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REM SLEEP MODULATION BY PERIFORNICAL OREXINERGIC INPUTS TO THE PEDUNCULO-PONTINE TEGMENTAL NEURONS IN RATS

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Abstract—Rapid eye movement sleep (REMS) is regulated by the interaction of the REM-ON and REM-OFF neurons located in the pedunculo-pontine-tegmentum (PPT) and the locus coeruleus (LC), respectively. Many other brain areas, particularly those controlling non-REMS (NREMS) and waking, modulate REMS by modulating these REMS-related neurons. Perifornical (PeF) orexin (Ox)-ergic neurons are reported to increase waking and reduce NREMS as well as REMS; dysfunction of the PeF neurons are related to REMS loss-associated disorders. Hence, we were interested in understanding the neural mechanism of PeF-induced REMS modulation. As a first step we have recently reported that PeF Ox-ergic neurons modulate REMS by influencing the LC neurons (site for REM-OFF neurons). Thereafter, in this *in vivo* study we have explored the role of PeF inputs on the PPT neurons (site for REM-ON neurons) for the regulation of REMS. Chronic male rats were surgically prepared with implanted bilateral cannulae in PeF and PPT and electrodes for recording sleep-waking patterns. After post-surgical recovery sleep-waking-REMS were recorded when bilateral PeF neurons were stimulated by glutamate and simultaneously bilateral PPT neurons were infused with either saline or orexin receptor1 (OX1R) antagonist. It was observed that PeF stimulation increased waking and decreased NREMS as well as REMS, which were prevented by OX1R antagonist into the PPT. We conclude that the PeF stimulation-induced reduction in REMS was likely to be due to inhibition of REM-ON neurons in the PPT. As waking and NREMS are inversely related, subject to confirmation, the reduction in NREMS could be due to increased waking or vice versa. Based on our findings from this and earlier studies we have proposed a model showing connections between PeF- and PPT-neurons for REMS regulation.

Key words: antagonist, chemical stimulation, GABA, glutamate, orexin receptor1, REM-ON and REM-OFF neurons.

INTRODUCTION

Rapid eye movement sleep (REMS) is an instinct behavior and does not have voluntary regulation. It has been identified by the presence of electrophysiological signals and has been detected in all mammals, including humans (Campbell and Tobler, 1984). Normally REMS is not expressed during waking and it appears only after the subject has spent some time in non-REMS (NREMS) (Jouvet, 1975; Gottesmann, 2001). The quantity of REMS gradually decreases through aging; however, it is never absent in life (Roffwarg et al., 1966; Campbell and Tobler, 1984). REMS is affected in most of the altered psycho-somato-patho-physiological states (Petit et al., 1993; Boeve et al., 2007) and many of the physiological processes are affected depending on the duration of experimental REMS deprivation (REMSD) (Kushida et al., 1989). Thus, quite understandably REMS regulation is extremely complex and it exerts global effect throughout the body.

Based on published data from this lab and that of others we have recently proposed a comprehensive model for REMS regulation (Mallick et al., 2012). Essentially the REM-OFF neurons in the locus coeruleus (LC) must cease firing and simultaneously the REM-ON neurons in the pedunculo-pontine area (PPT) must become active for the generation and continuation of REMS. These REM-OFF and REM-ON neurons are under the influence of many factors and brain areas, including those regulating NREMS and wakefulness (Thankachan et al., 2001; Mallick et al., 2004, 2012). Normally REMS does not appear during waking; however, in REMS-associated disorder, narcolepsy, REMS like symptom (atonia) appears during waking (Chemelli et al., 1999; Zeitzer et al., 2006). The postero-lateral perifornical area (PeF), the exclusive site of orexin (Ox)-ergic neurons in the brain, is involved in waking as well as narcolepsy (Chemelli et al., 1999; Estabrooke et al., 2001; Alam et al., 2002) suggesting its role in REMS regulation. Direct modulation of PeF neurons (Alam and Mallick, 2008; Sasaki et al., 2011; Choudhary et al., 2014), presence of Ox-ergic receptors (OX1R) on LC and PPT neurons (Peyron et al., 1998; Nambu et al., 1999) and modulation of REMS by infusion of Ox or OX1R-agonist or antagonist into the LC (Hagan et al., 1999; Horvath et al., 1999; Choudhary et al., 2014) or PPT (Koyama et al., 2004; Takakusaki et al., 2005; Kim et al., 2009) supports the role of Ox on REMS. The Ox-ergic PeF neurons are likely to modulate the REMS and the effect may be mediated by influencing either or both the LC and PPT neurons. In

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Abbreviations: EEG, electroencephalogram; EMG, electromyogram; EOG, electrooculogram; LC, locus coeruleus; NREMS, non-REMS; Ox, orexin; OX1R, orexin receptor1; PeF, perifornical area; PPT, pedunculo-pontine tegmentum; REMS, rapid eye movement sleep; REMSD, REMS deprivation.

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support, recently we have shown that the PeF-induced modulation of REMS is mediated through the LC (Choudhary et al., 2014). However, Ox activates (Hagan et al., 1999; Kim et al., 2009) both the LC (site of REM-OFF) and the PPT (site of REM-ON) neurons, which behave in an opposite manner in relation to REMS and Ox-level has been found to significantly increase in the LC but not in the PPT after REMSD (Mehta et al., 2015). Thus, although Ox is likely to modulate PPT-REM-ON neurons for modulation of REMS, it was necessary to study if PeF-induced modulation of REMS is mediated by Ox acting on the PPT neurons. Therefore, in this study we have investigated if chemical stimulation of the PeF neurons modulated REMS and if the effect was prevented by simultaneous blocking of OX1R antagonist into the PPT. We have observed that stimulation of the PeF Ox-ergic neurons decreased REMS and indeed the effect was prevented by simultaneous infusion of OX1R antagonist into the PPT. To our knowledge this is the first direct evidence confirming that the PeF stimulation influences the PPT neurons for modulating REMS.

EXPERIMENTAL PROCEDURES

Animals

Six adult, healthy, inbred male Wistar rats (250–300 g) were used in the study. All the rats had free access to rodent food and water *ad libitum* and were maintained in 12:12 light: dark cycle at 25 ± 1 °C control temperature. All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of the Jawaharlal Nehru University. All possible steps were taken to minimize sufferings to the rats and to use minimum number of rats to complete the study.

Chemicals used

L-Glutamic acid monosodium salt; SB-408124, an OX1R antagonist and Pontamine Sky Blue were procured from Sigma Aldrich, USA. Isofluorane gaseous anesthesia from Baxter Healthcare Corporation, Guayama, PR, USA was used during surgery.

Surgical procedure and post-surgical recovery

Gaseous anesthesia, isofluorane (Baxter, USA) was used to anesthetize the rats ($n = 6$) to undertake surgical procedures for implantation of electroencephalogram (EEG), electrooculogram (EOG) and electromyogram (EMG) recording electrodes (Singh and Mallick, 1996). Using standard stereotaxic surgical procedure and guided by Paxinos and Watson (Paxinos and Watson, 1998) rat brain atlas stainless steel guide cannulae were implanted targeting PeF at coordinates AP = −3.14, L = 1.4, DV = 8.4 (Choudhary et al., 2014) and targeting PPT at AP = −7.8, L = 1.8, DV = 7.4 (Fig. 1A) (Pal and Mallick, 2009). Briefly, as described earlier (Choudhary et al., 2014) the sterilized guide cannulae with obturators, designed and constructed in the lab using 24 gauge (G) needle, were stereotaxically introduced into the brain such that the tips reached 1 mm above the target area (s). The free ends of the EEG, EOG and EMG electrodes and the ground electrode were soldered to a 9-pin female plug, which along with the cannulae were secured on to the skull using dental acrylic cement. After surgery the rats were allowed to recover for at least a week with adequate post-operative care. During the later half of the recovery period the rats were acclimatized to the recording chamber, handling and mock microinjection. Mock injection was carried out by removal and reinsertion of the stainless-steel obturators to habituate the rats for intra-PeF and -PPT microinjections.

Microinjection of chemicals

A 33 G stainless steel injector cannula (Plastic One, USA) connected to a 2 μl Hamilton syringe by approximately 10 cms long polythene tubing was used for infusion of chemicals locally deep into the desired brain areas, either or both, PeF and PPT in this study. All microinjections were made bilaterally one at a time into the PeF and PPT. All solutions were prepared fresh prior to microinjection.

Chronic recording

Electrophysiological recording of the chronic rats under freely behaving condition was carried out in a semi sound-proof faraday-cage cubicle fitted with a commutator so that the recording wires did not get entangled discomforting the rats. The cage also had a camera to display the rat behavior on to a monitor outside the cubicle without disturbing the rats. Bipolar EEG, EOG and EMG signals were recorded both on paper and computer; the latter using Vital Recorder software (Kissei, Japan) and was later analyzed offline by SleepSign software (Kissei, Japan).

Chemical stimulation of the PeF along with microinjection of OX1R antagonist (SB-408124) into the PPT

The recordings were taken during the light phase for 8 h between 10:00 AM and 6:00 PM with or without microinjection either or both into the PeF and the PPT. Bipolar EEG, EMG and EOG were simultaneously and continuously recorded in a computer using Vital Recorder software as well as on paper using a Grass Polygraph Model 7H. On day1 baseline recording of sleep-wakefulness was taken without microinjection. On day2, 200 nl each of saline and glutamate (40 ng/200 nl) was bilaterally microinjected into the PPT and the PeF, respectively in the same animal and sleep-waking recorded. On day3 no recording was done, while on day4 SB-408124 (712 pg/200 nl) was microinjected bilaterally into the PPT, half an hour before microinjection of glutamate (40 ng/200 nl) into the PeF and recording continued for 8 h. As the baseline and treatments (microinjections) were conducted on the same animal, the same animal served as its own control. This minimized the individual animal variation and other non-specific confounds, if any, in addition to minimizing the use of number of animals in this study.