexins have been found to play a pivotal role in the emergence from anesthesia (Kelz et al., 2008; Shirasaka et al., 2011) by significantly shortening anesthesia time (Kushikata et al., 2003) and inducing electroencephalogram (EEG) arousal in these anesthetized rats (Dong et al., 2009, 2006). Together these findings have led to speculation that pharmacological manipulation of orexin system may open new avenue for the treatment of coma.

Abbreviations: PFC, prefrontal cortex; LC, locus coeruleus; TMN, tuberomammillary nuclei; DR, dorsal raphe; CSF, cerebrospinal fluid; EEG, electroencephalogram; i.c.v., intracerebroventricular; LORR, loss of righting reflex; OX_1R, orexin receptor 1; OX_2R, orexin receptor 2.

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doi:10.1016/j.neuropharm.2011.08.047

Coma is a state of continuous 'eyes-closed' unconsciousness characterized by the total absence of arousal and awareness, which is often caused by a variety of factors such as chemical intoxications, serious diseases, and physical injuries (Schiff and Plum, 2000). Among these, acute alcohol intoxication has been one of the most common reasons of coma because alcohol is an available, legal, and frequently used drug and intoxicant worldwide (Tokuda et al., 2003). At high concentrations, alcohol acts as an anesthetic and respiratory depressant on the central nervous system, which impairs cognitive processing and even results in coma (Givens, 1997). Prefrontal cortex (PFC), a brain region with higher EEG frequency during waking and critical for cognitive function (Lambe and Aghajanian, 2003; Yamasaki et al., 2002), is more vulnerable to the effects of alcoholism than other brain regions/systems (Oscar-Berman and Marinkovic, 2003). Expressions of orexin receptors have been observed in deeper layers of PFC (van den Pol, 1999), and our previous electrophysiological experiments have demonstrated orexin A exerts an excitatory effect on the firing activities of PFC neurons in rats *in vitro* (Li et al., 2010; Song et al., 2006, 2005; Xia et al., 2005a). Whether orexins exert a promoting-arousal effect in alcohol-induced unconscious rats and the effect of orexins on PFC neurons in unconscious rats *in vivo* is still unclear.

In the present study, we examined the effects of intracerebroventricular (i.c.v.) injection of orexin A on the behavior and EEG of unconscious rats induced by acute alcohol intoxication as well as the spontaneous activities of PFC neurons by single-unit recording *in vivo*. We then further explored the possible mechanism involved in the excitatory effects of orexin A on alcohol-induced unconscious rats by using orexin receptor 1 (OX_1R) antagonist SB 334867 and orexin receptor 2 (OX_2R) antagonist TCS OX2 29.

2. Materials and methods

2.1. Animals

Adult female Sprague-Dawley rats weighing 230–280 g were used in this study. Animals were individually housed on a 12-h light/12-h dark cycle. Experiments were performed during the light phase. Animal use and all experimental protocols were approved by the Third Military Medical University Animal Care Committee.

2.2. Loss of righting reflex test

According to an earlier report (El Yacoubi et al., 2003), alcohol was given i.p. at 4.48 g/kg in a v/v solution of 32% to induce coma. An animal was considered to have lost its righting reflex if it failed to correct its posture while lying on its back. Duration of loss of righting reflex (LORR) was evaluated by measuring the time interval between the appearance of LORR and recovery of righting reflex after alcohol administration as the criterion for the onset and termination of hypnosis, respectively.

2.3. EEG recordings

The animals were anesthetized by injection of pentobarbital sodium (50 mg/kg) into i.p. During the course of surgery, the heart rate and respiration of anesthetized rats were monitored continuously. Under deep anesthesia, rats were implanted with cortical EEG recording electrodes as previously described (Deboer and Tobler, 2003). Briefly, electrodes were screwed through the skull on the dura over the frontal cortex (3.5 mm lateral to the midline, 3 mm anterior to bregma) and the parietal cortex (3 mm lateral to the midline, 4 mm posterior to bregma) (Paxinos and Waston, 1997). All rats received injections of the analgesic Dipyrone (50% solution [0.11 ml, i.p.]) and the antibiotic Tribissen (24% solution [0.1 ml SC]). The rats were connected to the recording system by a flexible cable and allowed to recover for at least 7 days after surgery.

Recordings of EEG were scored by 10-s epochs for sleep-wake states (Louis et al., 2004). The degree of EEG slowing usually paralleled the degree of alteration of consciousness and indicates its severity. With progression from lethargy to coma, there is diffuse showing of background rhythms from alpha (8–13 Hz) activity to theta (4.5–8 Hz) activity and subsequently delta (1–4.5 Hz) activity in EEG (Young, 2000). Therefore, in this study, delta activity, as an important indicator for coma, was calculated per 5 min epoch and presented as a ratio of delta power in EEG after application of drugs per 5 min/delta power in EEG 5 min before application of drugs.

2.4. Single-unit recordings

In vivo extracellular single-unit recordings of PFC neurons in rats were carried out as described previously (Homayoun et al., 2005). In brief, unconscious rats were operated on by drilling holes in their skull (anterior +3.0 mm from bregma; medial lateral 0.7 mm and DV −3.5 mm) and opening their dura over the PFC area (Paxinos and Waston, 1997). Single-unit firing activities were recorded through the use of glass micropipettes (~1 μm tip) filled with 3 M KCl. After amplified and filtered by the Multiple Channels of Biology Data Acquisition and Processing System (RM6420, Chengdu Instrument Factory, China), the firing signals were acquired at 400 kHz for on-line viewing to verify the recorded activity from a single unit. Continuous data files were saved on a PC computer hard disk for off-line spike sorting. The unit activities were subsequently analyzed per 5-min epoch for averaging discharge rates (bin width, 20 s), which were normalized relative to a 30 min baseline. To avoid the transient effects of the injection, windows of analysis were started 5 min after the injection of the drugs of interest.

2.5. Drugs application

Most of the drugs were obtained from Sigma (St.Louis, Missouri, USA). Orexin A, orexin B, pyrilamine, and prazosin were dissolved in artificial CSF (ACSF) containing (in mM): 126 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 26 $NaHCO_3$, 2 $CaCl_2$ and 10 glucose, pH 7.3–7.4. Ritanserin, SB 334867 and TCS OX2 29 (Tocris bioscience, Bristol, UK) were dissolved in ACSF containing 10% dimethylsulfoxide (v/v). Under deep anesthesia, a stainless steel guide cannula with a dummy cannula (8 mm length), fixed to the skull with two screws and dental cement, were inserted vertically from the skull surface through a bore hole into the lateral ventricle using the following coordinates: 0.5 mm rostral to Bregma, laterality = 1.6 mm, height = −3.4 mm from dura. The volume of drugs was 2 μl and administered by i.c.v. injection to rats through the implanted cannula.

2.6. Statistical analysis

All data were presented as mean ± SEM. Data analyses were conducted with the Statistical Product and Service Solutions (SPSS) version 13 software packages. Statistical analysis was carried out using one-way or two-way repeated-measures analysis of variance with Kolmogorov–Smirnov test for normal distribution followed by post-hoc multiple comparison with Dunnett's or Newman–Keuls tests where appropriate. In all instances $p < 0.05$ was considered statistically significant.

3. Results

3.1. The inhibitory effects of alcohol on the duration of LORR and EEG of rats

The changes in behavior and EEG of unconscious rats were judged by the duration of LORR and the comparison of the percentage of delta wave in EEG before and after application of alcohol. LORR was observed shortly after injection (i.p.) of 32% ethanol solution and lasted for 3.07 ± 0.94 h ($n = 30$). Concurrently, the delta activity in EEG, a marker of slow wave sleep homeostasis (Borbely and Achermann, 1999), was assessed. As shown in Fig. 1A, no delta activities were observed in the waking rats, whereas after injection of alcohol, the delta power appeared. The normalized delta wave in EEG gradually increased (one-way ANOVA, $F_{(6,35)} = 21.54$, $p < 0.01$) and reached a peak level at 20–25 min after the application of alcohol, which was significantly different from that measured in the same animals 5 min before the administration of alcohol (Dunnett's test, $p < 0.01$, Fig. 1B). After the i.p. application of saline, the delta power showed no significant change compared to the baseline (delta wave measured 5 min before injection of saline) during the experiments ($n = 6$) (one-way ANOVA, $F_{(6,35)} = 0.58$, $p = 0.74$).

3.2. Arousal effects of orexins on the duration of LORR and EEG of unconscious rats

Next, we determined the effects of orexins on behavior and EEG of alcohol-induced unconscious rats. As illustrated in Fig. 2A, as soon as the appearance of LORR was observed in the test subjects, orexin A or orexin B (4 nmol, i.c.v.) was applied through the implanted cannula, and the duration of LORR was significantly shortened to 1.77 ± 0.42 h (orexin A, $n = 10$; Dunnett's test,