Role of Orexinergic Projections from Perifornical Hypothalamic Neurons on Locus Coeruleus Neurons in the Regulation of Sleep and Wakefulness

Dissertation
submitted to
Jawaharlal Nehru University
for the partial fulfillment of the award
of the degree of

Master of Philosophy

RISHABH CHARAN CHOUDHARY

School of Life Sciences
Jawaharlal Nehru University
New Delhi 110067, India
January 2010

School of Life Sciences

Jawaharlal Nehru University

New Delhi-110067, India

CERTIFICATE

This is to certify that the research work embodied in this dissertation titled ***"Role of orexinergic projections from perifornical hypothalamic neurons on locus coeruleus neurons in the regulation of sleep and wakefulness"*** has been carried out in School of Life Sciences, Jawaharlal Nehru University, New Delhi. It is being submitted in partial fulfillment of the requirement for the award of M.Phil degree in Jawaharlal Nehru University. This work is original and has not been submitted for any other degree or diploma in this or any other University.

Prof. Birendra N. Mallick
(Supervisor)

Rishabh Charan Choudhary
(Candidate)

Prof. R. Madhubala
(Dean)

ACKNOWLEDGEMENT

It was a dream which came true. A roller coaster ride with many ups and downs ultimately reached its destination. During the course of my thesis work, I was supported by many people. It's a best opportunity to express my gratitude to all of them.

First of all, the credit of taking me to this pinnacle goes to my supervisor Prof. B.N. Mallick. Therefore, I would like to express my deep sense of gratitude and indebtedness to Prof. B.N. Mallick who has always been a guardian, guide and the philosopher His keen interest and constrictive criticisms made his mirage turn into actuality.

I owe a debt of gratitude to Prof. B.N.MAllick for introducing me to the field of sleep-wakefulness and allowed me to work in his laboratory and whose affectionate patronage and encouragements always worked as a morale booster for me.

Word will never be enough to depict the support Prof. R Madhubala Dean, S.L.S. extended during the critical phases of this thesis. I am exceedingly grateful to Dr. Sushil kumar Jha. I also appreciate the help and thoughtfulness rendered by previous deans and all faculty members at School of Life Sciences.

I am also thankful to all the CIF staff, Librarian, and SLS office staff for their assistance in various day-to-day works.I am especially thankful to Meenu mam of SLS for her caring and friendly nature.

I must acknowledge my thanks to my lab seniors Dinesh, Manoj, Sudipta, Vibha, Gitanjali, Manorma, Abhishek, and Sudhuman for their support and guidance and all my friends especially Ravindra, Arojit, Amit, Rupesh, Megha, Vaibhav, Ravikant, Mahendra, Sukhveer, Gunjan, Kamlesh and Anshu for their support and making my time enjoyable during my working period. A special thaks goes to Mudasir for his support. Thank you very much to all of you.

I would also like to express my thanks to Sant Kumar, Ramesh Chand, Jitendra and Rohit for excellent support in the laboratory. I am also thankful to Animal house incharge and all the animal house staff for ensuring proper supply of animals for my research work.

I gratefully acknowledge the Department of Biotechnology (DBT) for financial assistance provided to me in the form of J.R.F. Help rendered by DST, ICMR and CSIR is acknowledged.

Last but not the least, I acknowledge MY PARENTS who had always been a constant source of loving, care and affection and stood by me in every decision that I made. My brother Jitendra, has been very understanding and never let me feel the responsibilities of the family during this tenure. My loving sisters Jaya and Puja, have always been supporting for me. My father has been great teacher for me and gave me the freedom regarding choosing my career and supporting me in my studies with limited resources. Thank you Papa and Mom ....

..... *Rishabh Charan Choudhary*

Contents

Abbreviations

Neurophysiological terms

REM	Rapid eye movement
NREM	Non rapid eye movement
EEG	Electoencephalogram
EOG	Electrooculogram
EMG	Electromyogram
PGO	Ponto-Geniculo-Occipital
LC	Locus coeruleus
PPT	Pedunculo pontine tegmentum
LDT	Laterodorsal tegmentum
DR	Dorsal raphae
TMN	Tubero mammilliary nucleus
Pef	Perifornical hypothalamus
LH	Lateral hypothalamus
Pno	Pontine reticular formation
OXR	Orexin
OXR1	Orexin receptor1
OXR2	Orexin receptor2
SWS	Slow wave sleep
RPO	nucleus reticularis pontis oralis
RPC	nucleus reticularis pontis caudalis
NA	Noradrenaline
Ach	Acetylcholine

Chemical terms

µ	micro
nL	nano litre
µL	micro litre
mL	milli litre
mM	milli molar
M	Molar
min	minute
h	hour
kg	kilo gram

Introduction

The response of an organism, usually upon exposure to the environmental stimulus is referred to as behavior. It can be conscious or unconscious, overt or covert, and voluntary or involuntary. In animals, behavior is largely controlled by the neuro-endocrine system. The study of behavior becomes challenging by the fact that any behavior, be it eating, expression of feelings or sleep-wakefulness, is a subjective phenomenon.

Sleep-wakefulness is a complex behavioral phenomenon, regulated by an assemblage of factors, viz., neural, and humoral. Wakefulness is considered as most attentive alert state having highest level of consciousness to environment where as during sleep consciousness remains in subdued state. Sleep has been classified into two states viz., non-REM (Non-rapid eye movement) and REM (Rapid eye movement) sleep each with its own distinctive, objective electrophysiological parameters. Various theories have been put forwarded to understand the mechanism REM sleep in which the classical electrophysiological and anatomical studies have outlined.

Neural regulation of REM sleep is maintained by the brainstem though other brain areas may modulate them. The mechanism that triggers REM sleep is located in the pontine reticular formation. The Locus coeruleus (LC) receives cholinergic as well as GABAergic projections from other parts of the brain and it has also GABAergic interneurons. Galaninergic and GABAergic neurons from ventrolateral preoptic area also project the LC. The noradrenergic neurons are clustered in the LC region of the brain, which is the primary site for supplying noradrenalin in the brain. The firing of noradrenergic neurons in the LC and serotonergic neurons in the mid brain raphe contributes to wakefulness and these neurons are silent or cease firing during REM sleep hence they are termed as **REM-OFF neurons**. On the other hand, cholinergic neurons in the Laterodorsal tegmentum (LDT) and the pedunculo pontine tegmentum (PPT) in the brain stem increase firing during REM sleep and hence known as **REM-ON neurons**. The anterior portion of hypothalamus including preoptic area is important for the regulation of sleep and the posterior portion regulates wakefulness. At least two distinct nuclear groups have been implicated for the generation of REM sleep. They are the cholinergic neurons in LDT and PPT and the noradrenergic neurons in the LC in the dorsolateral pons.

Orexins are a pair of excitatory neurotransmitters (neuropeptides) which is released from neurons in the perifornical (PeF) hypothalamic area that act on orexin receptors (OXR-1 and 2), present in the brain and are reported to modulate sleep and wakefulness (Peyron *et al.,* 1998).Orexinergic neurons gives it projection to almost all brain area in which, LC receives the heaviest projection, and tubero mammillary nucleus, dorsal raphe, basal forebrain receives moderate projections. Noradrenergic LC-REM-OFF and cholinergic LDT and PPT REM-ON neurons play important role in maintenance of REM sleep. LC-REM-OFF neurons remain active during waking but cease firing during REM sleep (Chu and Bloom, 1973). Orexin has been reported to increase waking (Hagan *et al.,* 1999) and glutamate stimulation of PeF increases waking while decreases REM sleep (Alam and Mallick, 2008). Since orexin is an excitatory neuropeptide and LC receives the heaviest projections, it is possible that orexinergic projection to LC keeps these neurons active and maintains wakefulness and prevents REM sleep occurrence during waking. Hence, in this study it has been proposed to stimulate PeF with glutamate and simultaneously block orexinergic receptors in the LC and record the effects on sleep-waking and REM sleep in freely moving normally behaving rat.

Review of literature:

SLEEP: AN OVERVIEW

Sleep-Wakefulness is a complex combination of physiological and behavioral processes regulated by different areas of brain. Sleep has been defined as a reversible state of perceptual disengagement and unresponsiveness to the environment. Sleep is usually accompanied by postural recumbence, quiescence and closed eyes. Sleep is a physical and mental resting state in which a person becomes relatively inactive and unaware of the environment. In essence, sleep is a partial detachment from the world, where most external stimuli are blocked from the senses.

QUANTIFICATION OF SLEEP

Sleep being as a subjective phenomenon objective quantification is done by using various electrophysiological parameters. They are-

1. ELECTROENCEPHALOGRAM (EEG) - EEG is the recording of electrical activity along the scalp produced by the firing of neurons within the brain.
This methodology is known as *electroencephclography*.The record of the signals is referred as the *electroencephalogram*

2. ELECTROOCULOGRAM (EOG) - Recording of the movements of the eyes.

3. ELECTROMYOGRAM (EMG) - Recording of the muscle tone from the antigravity muscles.

4. HIPPOCAMPAL THETA RHYTHM- Generators for the theta rhythm are located in the pyramidal cells of CA1 region, in the dentate gyrus and in the medial entorhinal cortex. Hippocampal waves are usually recorded from the CA1 region of the hippocampus.

5. PONTO-GENICULO-OCCIPITAL WAVES (PGO WAVES)-PGO waves originate in the pontine region and propagate rostrally through the lateral geniculate and other thalamic nuclei to the cortex. PGO waves are best characterized in cats and are recorded from pons, lateral geniculate body and occipital cortex.

REM sleep has been identified and quantified in many species including humans (Aserinsky and Kleitman, 1953; Rechtschaffen and Kales, 1968), cats (Dement 1958; Ursin and Sterman, 1981), dogs and monkeys (Reite *et al.*, 1965) rats (Michel *et al.*, 1961; Timo-Iaria *et al.*, 1970) and in mice (Higashi A, 1983). The total time spent in sleep is negatively correlated with the body size but has a positive correlation with the time spent in REM sleep (Zepelin, 1994). Therefore larger animals spend less time in NREM sleep and consequently experience less REM sleep. It has also been proposed that predators (e.g. cats) are usually good sleepers as compared to prey species (e.g. rabbit) with REM sleep occupying 15% or more time spent in sleep (Bert *et al.*, 1977).

Berger in 1929 first recorded EEG, the first electrophysiological correlate and showed in humans that its frequency and amplitude are inversely related to each other. On the basis of EEG, EOG, EMG, PGO waves and theta waves, sleep-wakefulness cycle in animals can be objectively classified into following five stages:

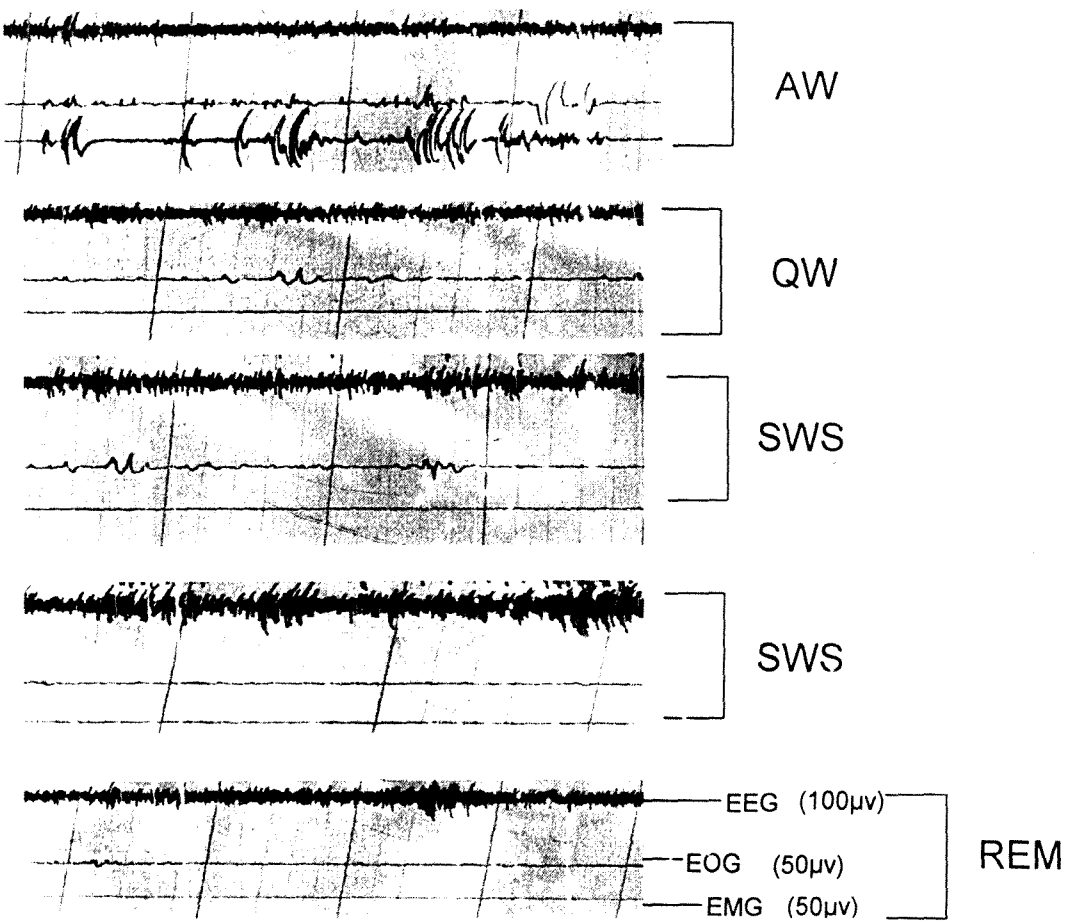


Fig: 1.Electrophysiological patterns of various sleep stages

Active Wakefulness (AW) - This is a state of awareness in which an individual is conscious of his or her surrounding environment and has the ability to interact with it. EEG is desynchronized posses low voltage and high frequency (14-30 Hz) known as beta rhythm in humans, and amplitude that can vary from 20-50 uV, among different species. Eye

movement and high muscle tone are present. Theta rhythm appears in the hippocampal waves. PGO waves do not show activity though occasional spikes may be present during eye movements. The EEG in rat shows bandwidth of 30-40 Hz and amplitude of 30μV approximately (Timo-Iaria., 1970).

Quite Wakefulness (QW) - The beta waves (present during active wakefulness) are replaced by spindle waves (waxing and waning of EEG in range of 7-14 Hz in cat (Steriade 1995) along with mostly low voltage fast waves. EOG and EMG activities are present but are remarkably reduced. Hippocampal waves show high amplitude spindle and PGO (in cat) waves are absent. In human this EEG state is called alpha rhythms. Both active wakefulness and quite wake fullness together comprise of about 31.5% of the time spent in the 24 hr day-night cycle in cat (Jouvet 1969).

Sleep onset is associated with slowing of the EEG activity and the rising EEG amplitude, followed in most mammals by the appearance of spindling and oscillation, delta waves and slow sleep oscillation.

Sleep has two distinct states, **non-REM** sleep and **REM** sleep

Non-REM sleep:

Slow Wave Sleep 1 (SWS1) - EEG is synchronized and spindle oscillations dominate. EEG show high amplitude (ranges 75-400 μV in different species) Spindles show a bandwidth of 2-5 Hz in dogs, 6-7 Hz in sloth, 8-11 Hz in opossum (Zepelin 1994). In rat spindle frequency varies from 6-12 Hz having amplitude ranging from 30-300 μV (Timo-Iaria *et al.*, 1970). PGO waves are absent in cat.

Slow Wave Sleep 2 (SWS2) – Spindles become reduced and progressively overhelmed by low frequency (1-4Hz) high voltage EEG waves (delta waves). Irregular high-voltage slow waves in the the hippocampal activity with lowering activity in EMG and no activity in EOG recorded. PGO waves are absent in cat.

The slow wave sleep and deep sleep comprises of 5205% of the total time spent in the 24hr day-night cycles (Jouvet, 1969).

Rapid Eye Movement (REM) Sleep – REM sleep is the deepest sleep state when the threshold for arousal is maximum and it mostly follows deep sleep state. EEG waves show low amplitude high frequency (4-8 Hz) theta waves referred as active or desynchronized sleep state in cat (Vertes, 1982). As compared to wakefulness, there is little increase in voltage in EEG viz ~40 μV in REM sleep compared to ~30 μV in wakefulness in rats (Timo-Iaria *et al.*, 1970).

Hobson reported in *Hyla* and *Rana* that fast frequencies EEG are suggestive of REM sleep-like states in *Anurans (Amphibians)* (Hobson, 1967).Very small amount of REM sleep like state also found in *squamata (Reptiles)* (Tauber, Roffwarg et al., 1966; Taubar, Rojas-Ramirez *et al.*, 1968; Huntley, Friedman et al., 1977; Ayala-Guerrero and Huitron-Resendiz, 1991; Ayala-Guerrero, Huitron-Resendiz, 1994). Aphibians evolved about 400 million years ago in the Devonian period and they radiate in the Carboniferous period, about 50 million years later (Sarnat and Netsky, 1981). Reptiles evolved in Carboniferous period, of Paleozoic era, approximately 350 million years ago and dominated in Permian period about 280million years ago (Sarnat and Netsky, 1981; Carrol, 1988), thus REM sleep is believed to have evolved at least as early as 300 million years ago. In Avian and mammalian species REM sleep phylogenetically evolved some 140 million years ago from a common ancestor of marsupial and placental mammals (Allison and van Twyver, 1980; Siegel 1996; 1999).

REM sleep with its characteristic features like the occurance of rapid eye movement during this phase (therefore may be called REM sleep) and its repetitive alteration with periods of cortical synchronization was described (Dement and Kleitman, 1957) after a similar cycle had been discovered in human sleep (Aserinsky and Kleitman, 1953).The desynchronized phase of human sleep was also found to be associated with rapid eye movements and with the psychological experience of dreaming (Dement and Kleitman, 1957). The individual may experience fluctuations in heart rate and rhythm during the stage. Desynchronization

of the cortical electrical activity in the sleeping cat was first reported in association with muscle twitch (Derbyshire *et al.,* 1936) and interpreted as second. "deep", stage of sleep (Klaue, 1937). Because of paradoxical nature of REM sleep state to wakefulness and sleep state, based on behavioral and electrophysiological characteristic, REM sleep has also been termed as "*sommeil paradoxical*" or desynchronized sleep or active sleep or dream sleep (Jouvet and Michel, 1959). Within REM sleep periods a distinction is made between the tonic and bursts of phasic REM state every 16-120 seconds and lasting from 2-9 seconds (Aserinsky and Kleitman, 1953). Phasic REM sleep is characterized bt bursting of rapid eye and middle ear movements and tonic REM sleep is characterized by cortical and hippocampal EEG patterns. Phasic REM sleep is preceded by, ponto-geniculo-occipital EEG waves (PGO spikes in animal preparations) originating bilaterally in the dorso-lateral pons and projecting rostrally through the lateral geniculate nucleus (LGN) and other thalamic nuclei (Hobson, 1969). During REM sleep, general atonia is maintained as a result of marked and sustained hyperpolarization of the motorneurons (Chase et al., 1989).

REM episodes repeat a number of times during sleep. In humans this stage recurs at an interval of approximately 90 minutes with its frequency of occurrence and duration stint increasing with passage of time in sleep. The time taken for first appearance of REM sleep after going to sleep is known as **REM Latency** and has clinical significance as it is altered in certain disorders. Consistent with the overall increase in neural activity during REM sleep, these levels becomes equal to those during waking state.

REM sleep is regulated by brain stem though other brain areas may modulate it as well. The neurons in the LC cease firing during REM sleep and if an experimental animal was not allowed to have REM sleep, these neurons in the LC continued firing incessantly leading to disturbance or loss of REM sleep. Alternatively, if these neurons were not allowed to cease firing either by continuous electrical stimulation (Mallick *et al.*, 1996) or by applying antagonist of the neurotransmitter that keep these neurons inhibited (Mallick *et al.*, 1997. Mallick *et al.*, 2004), REM sleep did not continue resulting in its reduction. Thus , the neurons in the LC must cease firing (as if it is a pre requisite) for the generation of REM sleep and non-cessation of these neurons caused reduction of REM sleep associated

with increased levels of nor-adrenalin (NA) in the brain which ultimately induces the effects associated to REM sleep deprivation /loss.

Localization of area(s) in brainstem responsible for the regulation of REM sleep- Moruzzi and Magoun were the first to demonstrate the involvement of medial aspects of the brain stem reticular formation in the induction and maintenance of cortical desynchronization. Later it was shown that nucleus pontis caudalis (Jouvet 1962) as well as nucleus pontis oralis (Carli and Zenchetti, 1965) are involved in cortical activation. The activity of the LC NA-ergic system (Jouvet, 1972; Berridge and Foote, 1996; Shouse *et al.*, 2000) is causally and positively related with behavioral and EEG indices of arousal. Also, the PPT neurons through their projections to the intralaminar and midline thalamic nuclei (Lavoie and Parent, 1994) function as a part of the non-specific activating system responsible for cortical desynchronization and regulation of sleep- wake cycle (Steriade *et al.*, 1990). It is known that EEG desynchronization occur during waking and REM sleep and that NA-ergic LC as well as cholinergic LDT/PPT are known to be responsible for EEG desynchronization through their projections to thalamus. However it has been reported that there are separate groups of neurons in the pontine region responsible for EEG desynchronization during wakefulness and REM sleep (Mallick *et al.*, 1998). Initial studies to localize anatomical structure in the brain responsible for the regulation of REM sleep generation started with transection and lesion experiments. A transection is a complete separation of one brain region from another by using surgical technique. A lesion is a localized destruction of a group of neurons or a group of nuclei. Concept behind such study was that if any normal manifestation, behavioral or otherwise, of a living organism continued to be expressed even after the destruction of certain brain area (s), the damaged area of the brain is possibly not essential for normal manifestation of the function under consideration. In 1935, Bremer conducted the now classical *cerveau isole* and *encephale isole* experiments on cats. Bremer showed that intercollicular transection (*cerveau isole)* that separates brainstem from forebrain at the level of upper midbrain resulted in EEG pattern reflecting a continuous state of sleep (Bremer, 1935). When the transection was made between C1 and C2 segments of spinal cord (*encephale isole*), thus separating the brain from spinal cord inputs, the sleep-wake cycle persisted (Bremer 1936).

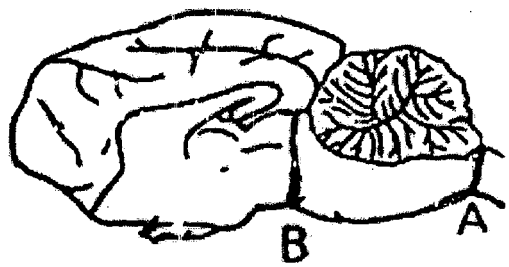


Fig: 2. A. Encaphale isole B. Cerveau isole (cat)

(Taken from:" *Brain control of wakefulness and sleep*",By Mircea Steriade, Robert W. McCarley)

Bremer put froth the argument that the sensory input in *cerveau isole* is not sufficient for forebrain arousal and hence, a state of permanent synchronized EEG that is similar to sleep state occurs. However in *encephale isole* preparation, the sensory input from the cranial nerves particularly from the fifth (trigeminal) and eighth (vestibulocochlear) nerve are preserved, which provide sufficient sensory stimulation to maintain the sleep wake rhythm. Morruzi and Magoun in 1949 and Morruzi, 1972 proposed that there is an *ascending reticular activating system* (ARAS), which maintains the arousal levels required to keep the brain awake. Therefore, the sensory afferents are not directly responsible for waking process (as interpreted by Bremer). Instead these afferents activate the reticular formation via their collaterals (Starzl *et al.*, 1951).Thus sleep was considered as a state resulting from functional de-afferentation of the ARAS or putting it simply, sleep is a passive phenomenon that results from blocking of external sensory input to the brain. Morruzi's group further hypothesized that caudal brainstem is involved in sleep induction (Batini *et al.*, 1959). After bilateral electrolytic lesions in the dorsolateral pontine tegementum affecting LC suggested the role of this area in the REM sleep (Jouvet and Delrome 1965). In an attempt to further localize the neurons generating REM sleep, various lesion and transaction studies carried out. Carli and Zenchetti's 1965 identified that nucleus pontis oralis (RPO) is consistently correlated with REM sleep. Jouvet in his initial studies (1962) identified nucleus reticularis pontis caudalis (RPC), as the structure crucial for REM sleep, which also overlapped with Carli and Zanchetti's definition of raphe pontis oralis (RPO).

However further studies by Jouvet's group (Jouvet, 1972) pointed to the LC, not RPC as the crucial structure for REM sleep.

Pontine region and regulation of REM sleep:

The pontine region contains noradrenergic, cholinergic as well as GABA-ergic neurons. The noradrenergic neurons are clustered in the LC, which is the primary site for supplying NA in the brain. The functional characteristics of these NA-ergic neurons in the LC are that they cease firing during REM sleep (Bloom *et al.*, 1981, Jacob *et al.*, 1986) and hence they have been termed as REM-OFF neurons. Monaminergic REM-OFF neurons are present in LC as noradrenergic neurons (Chu and Bloom, 1973; Hobson *et al.*, 1975; Aston-Jones and Bloom, 1981), in dorsal raphe (DR) as serotonergic neurons (McGinty and Harper,1976), in tuberomammilary nucleus(TMN) of hypothalamus as histaminergic neurons (Sherin *et al.*,1998). On the other hand, the cholinergic neurons in the laterodorsal tegmentum LDT and PPT in the brainstem increase firing during REM sleep and they have been termed as REM-ON neurons. (Sakai *et al.*, 1989; Steriade *et al.*, 1990). The present knowledge indicates that interplay between REM-ON and REM –OFF neurons located in these nuclei in the pontine region are responsible for the generation and regulation of REM sleep (Hobson *et al.*, 1975; Sakai, 1988).

Locus coeruleus; an anatomical description:

The LC is small clusters of neurons situated in the pontine region near the wall of 4th ventricle and is one of the few pigmented structures in the brain. Depending on the size of the cells and their organization, the LC has further been subdivided into LC-principal, LC-α, peri LC- α and sub coeruleus by some sleep researchers (Sakai *et al.*, 1981). The LC or its analogue, projecting to the forebrain is not found in reptiles (Parent and Poitras 1974) and avians (Hashimoto *et al.*, 1974), though some catecholaminergic neurons projecting to the cerebellum and nearby tegmentum has been reported in teleosts and amphibian (Shimizu *et al.*, 1976). The number of neurons in LC increases from 200 in parakeet to

1600 in rats and 20,000 in human (Singewald and Philippu 1998).Projections from these neurons divide into ascending and descending branches and innervate almost all the areas in the brain, spinal cord (Skagerberg *et al.*, 1982, Lyons and Fritschy, 1989) and brainstem (Fritschy and Grazanna 1990). The LC receives cholinergic (Luppi *et al.*, 1995, Jones 1990) as well as GABA-ergic projections (Aston-Jones *et al.*, 1989 and 1991) from other parts of the brain and it also has GABA-ergic interneurons (Iijima and Ohtomo 1988). Galanin-ergic and GABA-ergic neurons from ventrolateral preoptic area also project to the LC (Sherin et al., 1998, McGinty et al., 2001).

Locus coeruleus and REM sleep:

There is ample evidence that brain noradrenergic system plays a significant role in the regulation of REM sleep. Several techniques including electrical as well as chemical lesion, stimulation and microinjection have been extensively used to explore the role of LC in regulating REM sleep. Electrical destruction of the dorsal part of LC did not suppress the occurence of REM sleep. (Jones and Harper, 1977). Similarly, destruction of ventral part of LC (LCα and peri LC- α), was followed by irreversible disappearance of REM sleep atonia (Henly and Morrison 1974). However, destruction of LCp and LCα along with peri- LCα suppressed REM sleep during the two post lesion months (Sakai *et al.*, 1978). Electrolytic lesions of the dorsal noradrenergic bundle that ascends from the LCp (Jouvet *et al.*, 1973) resulted in increase in both non-REM sleep and REM sleep (Sakai et al., 1975). The firing rate of the neurons in the LCp is maximum during wakefulness, decreases during non-REM sleep and almost cease during REM sleep (Aston-Jones and Bloom 1981, Jacob *et al.*, 1986, Hobson *et al.*, 1975) while that of the neurons located ventrally increase their firing rate (almost exclusively) during REM sleep (Sakai *et al.*, 1981). The activity of the NA-ergic neurons in the LC has been positively correlated with activation of the sympathetic nervous system (Reiner 1986). Sympathetic activation is normally accompanied by EEG desynchronization and according to Reiner, the activity of the LC-NA-ergic neurons increases with an increase in discharge in the sympathetic nervous system. The effect of deprivation of REM sleep at the single neuronal level has been studied in cats and it was observed that REM-OFF neurons do not cease firing during REM sleep and they continued

firing during REM sleep deprivation. This and various other studies further led Mallick and coworkers to hypothesize that cessation of LC neurons is pre-requisite for the generation of REM sleep (Pal *et al.*, 2005). The hypothesis was confirmed by the results of a study where chronic preparation of normally behaving, free moving rats received mild, low frequency but long term bilateral stimulation in the LC which reduced REM sleep significantly by reducing the frequency of generation of REM sleep during the post-stimulation period (Singh and Mallick,1996). This suggested that if the LC neurons could be kept active and not allowed to stop firing, REM sleep would not be generated. Later it was proposed that the cessation of LC neurons is necessary for the onset of REM sleep and a detailed mechanism for the same is reviewed (Pal et al., 2005; 2007).

Activation of LC neurons during Wakefulness:

Normally REM sleep does not appear during wakefulness or immediately after going to sleep. It appears after certain period of non rapid eye movement (NREM) sleep. At least in humans, the duration and number of REM sleep episodes increase with progress and depth of sleep through the night. Although it was known that REM sleep can not be initiated as long as the LC noradrenergic REM-OFF neurons continued firing, the cellular mechanism of sleep wake state dependent changes on the LC neuronal firing from highly active state during wakefulness to slowing down during NREM sleep and finally cessation of firing during REM sleep was not known. Mallick's group proposed that the wakefulness inducing area the midbrain reticular formation (MRF) possibly exerted opposite influence on REM–OFF and REM–ON neurons and hypothesized that MRF would excite REM-OFF and inhibit REM-ON neurons during wakefulness. In a combined single unit recording and MRF stimulation study carried out in freely moving normally behaving cats, it was observed whose firing rate increased during spontaneous wakefulness, including the REM–OFF neurons, were excited, while the REM-ON neurons were inhibited by the MRF wakefulness inducing area. These results supported hypothesis and suggested that the wake active neurons and MRF continuously excite the NA-ergic REM-OFF neurons in the LC and inhibit the REM-ON neurons through out the waking period. This view may also be supported any the fact the activation of the REM-OFF neurons is reported to prevent REM

sleep and is likely to increase the level of NA in the brain causing cortical activation and desynchronization of EEG. Therefore, it is likely that continuous activation of noradrenergic REM–OFF neurons contributes to EEG desynchronization associated with wakefulness but not with that of REM sleep. This view may be supported by the power spectrum analysis study in the freely moving cats that's the adrenergic and cholinergic antagonist affected different higher frequency bands of the desynchronized EEG. Additionally, as mentioned above, MRF wakefulness inducing area inhibited the REM-ON neurons and this may be the cause for non activation of REM-ON neurons during waking period. It may be supported by the fact that activation of area containing REM-ON neurons increases REM sleep.

The perifornical (PeF) area in the posterior lateral hypothalamus has been implicated in regulation of sleep–wakefulness (Hagan et al., 1999; Siegel, 2004) by giving its projection to the wake promoting area. Electrical stimulation of the PeF area evokes locomotor activity and electroencephalogram (EEG) activation as well as autonomic effects such as higher blood pressure and heart rate (Krolicki et al., 1985). Most of the neurons within the PeF area Increase their firing rate during wakefulness and decrease their activity during slow-wave sleep (Koyama et al., 2003; Lee et al., 2005). Orexin excite dorsal raphe, locus coeruleus, tuberomammillary and basal forebrain cholinergic neurons by activating postsynaptic receptors in these regions (Hagan et al., 1999; Eggermann et al., 2001). LC posses the densest concentration of OXR1 (Peyron 1998).). Activation of neurons in and around the PeF including those of orexin neurons contribute to the promotion of arousal and suppression of non-REM and REM sleep (Alam et *al.*, 2008).

Dorsal Raphe (DR) and REM sleep:

The dorsal raphe (DR) is located in the midbrain and pontine central gray. It has serotonergic, peptidergic, (Cholecystokinin and neuropeptide Y) and dopaminergic neurons. (Monti 2000). DR has reciprocal connection with cerebral cortex , limbic system, basal forebrain, hypothalamus, raphe nuclei, LC, pontine reticular formation (Semba and Fibiger,1992) and LDT/PPT (Bockstaele *et al.*,1993; Losier and Semba, 1993; Iwakiri *et al.*, 1993; Honda and Semba, 1994; Vertes and Kocsis, 1994; Gonzalo-Ruiz *et al.*, 1995;

Peyron *et al.*, 1998; Monti,2000).The activity of DR neurons is highest during wakefulness, diminishes during NREM sleep and completely cease during REM sleep (McGinty and Harper,1976; Trulson and Jacobs,1979).The seretonergic neurons in DR are REM-OFF neurons. The DR along with medial raphe sends heaviest seretonergic projections to REM sleep induction zone in pontine reticular formation (Semba, 1993).Seretonin has also been shown to inhibitory effect on cholinergic neurons (Strecker *et al.*, 1994; Amici *et al.*, 2004;).

Tuberomammilary nucleus (TMN) and REM sleep:

The tuberomammilary nucleus (TMN) in the posterior lateral hypothalamus is the sole source of histamine in the brain (Lin *et al.*, 1988; Saper, 2000). The histaminergic neurons are clustered in two main groups; one is situated ventrolaterally along the edge of brain and other dorsolaterally along the edge of the mammilary of the third ventricle (Saper, 2000). Histaminergic neurons are exclusively localized in the TMN (Watanable *et al.*, 1984), and projects to practically all brain regions, with especially dense innervations in the hypothalamus, basal forebrain and amygdala (Panula and Costa 1984, Takeda et al., 1984). TMN has robust projection to the DR and LC (Lee et al., 2005). The firing rate of histaminergic neurons vary across the sleep/wake cycle. These neurons fire maximally during wakefulness slow down during NREM sleep and cease their firing during REM sleep (Sherin *et al.*, 1996). Histaminergic neurons cease their firing during cataplexic episode (John *et al.*, 2004).

Role of Hypothalamus, Hypocretin/Orexin in REM sleep:

Von Economo (1930) believed that the posterior hypothalamus contained a "wake centre". The hypocretin-producing cell bodies are specific to the hypothalamus and have widespread anatomical projections within the central nervous system of the rat with the densest extra-hypothalamic projection to the noradrenergic Locus Coeruleus (LC) and lesser projections to the basal ganglia, thalamic regions, the medullary reticular formation ,and the nucleus of solitary tract.There are minor projections to the cortical regions,central

and anterior amygdala nuclei, and olfactory bulb (de Lecea *et al.*, 1996, Mignot, 2000, Peyron *et al.*, 1998). Orexin producing neurons ,localized in the lateral hypothalamic area (LHA), send projections to all over the brain region except the cerebellum. Especially the orexinergic fibers are found in monoaminergic and cholinergic nuclei in the brain stem region (Marcus *et al.*,2001).Orexins are a pair of excitatory neurotransmitter acts on their receptor (ORXR-1 and ORXR-2), present in brain and modulate sleep and wakefulness (Peyron *et al.*,1998).Orexin has been reported to increase wakefulness (Hagan *et al.*,1999).

The Orexin/Hypocretin system:

In 1996, Gautvik, de Lecea, and colleagues reported the discovery of several genes in the rat brain, including one they dubbed "clone 35." Their work showed that clone 35 expression was limited to the lateral hypothalamus (de Lecea L, *et. al.* 1996).They discovered a hypothalamic-specific mRNA by using substractive RNA hybridizaton technique that encodes a substance they named preprohypocretin,the precursor of two neuropeptide named hypocretin-1 and hypocretin-2 Luis de Lecea, Thomas Kilduff, and colleagues reported discovery of these peptides, termed them *hypocretins* to indicate that they are synthesized in the hypothalamus and to reflect their structural similarity to the hormone secretin (i.e., *hypo*thalamic se*cretin*). They mapped the location of the peptides and concluded that they were restricted to neuronal cell bodies in the hypothalamus,both peptides are found in same neuron (de Lecea L, *et. al.* 1996, 1998). Masashi Yanagisawa and colleagues at the University of Texas Southwestern Medical Center at Dallas, found t two peptides in hypothalamic extracts orexin-A and orexin-B while they were looking for ligands for a number of orphan receptors and coined the term *orexin* after adminstration of these peptide ito rat lateral ventricle and found that eating was increased and because of this they named "orexins," from the Greek word for apetite (appetite-stimulating) activity of these hormones (Yanagisawa M, *et al.*,1998).

Structure of Orexin/Hypocretin:

The human preproorexin gene is located on chromosome17q21 and is composed of 616 nucleotides contained in 2 exons and 1 intron. The first exon contains the initial part of the secretory signal sequence, with the somewhat smaller second exon containing the rest of the signal sequence as well as the coding region of orexin (Sakurai T, 1999). A 3.2-kb promoter region directs expression specifically to the lateral hypothalamus. The orexin prohormone is proteolytically cleaved into 2 peptides, orexin A and orexin B, which are coexpressed in neurons of the LH/PFA. Orexin A is comprised of 33 amino acids and is highly conserved across mammalian species (Sakurai T, 1998). The 28-residue orexin B peptide is identical in the rat and the mouse, with human orexin B displaying 2 amino acid substitutions (Sakurai T, 1998 and Sakurai T, 1999). Two orexin receptors, designated OX1R and OX2R, have been identified (Sakurai T, 1998).Both are G protein–coupled receptors and have significant homology at the transcript level.OX1R signals via activation of phospholipase C (PLC) and increases intracellular Ca^{2+}. In contrast, OX2R couples to Gq and Gi/o, and the activation of the OX2R results in increased PLC and decreased adenylyl cyclase activity (Zhu Y., 2003). Both orexins A and B have affinities of approximately 30 nM for the OX2R. The 2 orexin receptors differ critically in that orexin A also binds the OX1R at approximately 30 nM, whereas orexin B has a very low affinity (approximately 420 nM) at the OX1R (Sakurai T, 1998).

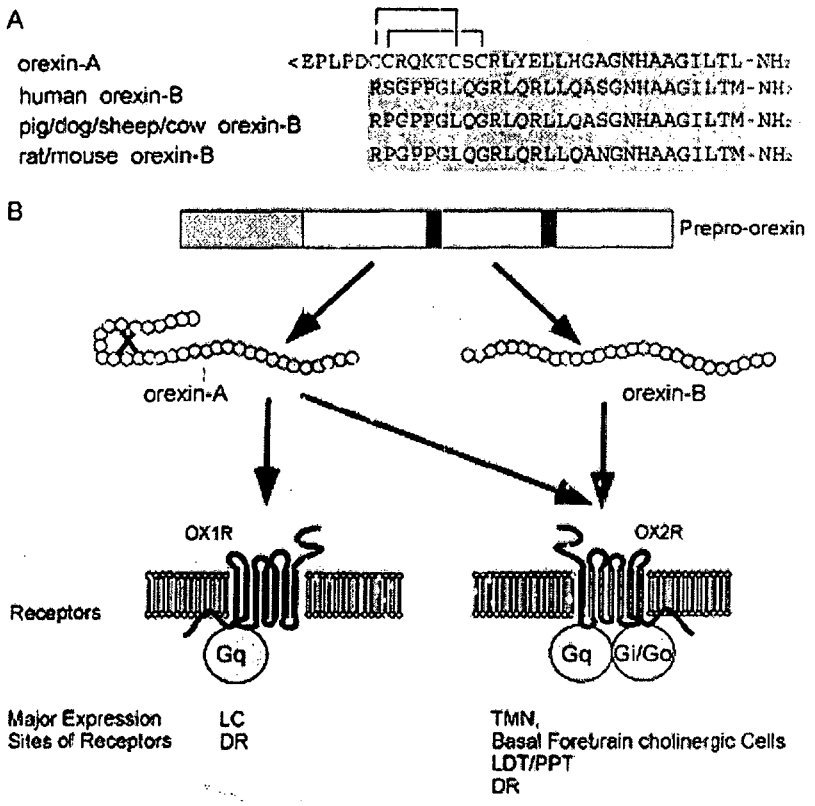


Fig: 3. Taken from- *Physiological Review, Roles of orexin/hypocretin in regulation of sleep/wakefulness and energy homeostasis (Sakurai T., Sleep Medicine Review, 2005 9, 231-241).*

The overview of the orexin system: (A) sequence (In figure 3) of mature orexin-A and -B peptides. The topology of the two intrachain disulfide bonds in orexin-A is indicated above the sequence. Shadow indicates amino acid identities.Mammalian orexin-A sequences thus far identified (human, rat, mouse, pig, dog, sheep, cow) are all identical. (B) Schematic representation of the orexin system. Orexin-A and -B are derived from a common precursor peptide, prepro-orexin. The actions of orexins are mediated via two G protein-coupled receptors named orexin-1 (OX1R) andorexin-2 (OX2R) receptors. OX1R is selective for orexin-A, whereas OX2R is a non-selective receptor for both orexin-A and orexin-B. OX1R is coupled exclusively to the Gq subclass of heterotrimeric G proteins, whereas OX2R may couple to Gi/o, and/or Gq.

Anatomical organization of Orexin/hypocretin:

Neurons expressing preproorexin mRNA are restricted to the LH/PFA. Immunohistochemical studies indicate that orexins are expressed in only about 70,000 cells in humans (Thannical *et al.*, 2000), with about 3000 orexin neurons in the rat (de Lecea L, *et. al.*2006). Despite the small number of orexin cells, orexin axons are found in almost all areas of brain, a notable exception being the cerebellar cortex. The widespread distribution of orexin axons can be attributed to the fact that single orexin neurons often collateralize to innervate multiple targets (Berridge *et al.*,2005). Although orexin axons are found in most of the brain, the relative density of orexin fibers varies considerably, with particularly high densities seen in the LC, paraventricular nucleus of the thalamus, septum and diagonal band complex, and the infralimbic and prelimbic aspects of the prefrontal cortex. (Peyron *et al.*,1998). Orexin receptors are distributed in a pattern consistent with orexin projections.mRNAs for ORX1R and ORX2R are differentially expressed throughout the brain (Marcus et al., 2001). ORX1R mRNA is enriched in neurons of the hypothalamus, thalamus, locus coeruleus, cerebral cortex, hippocampal formation, and basal ganglia (Harrison *et al.*,2001) with the distribution of OX1R-binding sites and immunoreactivity paralleling the distribution of OX1R mRNA. OX2R mRNA is enriched in the pons, medulla oblongata, hypothalamus, and thalamus (Trivedi *et al.*, 1998) with highest distribution in TMN neurons (Marcus *et al.*, 2001).

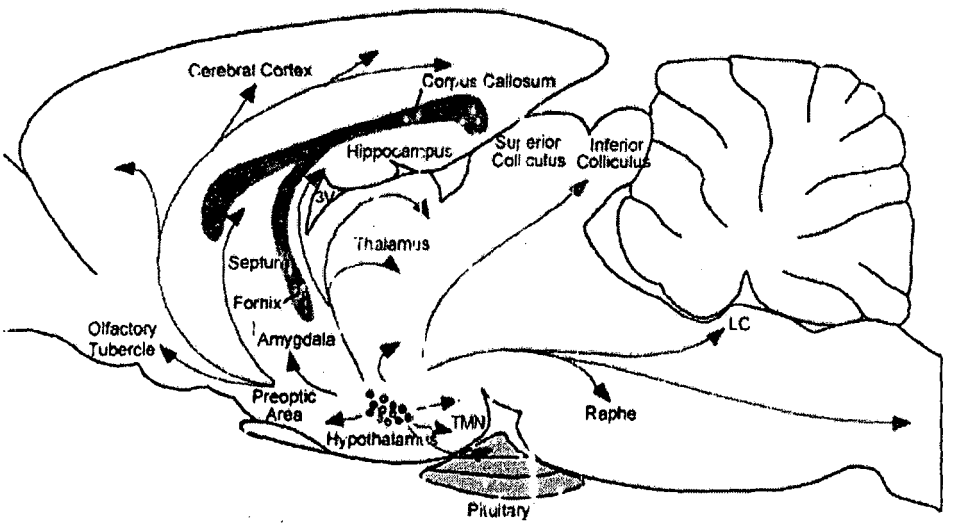


Fig: 4. *Schematic drawing of saggital section through the rat brain, summarizing the organization of the orexin neuronal system, Orexin neurons giving their projection to the different neurons.*

Taken form: *Physiological Review. Roles of orexin/hypocretin in regulation of sleep/wakefulness and energy homeostasis (Sakurai T., Sleep Medicine Review, 2005 9, 231-241).*

Hypothalamic OX1R distribution:

Low level of mRNA expression is present in the preoptic nuclei, with more moderate levels in the medial preoptic nucleus. Robust expression is seen in the anterior hypothalamic nucleus and the dorsomedial portion of the VMH. Moderate levels of OX1R mRNA are seen in the dorsal hypothalamus, ventral premammillary nucleus, posterior hypothalamus, and supramammillary nucleus. Diffuse expression is present in the LHA and the expression is absent in paraventricular, arcuate, suprachiasmatic, and tuberomammilary nuclei. (Marcus J, 2006).

Hypothalamic OX2R Distribution:

Tuberomammilary nucleus (TMN) contains the most densest OX2R expression in the hypothalamus.Supramammilary nucleus,and posterior hypothalamus express OX2R mRNA. OX2R mRNA is less robust than OX1R mRNA in the ventromedial nucleus and dorsal hypothalamus. Arcuate, dorsomedial, parvocellular paraventricular, medial mammillary, lateral mammillary nuclei also contain mRNA expression for OX2R.Diffuse OX2R mRNA is also present in the lateral hypothalamic area (Marcus J, 2006).

Forebrain OX1R distribution:

In cortical regions, OX1R mRNA is prominent in layer 5 and 6 of the cingulate,infralimbic, and prefrontal cortices, with less present in layer2. With in the basal fore brain,OX1R mRNA can be seen in nucleus of diagonal band.In hippocampus , OX1R mRNA is primarily located in the dentate gyrus, and in the CA1 and CA2 region of Ammon'a horn. In the thalamus, the paraventricular nucleus contains robust expression of OX1R mRNA, where as low level of expression are seen in other midline thalamic nuclei (Marcus J, 2006).

Forebrain OX2R distribution:

mRNA for OX2R are expressed at moderate levels in brain are the bed nucleus of tha stria terminalis and within the cortical nucleus of the amygdala. The basal forebrain contains robust OX2R mRNA expresion in the medial septal nucleus and both the vertical and horizontal limbs of the nucleus of the diagonal band In the hippocampus, OX2R mRNA is seen in the CA3 region of Amman's horn .In addition to other midline thalamic nuclei the

rhomboid and paraventricular thalamic nuclei contain small amount of OX2R mRNA expression in thalamus (Marcus J, 2006).

Midbrain,Pons, and Medulla OX1R mRNA:

Locus coeruleus and A5 cell group most robustly expressed mRNA for OX1R .The A4 and A7 noradrenergic cell group are also contain high levels of OX1R mRNA expression. More moderate levels of mRNA expression are seen in the ventral tegmental area, the substantia nigra, periaqueductal gray, and zona incerta. The PPT and LDT nuclei both express OX1R mRNA. The raphe nuclei, including the DR, medial raphe, and raphe obscurus have moderate levels of OX1R mRNA expression.In the hind brain, there is moderate expression of OX1R mRNA in the dorsal motor nucleus of the vagus,the nucleus of the solitary tract, and the reticular formation, extending to possible A1/C1 neurons in the ventrolateral medulla (Marcus J, 2006).

Midbrain, Pons, and Medulla OX2R mRNA:

Caudal to hypothalamus, OX2R mRNA is also expressed in a wide variety of brain regions. Low levels of diffuse mRNA expression are present in the nucleus of solitary tract, nucleus ambiguous, and parabrachial nucleus. Pontine gray, dorsal and medial raphe nuclei, dorsal motor nucleus of vagus, spinal trigeminal nucleus, hypoglossal nucleus, and facial motor nucleus has robust expression of mRNA for OX2R. OX2R mRNA is present in sensory nuclei such as the principal trigeminal sensory nucleus.The midbrain reticular formation and the lateral reticular formation also have moderate levels of OX2R mRNA expression. (Marcus J, 2006).

Hypocretin/Orexin in sleep and wakefulness:

The state of wakefulness depends upon interaction between different groups of neurons. Ascending input from brainstem monoaminergic (Serotonergic, noradrenergic, adrenergic, histaminergic, and dopaminergic) neurons are generally thought to promote wakefulness, alertness and desynchronized activity in the cerebral cortex, as reflected in the form of EEG. Monoaminergic neurons show their highest rate of firing during wakefulness and inhibit the 'REM-on' neurons in the LDT and PPT. These LDT and PPT cholinergic neurons are responsible to maintain REM sleep thus are also known to be 'REM-on' neurons. Orexinergic neurons in the Pef promote waking and suppress non-REM /REM sleep (Sakurai, 2007 and Siegel J, 2004) through interaction between different groups of neurons. Orexinergic neurons are most active during waking and are quiescent during non-REM/REM sleep (Yanagisawa, 2001, Jones 2005 and Siegel 2005). Since mRNA expression for both OX1R and OX2R vary across the different brain region, LC shows abundantly mRNA expression for OX1R (Harrison *et al.*, 2001) and TMN for OX2R (Marcus *et al.*, 2001) Hypocretin/orexin is excitatory neurotransmitter and gives the excitatory projection to different group of neurons like: noradrenergic LC neurons (Bourgin *et al.*,2000, Hagan *et al.*, 1999, Horvath *et al.*, 1999, Ivanov and Aston-Jones 2000,van den Pol *et al.*,2002), serotonergic DR (Liu *et al.*,2002), histaminergic TMN (Bayer *et al.*,2001) , and cholinergic basal fore brain nuclei (Eggermann *et al.*,2003).These orexinergic neurons also gives the excitatory projection to thalamic nuclei (Bayer *et al* 2002) and all these neurons are responsible to cortical desynchronization thus orexin/hypocretin promotes wakefulness. Further the glutamic acid stimulation of the perifornical-lateral hypothalamic area promotes arousal and inhibits non-REM/REM sleep (Mallick *et al.*, 2008).

Orexin/Hypocretin in Narcolepsy:

Narcolepsy is characterized by excessive day time somnolence, disturbance in sleep architecture and cataplexy (Scammell T E, 2003, and Siegel J M, 2004), rapid-onset REM sleep episodes and hypnagogic hallucinations (Aldrich, 1991). Narcolepsy is neurodegenerative disorder and caused by loss of neurons containing the neuropeptide

orexin/hypo cretin (Peyron *et al.*, 2000; Thannickal *et al.*, 2000). Along with the neuronal loss, patients with narcolepsy have low to negligible levels of Orexin-A in the cerebrospinal fluid (Nishino *et al.*, 2001).Two other peptides, dynorphin and pentraxin (Neuronal activity –related peptide) colocalize with orexin are also absent in human *post mortem* tissue (Blouin *et al.*, 2005; Crocker *et al.*, 2005), indicating death in the orexinergic neurons rather than reduction of peptide. Targeted deletion of prepro-orexin gene in mice shows behavioral similarity that of human narcolepsy (Chemelli *et al.*, 1999), the orexinergic neurons is destroyed in narcolepsy (Geraschenko *et al.*, 2001; Hara *et al.*, 2001; Beuckmann *et al.*, 2004), and null mutation in OX2R were found in familial narcoleptic dog stated that narcolepsy is the result of mutation in OX2R (Lin *et al.*, 1999).

Orexin/Hypocretin in feeding behavior:

The orexin in perifornical (PeF) area in the posterior lateral hypothalamus has been implicated in several physiological functions including the regulation of food intake, energy homeostasis, locomotor activity and regulation of sleep–wakefulness (Hagan et al., 1999; Siegel, 2004). Microinjection of orexin/Hypocretin neuropeptide into rat lateral ventricle increases feeding behavior. (Sakurai 1998). Administration of orexin by i.c.v. injection induced a significant increase of food intake and locomotor activity, whereas i.p. injection of glucose or i.c.v. injection of anti-orexin serum decreased food consumption. These results indicate that the orexin functions as feeding behavior in gold fish (Sakurai, 2006). The effects of mammalian orexin 1 and 2 on feeding in goldfish have been studied. Analysis of feeding has been performed following i.c.v. injection of the peptides directly into the third ventricle of anaesthetized fish either by analyzing feeding acts (Volkoff 1999) or by measuring food consumption per body weight (Volkoff 1999). In these assays, orexin 1 was more effective than hcrt 2. In ornate wrasse *Thalassoma pavo* intraperitoneal mammalian orexin 1 also significantly increases locomotion and feeding (Facciolo 2009). Fasting has also significant effects on brain hcrt expression in both zebrafish and goldfish: proionged fasting significantly increases brain hcrt mRNA in both goldfish (Nakamachi et al. 2006) and zebrafish (Novak et al. 2005 Hypothalamic orexin plays an important role in glucose homeostasis in rat (Kalsbeek, 2009). Hypothalamic orexin stimulates feeding-

associated glucose utilization in skeletal muscle via sympathetic nervous system (Shiuchi, 2009).

Hypothesis regarding generation of REM sleep:

Monoaminergic theory:

Proposed by Jouvet; this hypothesis stated that REM sleep is primed by serotonergic neurons which act upon cholinergic 'relay' which in turn triggers 'REM executive' neurons in LC region. This theory was put in doubt by the finding of other workers that selective lesioning of LC noradrenergic neurons did not disrupt REM sleep (Henley and Morrison, 1974; Jones *et al.*, 1977).

Reciprocal interaction theory:

Proposed by Hobson and colleagues (Hobson *et al.*, 1975). According to this theory the REM-off neurons in LC are inhibitory to REM-On neuronal populations and also to themselves where as REM-ON neurons are excitatory to the REM-OFF neurons of LC and also to themselves. It also stated that inactivation of putative monoaminergic REM-OFF neurons plays a critical role in the generation and maintenance of REM sleep. This theory fails to explain the mechanism of activation of REM-ON neurons.

Mutual inhibitory theory:

Proposed by Sakai (Sakai 1988). According to this hypothesis cessation of firing of the REM-OFF neurons excites the REM-ON neurons by disinhibition while the excitation of the REM-ON neurons inhibit the REM-OFF neurons.Therefore,REM sleep can appear either by excitation of the REM-ON neurons or by inhibition of the REM-OFF neurons. This hypothesis stated that for the generation of REM sleep, cholinergic neurons directly inhibit NA-ergic REM-OFF neurons. Kodama and co-workers in 1990 shown that ACh

increased around LC during spontaneous REM sleep .Also microinjection of ACh agonist into LC increased REM sleep (Baghdoyan et al., 1984). But Ach did not hyperpolarize LC neurons (Egan and North, 1986). Based on the above findings that ACh may be an initiating factor in inhibition of REM-OFF neurons, but the actual inhibition of REM-OFF neurons in LC might be induced by an inhibitory neurotransmitter mechanism for the regulation of REM sleep (Ali, 1999).

GABA-ergic interneuron based model:

Proposed by Mallick *et al.*, 1998. According to this model the cholinergic input from REM-ON neurons excite the GABA-ergic interneuron in an around LC, which inturn inhibit the NA-ergic LC neurons facilitating the generation of REM sleep. It was mentioned earlier that the REM-OFF neurons in the LC must stop firing during REM sleep but on the other hand, the cholinergic REM-ON neurons simultaneously increased firing during REM sleep (Hobson *et al.*, 1975., Mallick *et al.*, 1998). Acetylcholine increased around LC during REM sleep (Kodama, 1990) and microinjection of acetylcholine agonist in LC increased REM sleep(Beghdoyan *et al.*,1984; Vanni-Mercier *et al*,.1989; Quattrochi *et al.*,1998).Since ACh did not hyperpolarize the LC neurons (Egan and North, 1986), it was proposed by Mallick and group (Ali, 1999) that Ach might trigger the action , the actual inhibition of REM-OFF neurons in LC could be induced by an inhibitory neurotransmitter leading to the generation and regulation of REM sleep. A GABA-ergic interneuron based model is shown as in fig 5.

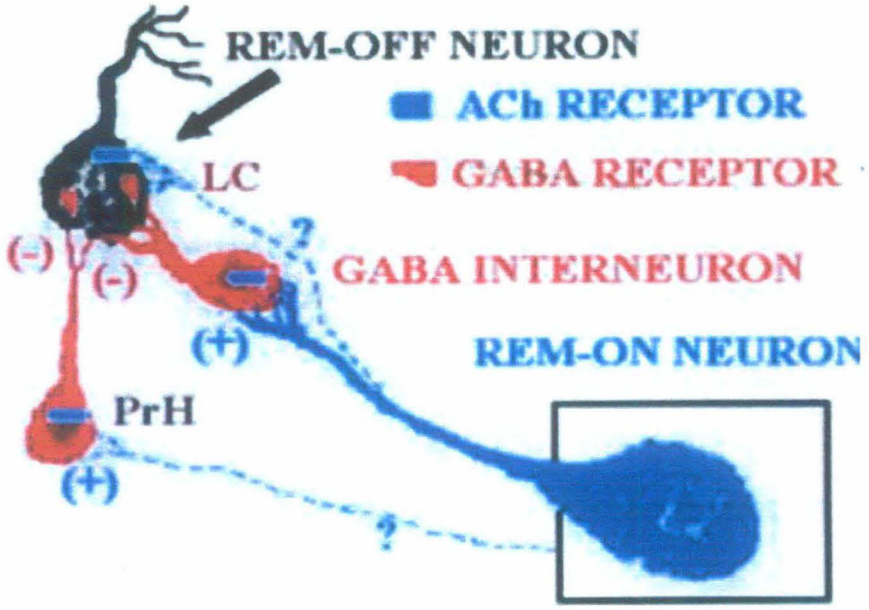


Fig: 5. Showing Possible interaction between REM-ON and REM-OFF neuron (Taken from Mallick *et al.*, 2002)

Lacunae

1. The PeF area contains several cell types including those expressing hypocretins (also known as orexins), melanin-concentrating hormone, γ-aminobutyric acid (GABA) and glutamate (Broberger et al., 1998; de Lecea et al., 1998; Peyron et al., 1998; Sakurai et al., 1998). Role of orexinergic neuron in Pef in LC mediated regulation of Sleep and wakefulness is not clear.
2. Orexinergic neurons excite dorsal raphe, locus coeruleus, tuberomammillary and basal forebrain cholinergic neurons by activating postsynaptic receptors in these regions (Hagan et al., 1999; Bourgin et al., 2000; Ivanov & Aston-jones, 2000; Eggermann et al., 2001; Eriksson et al., 2004) It was not known whether orexinergic neurons directly projects LC and excite through orexin receptor 1.

Objectives

1. To study the effect of glutamate microinjection in Pef on sleep wakefulness in chronic preparation of rats (freely moving normally behaving).
2. To study role of locus coeruleus Orexinergic receptor1 in regulation of sleep wakefulness through PeF orexinergic neurons.

Materials and methods

Animals:

We used seven inbred male Wistar rats (250-300 g) which were maintained on 12:12 light: dark cycle (Lights on at 7:30 am and off at 7:30 pm) at controlled temperature (25±1°C) that had freely access to food and water. Animals for experiment were taken from University Animal House Facility. All experimental procedures involving animals were conducted with the approval of Institutional Ethics Committee. Our full efforts were made to minimize animal suffering and the number of animals used.

Stereotaxic surgery:

Rats (250-300 g) were surgically implanted with EEG, EOG, and EMG electrodes for bipolar recording of sleep-wakefulness and two guide cannulae in Pef (Perifornical-lateral hypothalamic area) and LC (Locus Coeruleus) for bilateral microinjections under surgical gas anesthesia (Iso-fluorane). In brief, for bipolar EEG two stainless steel screw electrodes were fixed in the skull (2 mm anterior and 4 mm lateral to bregma). Another screw electrode was fixed on the midline over the frontal sinus which was taken as animal ground. For EMG a pair of flexible, stainless steel insulated wire (except at the tip) was inserted bilaterally in to the dorsal neck muscle, while for EOG recording another pair of wires were connected to external canthus of eyes. A pair of guide cannulae (22G stainless steel tube) with blocker were implanted targeting Pef bilaterally (AP= -3.14, L= 1.4, H= 8.4), another pair of guide cannula (22G stainless steel tube) with blocker were implanted targeting LC bilaterally (AP= -9.68, L= 1.3, H= 7.3), in such a way so that their tips remained 2 mm above the target area (Paxinos and Watson, 1997). The recording electrodes including ground were connected to nine pin female plugs, and fixed onto the skull using dental acrylic, along with guide cannula.

Brain atlas:

The atlas by Paxinos and Watson (1997) was used in this study. It is based on flat skull position of the rat in the stereotaxic apparatus. The flat skull position was achieved by lowering the incisor bar 3.3±0.4 mm below horizontal zero. The atlas refers to bregma and

interaural line as reference points. Bregma is defined as the point of intersection of the sagittal suture with the curve of the best fit along the coronal suture. When the both sides of the coronal suture meet at the sagittal suture at different points, bregma usually fall midway between the two junctions. Another reference point is lambda, and is defined as the mid point of the curve of best fit along the lamboid suture. Lamboid is located 0.3±0.3 mm anterior to the interaural line. The top of the skull at the bregma and lambda is 10.0+0.2 mm dorsal to interaural zero plane.

In this study, stereotaxic instrument manufactured by INCO (Ms INCO PVT LTD, Ambala, India).

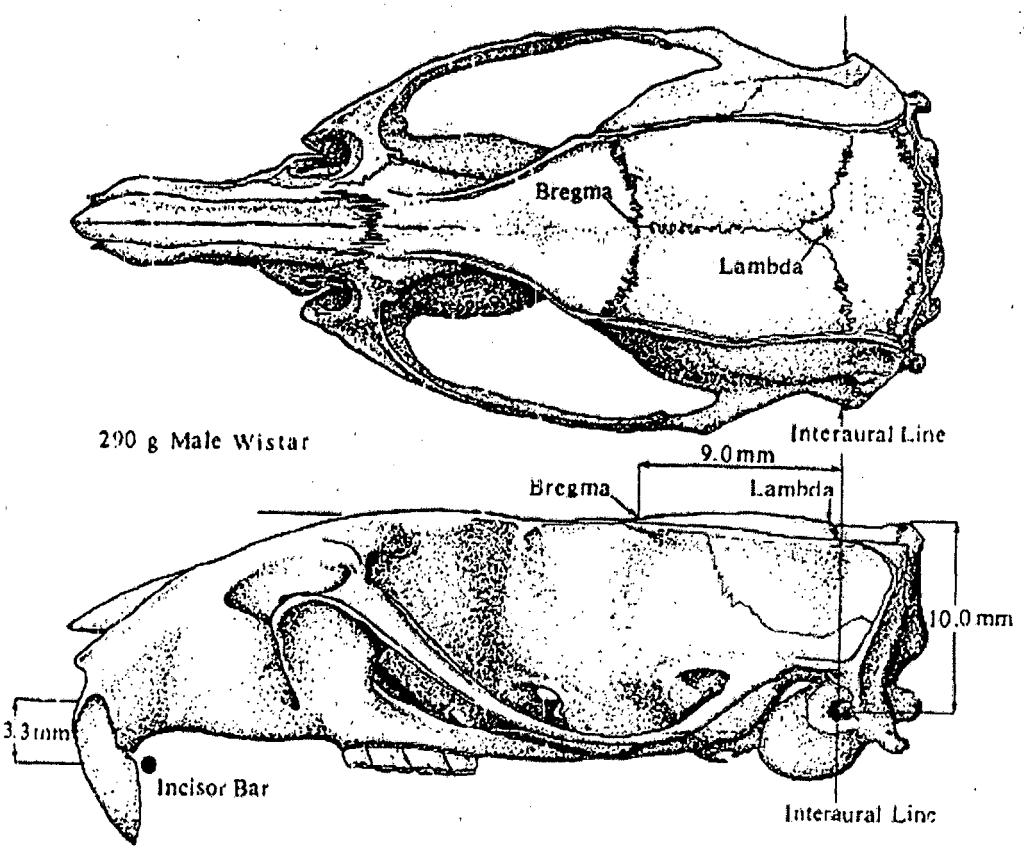


Fig: 6. Dorsal and ventral view of skull (290g) of a male Wistar rat .The positions of coronal suture, sagittal suture, lamboid, suture,Bregma,Lambda and plane of inter aural line are shown. The reference position in this study was taken from bregma.

Polygraph:

Grass Polygraph Model 7H used in this study of electrophysiological signals.

Electrodes:

I. **EEG electrodes:** Radio wires were soldered to small stainless steel EEG screws. These electrodes were then screwed on the animal skull. For EEG recording the frequency had filter setting on the polygraph was set at 1 Hz (Low cut off) and 100 Hz (high cut off) and the 50 Hz filter was recorded between 1-100 Hz with 50 Hz filter out.

II. **EOG electrodes:** Radio wires were stripped on one end and a wire loop was made from the exposed surface. The loop ends of the wire were soldered and were connected to the external canthus muscle of the eye of the animal. EOG was recorded between 1-30 Hz (Low and high) with 50 Hz filter in use.

III. **EMG electrodes:** Radio wires were stripped on one end and a wire loop was made from the exposed surface. The loop ends of the wire were soldered. The wire was passed through a hypodermic needle (20 G) with the loop end inside the dermis and connected to the dorsal neck muscle. EMG was recorded between 1(Low cut off) and 100 Hz (high cut off) with 50 Hz filter in.

Guide cannula:

Two 22 Gauge stainless steel cannula (22 mm) were soldered parallel to a lateral distance of 2.8 mm for Pef and 2.6 mm for LC. The cannula of desired length was made by cutting 22 Gauge hypodermic needles. Blockers were made from stainless steel wire of 28 Gauge.

Injector cannula:

28 Gauge stainless steel cannula (24 mm), was fitted with polyethylene (PE 20, Plastics One Inc., USA) tubing at one end. The tubing was connected to Hamilton syringe for microinjection manually. The cannula end was inserted into the animal brain passing through guide cannula.

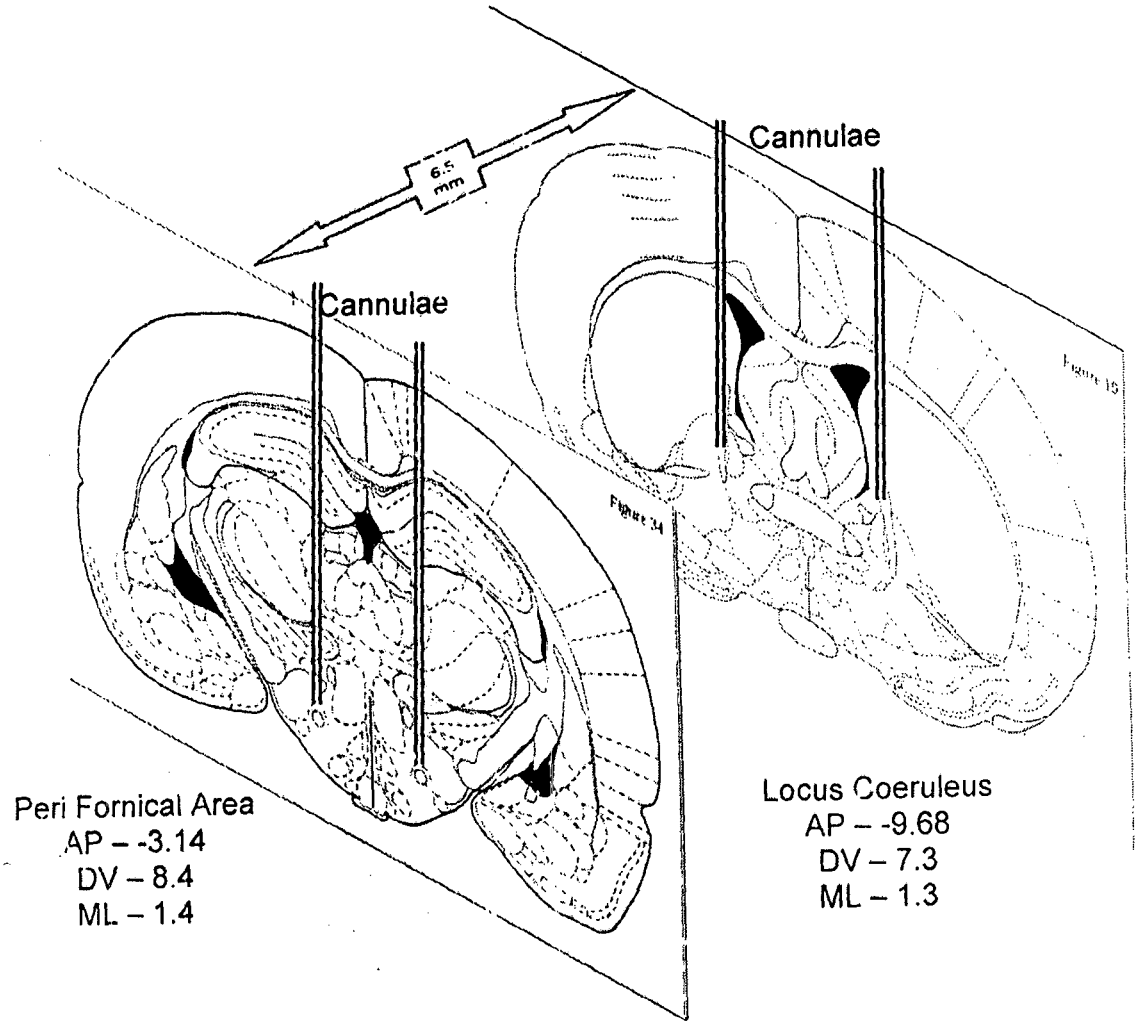


Fig: 7. Showing Guide Cannula targeting Pef and LC

Post operative care and habituation:

Operated rats were kept for 7 days with post operative care to recover from surgical trauma. After four days of recovery rats were acclimatized to the recording chamber.

Post operative care includes:

1. Intramuscular injection of gentamycin (antibiotic) for 5 days from the day of surgery [(2.5 mg/kg body wt) (Deboer *et al.*, 1998)].
2. Intramuscular injection of dexamethasone (anti-inflammatory) for 3 days from the day of surgery [(2 mg/kg body wt) (http:// ratguide.com/meds/)].
3. 1 ml of sterile, dextrose normal saline was given intraperitoneally.
4. Daily cleaning with betadine (antimicrobial) solution till the wound healed.
5. Nebasulf powder (antimicrobial) was applied till the wound healed.

Recording and microinjection:

Recordings were taken on light phase for 8 h between 9:00 am and 7:00 pm with or without microinjection in Pef as well as in LC. EEG, EMG and EOG recording were taken on Grass Polygraph Model 7H. Baseline recording for sleep-wakefullness was taken on the first day without any microinjection. After first day onwards, rats were microinjected with either saline or drug. On the second day 200 nl of saline was microinjected bilaterally into the Pef as well as in LC. On the third day 200 nl of saline was microinjected bilaterally into the LC and 200 nl saline containing 40 ng glutamate in Pef. On the fourth day of recording 200 nl of saline containing (0.356µg/ml) orexin-1 receptor antagonist (SB-408124) was microinjected bilaterally into the LC, half an hour before microinjection of 200 nl saline containing 40 ng glutamate in Pef. After microinjection recoding were continued for 8 h.

Data analysis:

After data acquisition, the records were manually scored in 10 sec epochs and scored in term of waking that included both active waking and quiet waking, non-REM sleep that included both slow wave sleep-1 and slow wave sleep-2 and REM sleep. The percentage of waking, non-REM and REM sleep, were calculated for each animal during baseline, saline, glutamate and glutamate and orexin-1 receptor antagonist (SB-408124). Each parameter during all four conditions was statistically compared using one way RMANOVA (Repeated measure analysis of variance) followed by pair wise multiple comparison test.

Confirmation of microinjection site:

Perfusion:

After all the recordings were completed, rats were anaesthetized with high dose of anesthesia Ketamine 80 mg/kg body wt and Xylazine 10 mg/kg body wt (Paul *et al.*, 1997) in combination and 200 nL of 2% pontamine sky blue dye (also known as Chicago blue dissolved in 0.5 M Sodium acetate) was injected manually. After that rats were perfused intracardially. During perfusion the thoracic cavity was opened to expose the heart. Flexible polyethylene tubing connected to a reservoir containing 0.1 M sodium phosphate buffer saline (PBS, pH=7.4) was introduced into the left ventricle and a cut was made on the right auricle. PBS 100 ml entering was followed by 100 ml of 4% paraformaldehyde. After the completion of perfusion, cranium was removed and brain was taken out carefully. The brain was kept in 4% paraformaldehyde at least for 24 hrs for fixation and after that transferred to 10% sucrose solution prepared in 0.1 M Phosphate buffer. After the brain sample in 10% sucrose solution, it was transferred to 30% sucrose solution prepared in 0.1 M Phosphate buffer.

Sectioning:

Using Leica Cryostat, 40 µm sections for LC and 40 µm sections for Pef was cut. The temperature of chuck holder (holding the brain) was maintained at -15 °C and the temperature of the blade and cryostat chamber was maintained at -30 °C. Area of interest was directly taken on slide (prepared in subbing solution) for neutral red staining and in PBS for immunostaining.

Neutral red staining:

The sections were allowed to dry and stick properly onto the glass slides for 2-3 days. Thereafter, the staining was done as follows:

i. The glass slides with sections were rinsed in distilled water.

ii. Slides kept in neutral red stain for 5-10 minutes.

iii. The sections were dehydrated in ascending alcohol grades (30%, 50%, 70%, 90% and 100%) and cleared in xylene.

iv. Mounted in DPX.

Subbing solution:

The coating of glass slides with gelatin is called subbing.

Composition of subbing solution:

Gelatin	- 1 g
Distilled water	- 200 ml
Chrome alum	- 0.1 g (Chromic potassium sulphate)

The above compounds were mixed and heated at 60 °C under continuous stirring, till the solutes dissolved completely. Glass slides were dipped into subbing solution and then left over night for drying.

RESULT

We used total nine rats for this study, in which only five rats received the treatment at the desired targets i.e LC and Pef, and so, only five animals data were used for analysis. Among four rats which were off-targeted for the desired site, two rats didn't get the confined location for LC and the other two were not targeted by Pef.

Effect of glutamate in Pef on sleep and wakefulness:

Significant reduction in REM sleep percentage [Mean ± SEM (5.1 ± 0.2)] was observed, as compared to baseline (7.6 ± 0.5)] and saline microinjection (7.5 ± 0.6) which were taken as control. Also, significant decrease in SWS2 percentage (14.9 ± 1.0) was observed as compared to saline microinjection (18.5 ± 1.1). AW, QW and SWS1 were not significantly affected, but total waking percentage (AW + QW) was significantly increased as compared to saline (38.1 ± 2.8). Significant decrease were also observed in total NREM sleep (SWS1 + SWS2) after glutamate microinjection in Pef (45.4 ± 1.5) as compared to saline (54.3 ± 3.1).

Although significant decrease in REM sleep duration(s)/ hr in glutamate microinjection in Pef (187.0 ± 8.8) was observed as compared to saline (270.2 ± 24.8) and baseline (277.5 ± 18.5), but decrease in REM sleep frequency was insignificant.

In the other two rats the administrations were off target and hence, no significant difference was observed compare to controls.

Effects of orexin receptor1 (OXR1) antagonist (SB-408124) into LC and simultaneous glutamate microinjection in Pef:

No significant differences were observed when five stages *viz.* AW, QW, SWS1, SWS2 and REM sleep were analyzed separately among different drug treatment (n=5). But total waking percentage (AW+QW) was found to be significantly increased in glutamate microinjection (49.2 ± 1.4) as compared to glutamate injection in Pef and OXR1 antagonist in LC (35.6 ± 4.0) and there were no difference observed among saline (38.1 ± 2.8) and

baseline (40.1 ± 3.8) waking percentage. Although there was significant decrease in NREM sleep (SWS1 + SWS2) also observed in glutamate microinjection (45.4 ± 1.5) as compared to glutamate microinjection in Pef and OXR1 antagonist (57.0 ± 4.2) in LC.

The difference in decrease in REM sleep duration/hr and REM sleep frequency in antagonist treatment in LC with simultaneous glutamate microinjection in Pef was not found to be significant when compared with glutamate microinjection in Pef.

In those rats (n=2) in which, OXR1 antagonist microinjection were not localized in LC the changes in REM sleep percentage was observed equals to the glutamate microinjection in Pef.

Table -1

MEAN (± SEM) PERCENT DURATION OF S-W-REM SLEEP STAGES

S. No.	GROUP	AW	QW	SW1	SW2	REM SLEEP
1	BASELINE	22.6 ± 2.6	17.5± 2.4	33.7 ± 1.7	18.3± 2.4	7.6 ± 0.5
2	SALINE	18.5 ± 3.8	19.5± 1.8	35.8± 2.4	18.5 ± 1.1	7.5± 0.6
3	SALINE + GLUTAMATE	26.9± 3.2	22.2 ± 2.2	30.6± 1.5	14.9 ± 1.0	5.1 ± 0.2
4	GLUTAMATE + OXR1 ANTAGONIST	17.3 ± 3.8	18.2 ± 2.8	39.5 ± 5.4	17.4 ± 2.4	7.36± 0.8

Table -2

MEAN (± SEM) PERCENT DURATION OF W-NREM-REM SLEEP STAGES

S. No.	GROUP	WAKING	NREM	REM
1	BASELINE	40.1± 3.8	52.0 ± 3.4	7.6 ± 0.5
2	SALINE	38.1 ± 2.8	54.3 ± 3.1	7.5 ± 0.6
3	SALINE + GLUTAMATE	49.2 ± 1.4	45.4 ± 1.5	5.1 ± 0.2
4	GLUTAMATE + OXR1 ANTAGONIST	35.6 ± 4.0	57.0 ± 4.2	7.3 ± 0.8

Table-3

EFFECT OF MICRO INJECTION OF GLUTAMATE AND OXR1 ANTAGONIST ON REM SLEEP FREQUENCY/HR AND REM SLEEP DURATION (MEAN ± SEM)

S. No.	GROUP	REM SLEEP FREQUENCY/HR	REM SLEEP DURATION/HR
1	BASELINE	9.4 ± 1.1	277.5 ± 18.5
2	SALINE	9.4 ± 1.1	270.2 ± 24.8
3	SALINE + GLUTAMATE	7.8 ± 0.7	187. 0 ± 8.8
4	GLUTAMATE + OXR1 ANTAGONIST	10.1± 0.6	264.2 ± 29.2

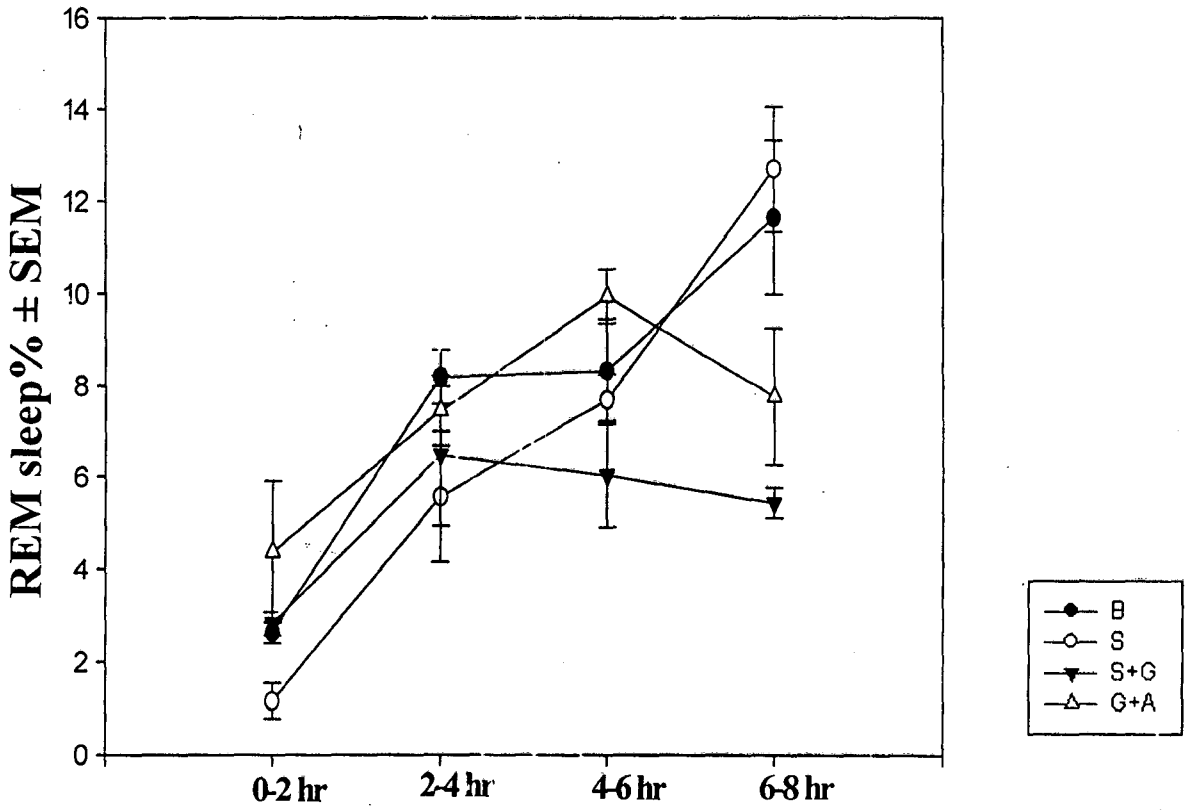


Fig: 8- Percentage of REM sleep during different time interval

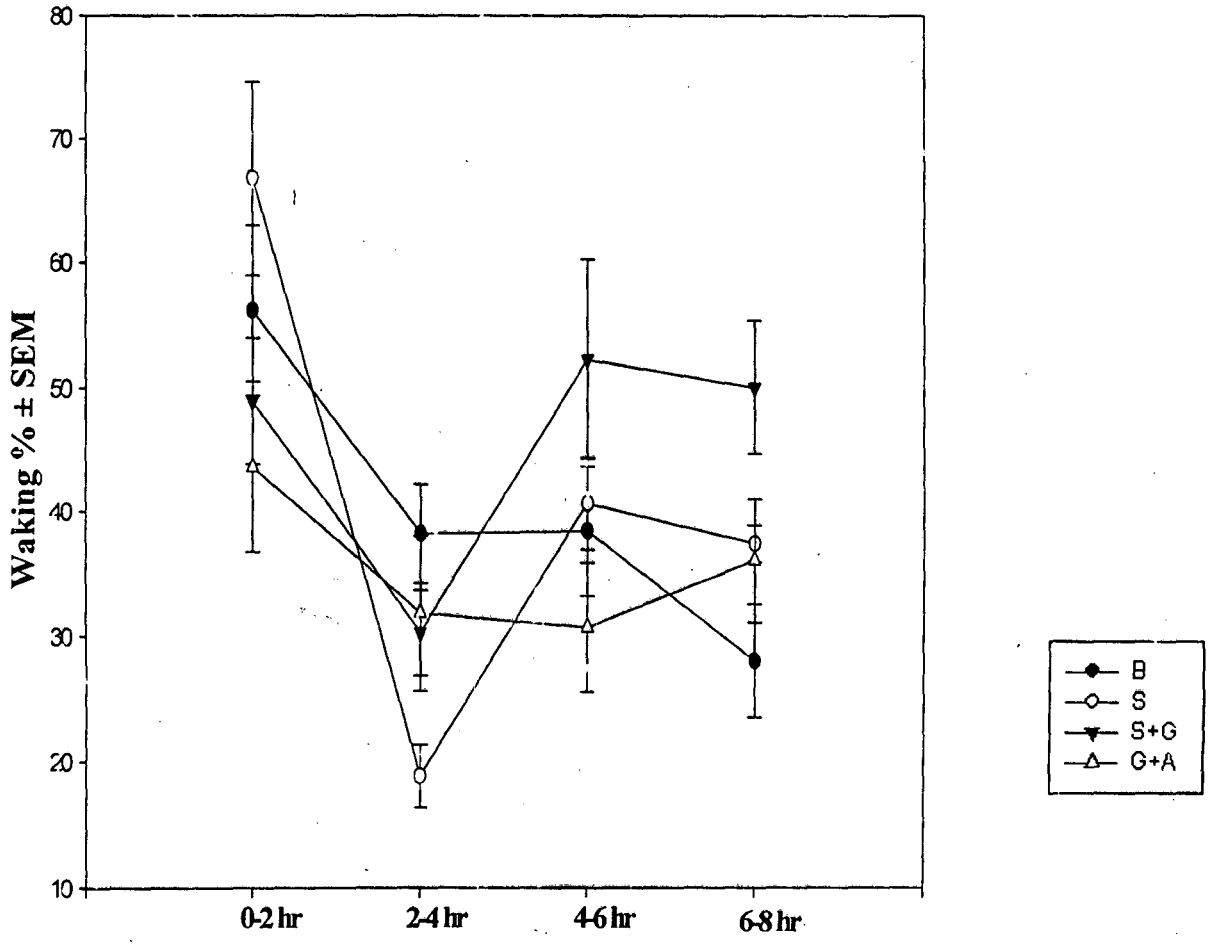


Fig: 9- Percentage of Wake during different time interval

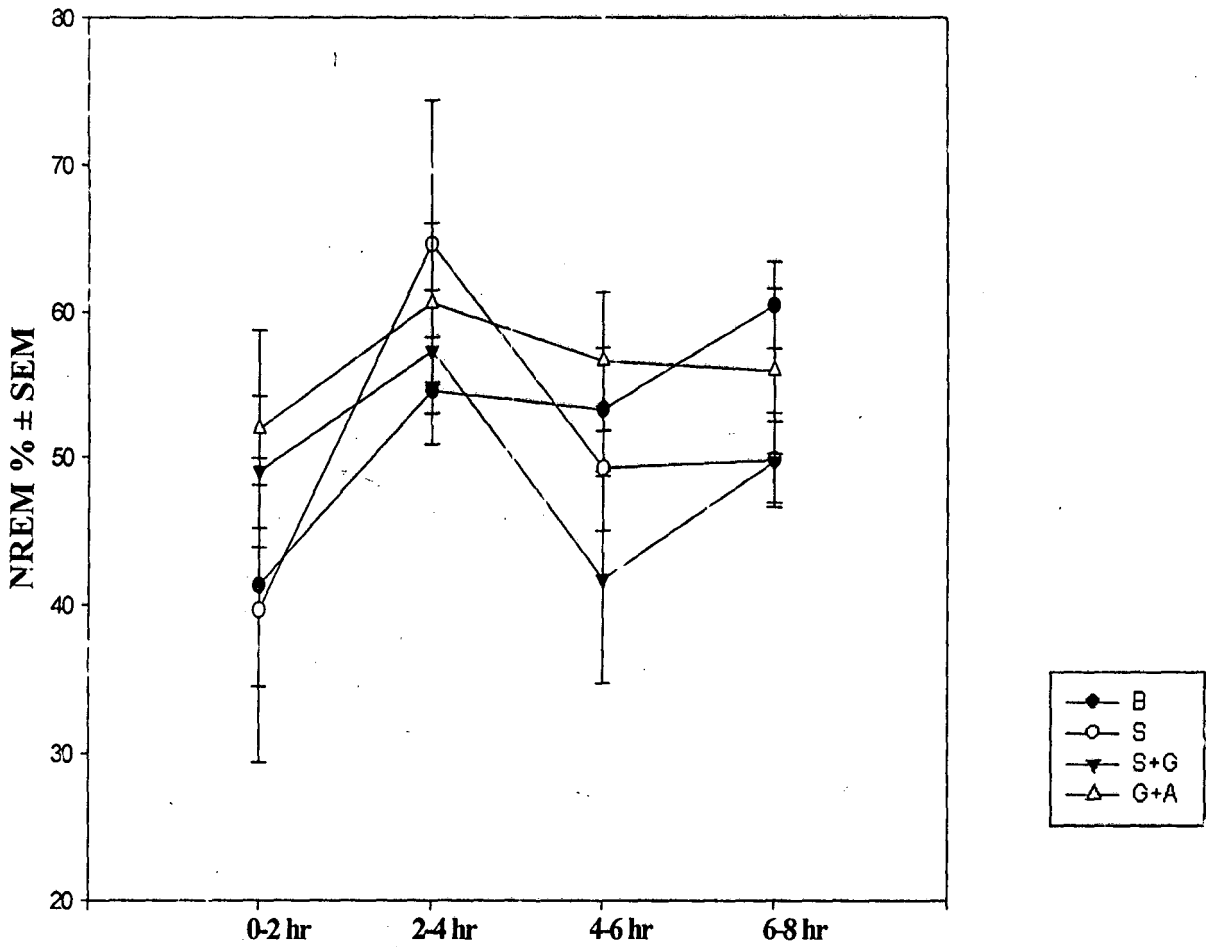


Fig: 10- Percentage of NREM during different time interval

* = p < .05

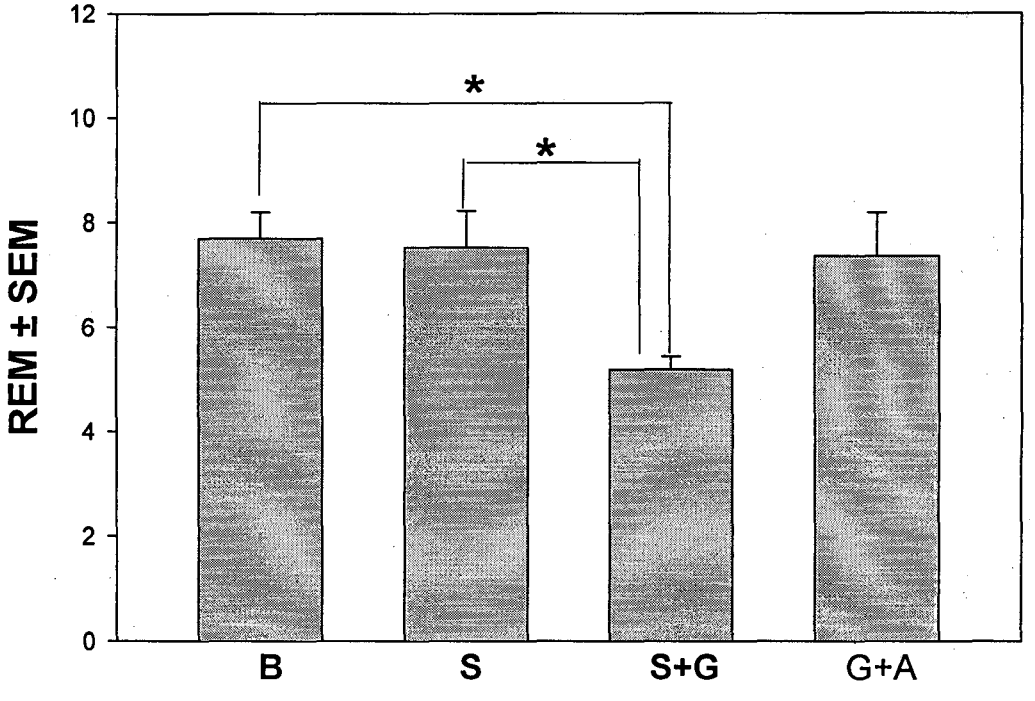


Fig: 11- Percentage of REM sleep by treatment of different drugs

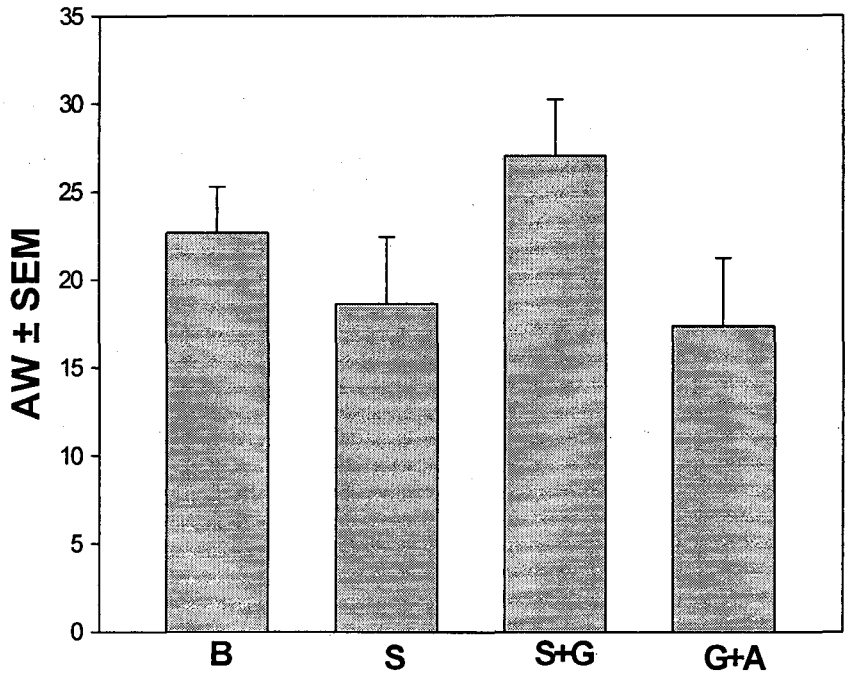


Fig: 12 (A) - Percentage of AW by treatment of different drugs

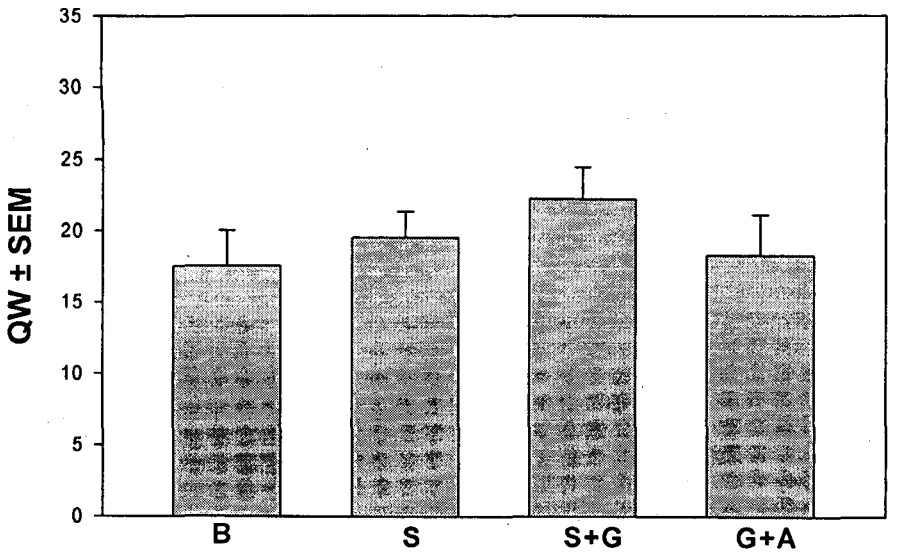


Fig: 12(B)- Percentage of QW by treatment of different drugs

* = p < .05

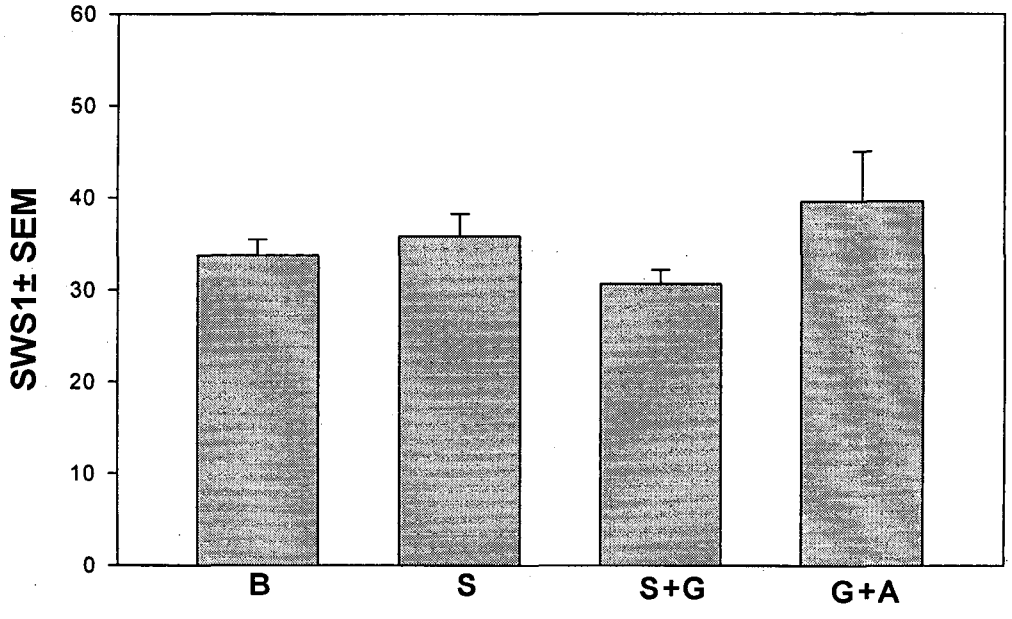


Fig: 13 (A) - Percentage of SWS1 by treatment of different drugs

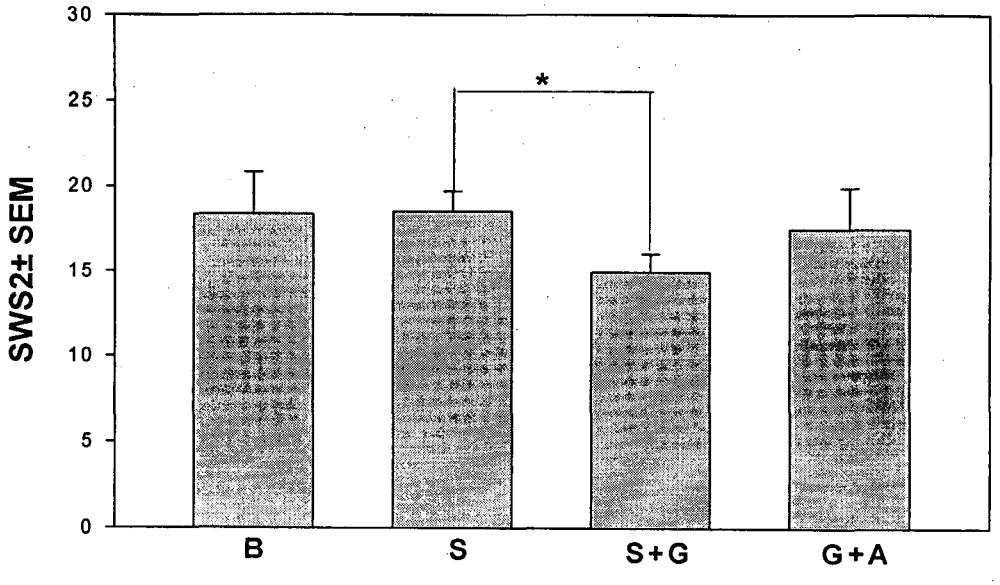


Fig: 13(B)- Percentage of SWS2 by treatment of different drugs

* = p < .05

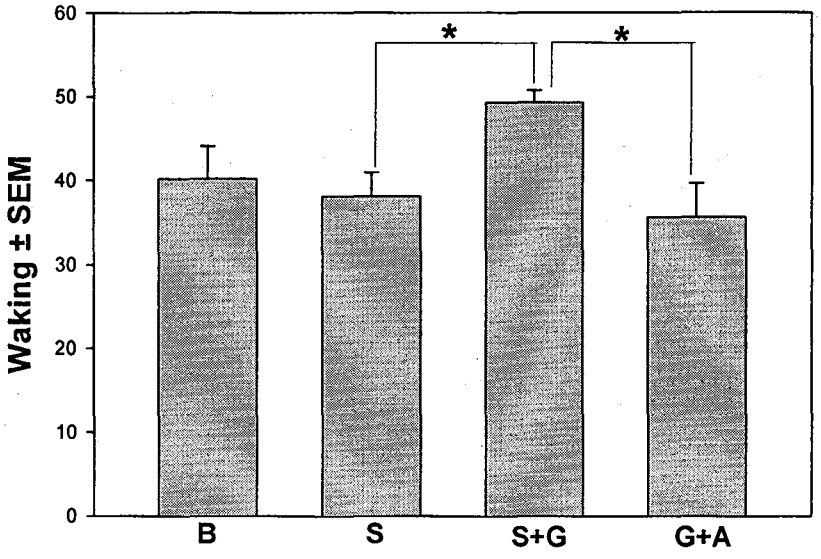


Fig: 14(A)- Percentage of Waking by treatment of different drugs

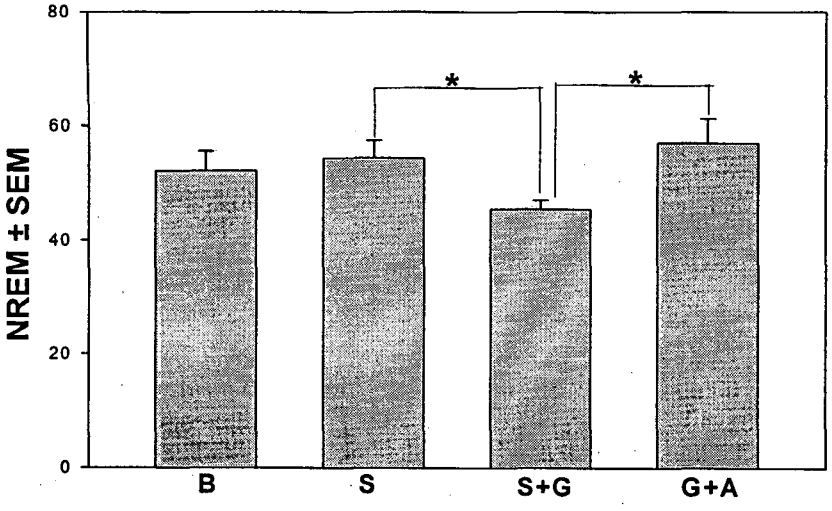


Fig: 14(B)- Percentage of NREM by treatment of different drugs

* = p < .05

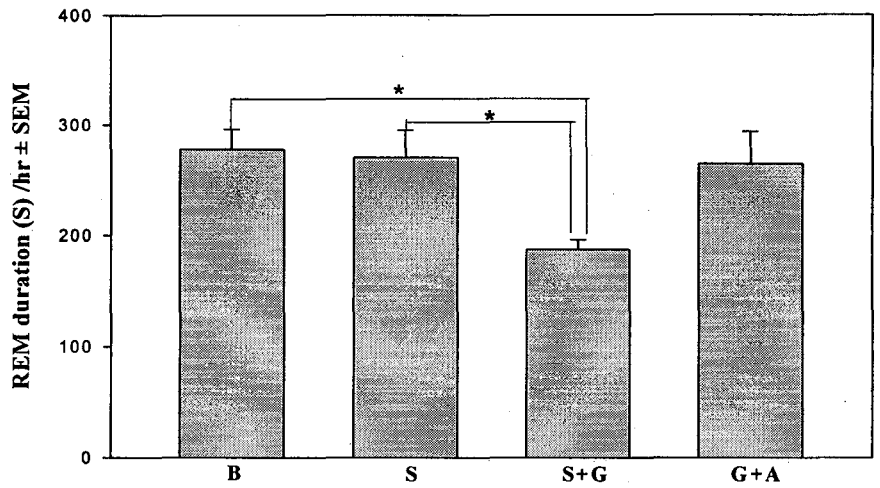


Fig: 15(A) . Percentage of REM duration/hr by treatment of different drugs

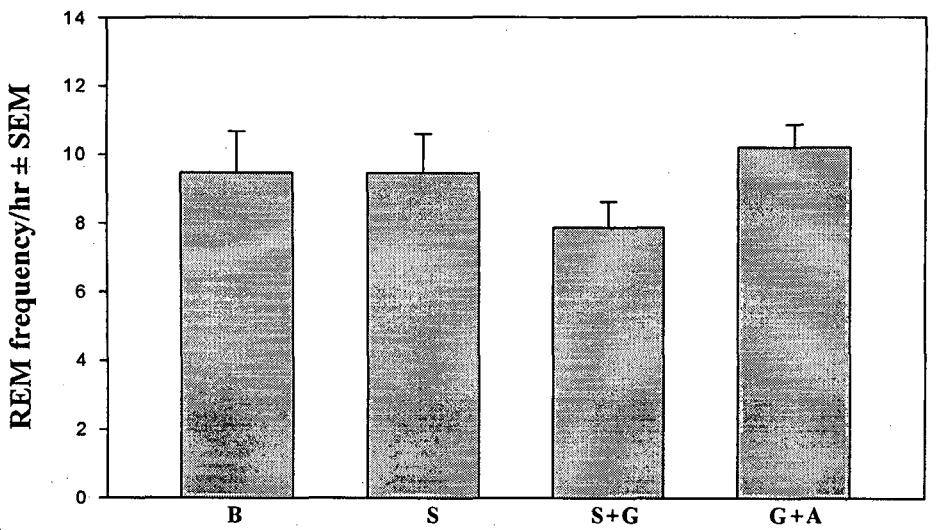


Fig: 15(B). Percentage of REM frequency by treatment of different drugs

***Note:**

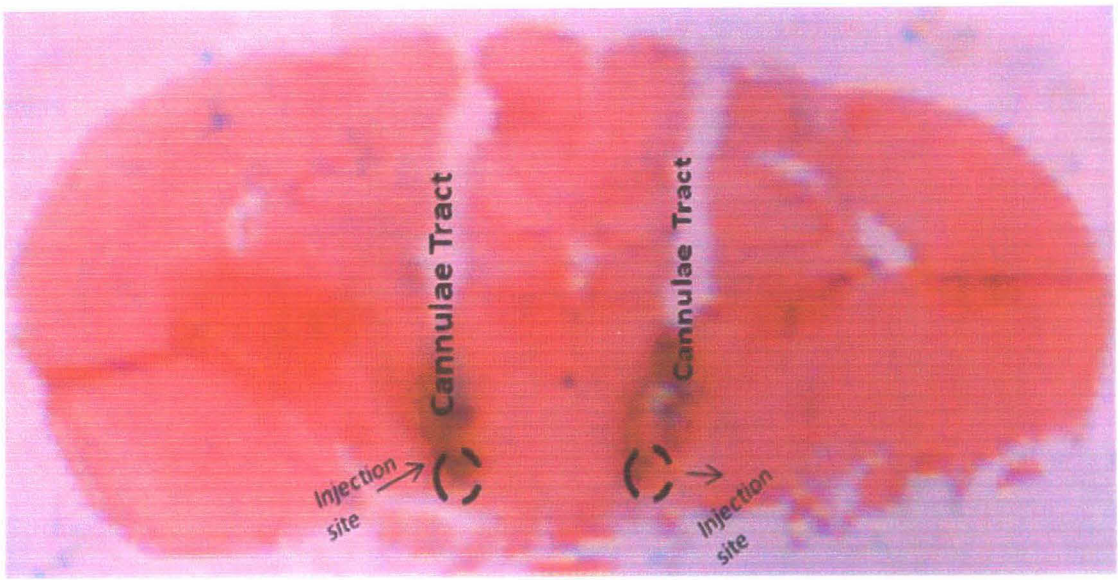
B = Baseline

S = Microinjection of saline.

S + G = Microinjection of Glutamate dissolved in saline

G + A* = Microinjection of Glutamate in Pef and Antagonist in LC dissolved in saline

A*- Orexin receptor1 antagonist



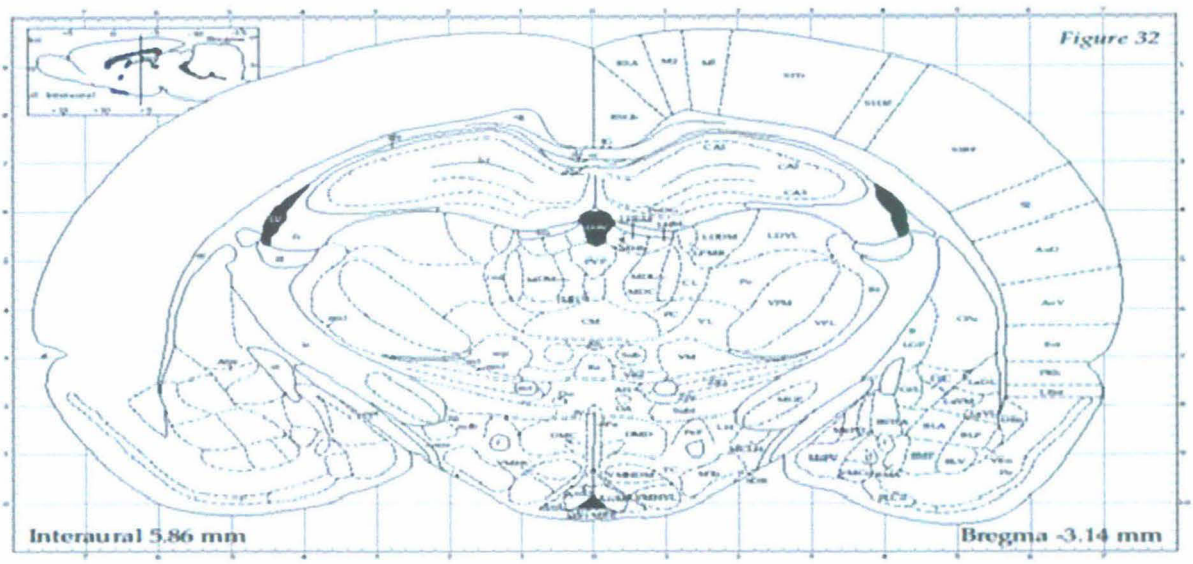


Fig:16. Representative histological section showing cannulae in Pef and injection site

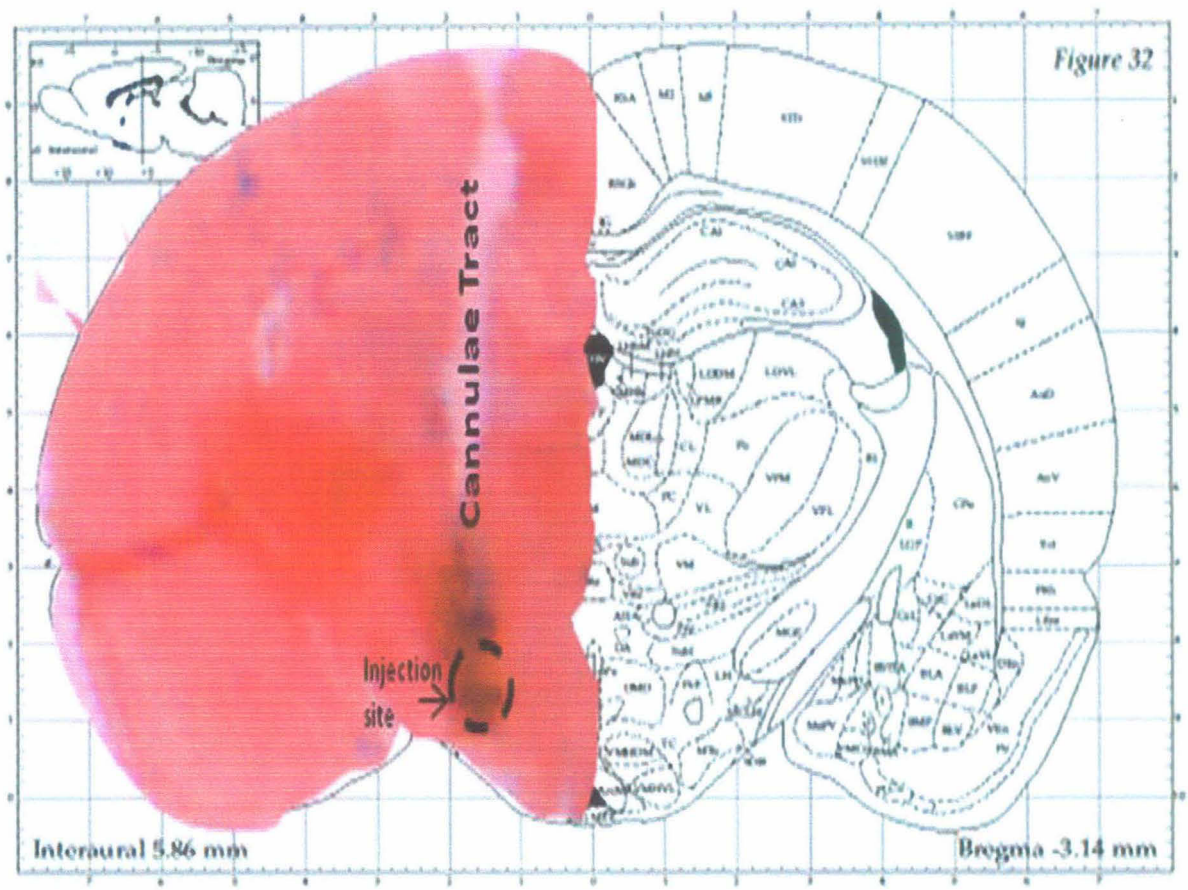


Fig: 17. Comparative histological section showing cannulae in Pef and injection site

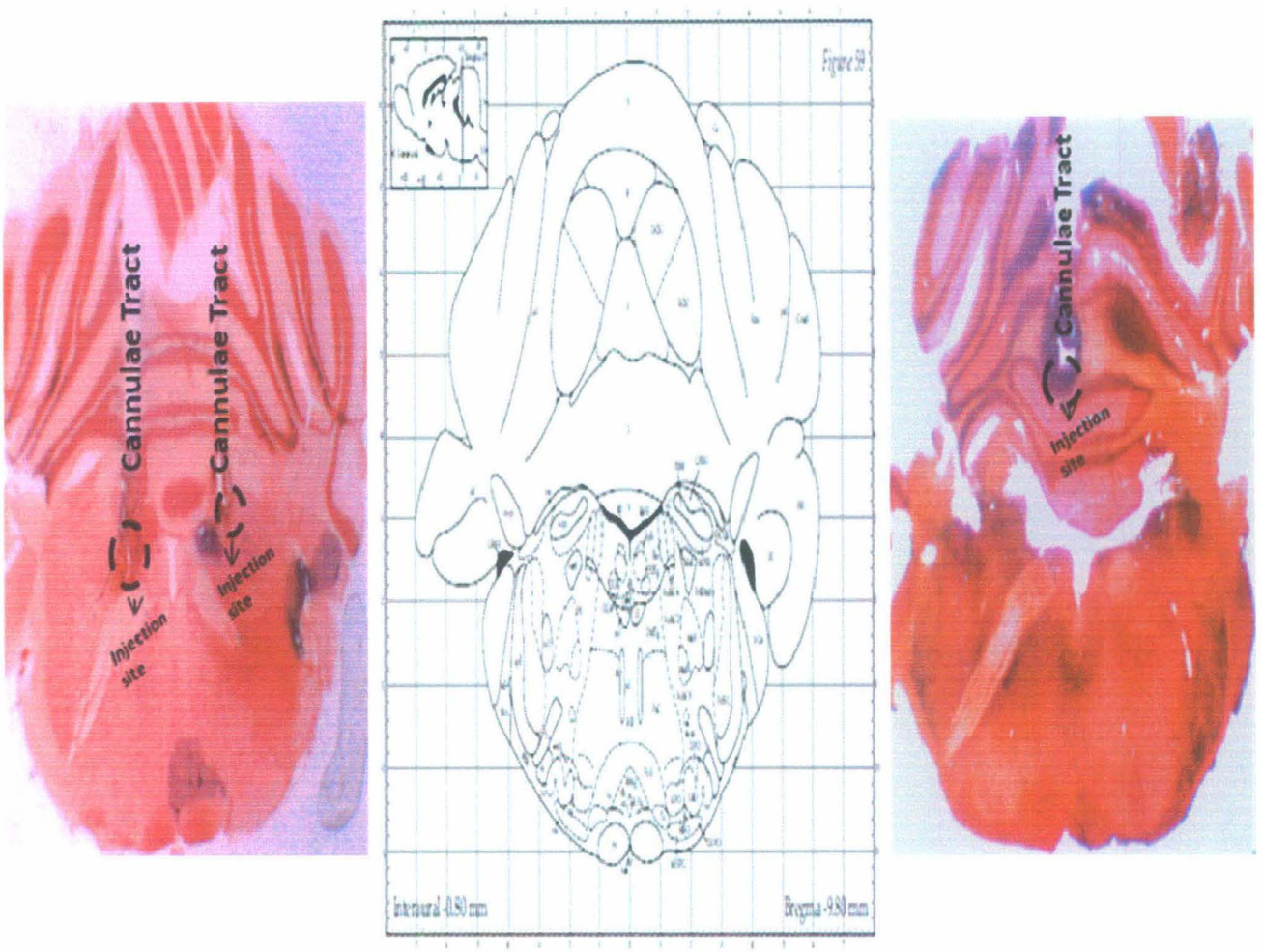


Fig: 18. Representative histological section showing cannulae in Pef and injection site

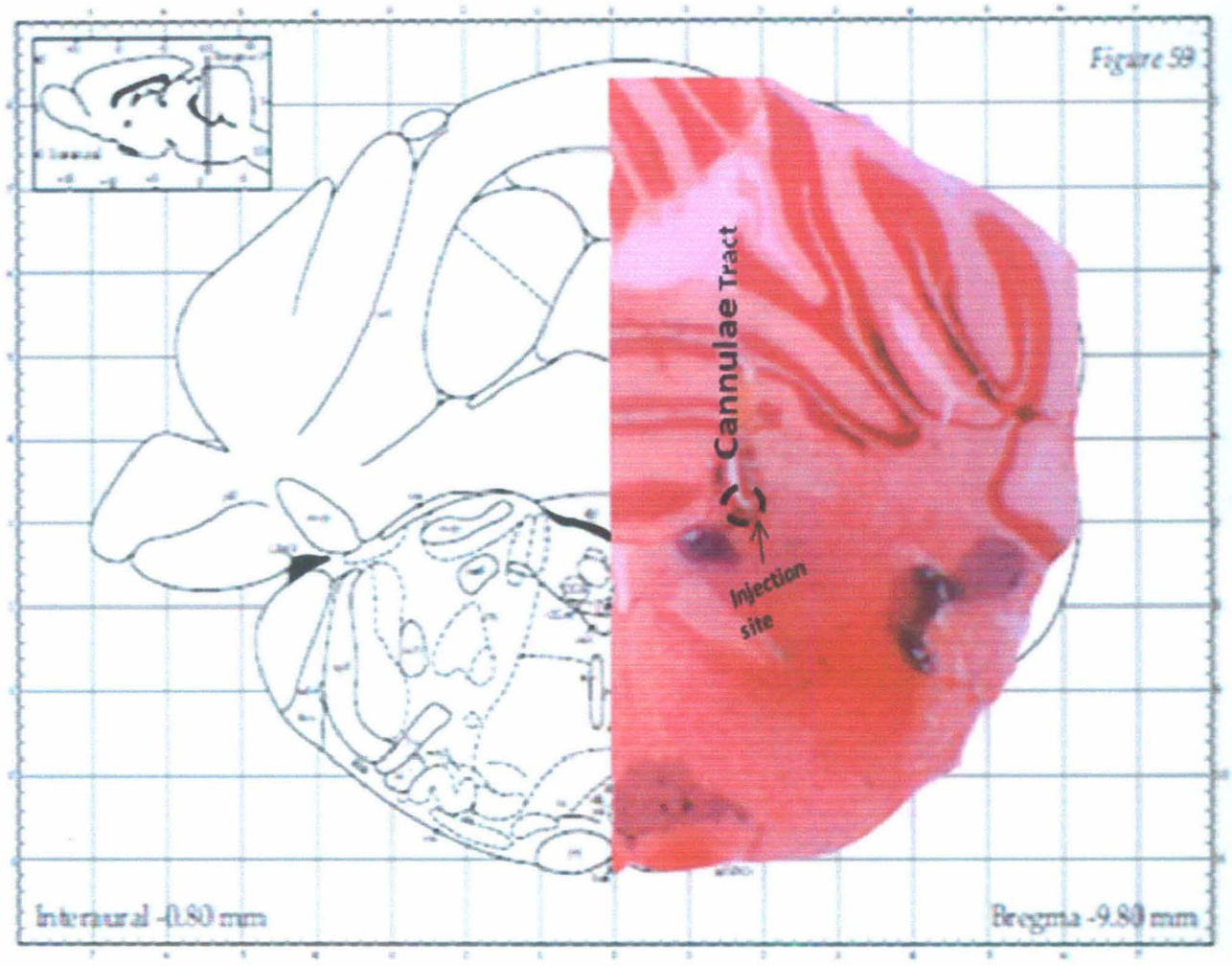


Fig:19.Comparativetive histological section showing cannulae in Pef and injection site

Discussion

Various studies like transection and lesion studies were carried out by different group of scientist to identify and define the mechanism of REM sleep, after the discovery of REM sleep by Aserinsky and Kleitman in 1953. Single unit recording study suggested that NA-ergic LC (REM-OFF) neuron in pons cease firing (Chu and Bloom, 1974; Aston-Jones and Bloom, 1981) while cholinergic LDT/PPT (REM-ON) increase their firing during REM sleep. A reciprocal interaction between these REM-OFF and REM-ON neurons has been suggested for the generation of REM sleep (Hobson *et al.*, 1975; Sakai, 1988).

The interaction between these neurons takes place through various neurotransmitters, and plays a key role in REM sleep generation and maintenance. One of the key neurotransmitters in recent studies is orexin. The actions of orexins are mediated via two G protein couple receptor OX1R and OX2R, and several studies have indicated that the effect of orexin is largely or partially mediated by activation of the histaminergic H1R (Histamin1 receptor) in the downstream of OX2R in the TMN (Eriksson *et al.*, 2001, Huang *et al.*, 2001, Yamanaka *et al.*, 2002, and Shigemoto *et al.*, 2004) as OX2R mRNA is most abundantly expressed in TMN (Marcus *et al.*, 2001) where as LC has densest concentration of OXR1 (Peyron *et al.*, 1998). Orexinergic neurons directly projects to LC that can functionally regulate REM-off neurons in LC. It has been already shown that glutamatergic neurons within Pef may be involved in the suppression of non-REM/REM sleep by activating orexinergic neurons (Sakurai T, 2002.) and orexinergic neurons also co-localize with glutamate (Yanagisawa M, 2003). Further glutamic acid stimulation of Pef promotes arousal and inhibits non-REM/REM sleep (Mallick, 2008), and electrical stimulation of the Pef evokes locomotor activity and its lesion leads to hypersomnia Shiromani, 2003. and Sinnamon, 1999) moreover, administration of orexin-1 in pontine reticular formation (Pno) increases wakefulness (Watson, 2008).

In our study of sleep wakefulness, we targeted the Pef and LC area for bilateral microinjection, in which Pef was chemically stimulated by glutamate and LC was blocked by OXR1 antagonist. The recording in laboratory conditions reported decrease in total percentage of REM sleep and increase in waking after glutamate microinjection. The significant decrease in REM sleep percentage found in rats with glutamate microinjection

in Pef and saline in LC, when compared to saline microinjection (both in LC and Pef) and baseline (having no any treatment) which were taken as a control. This is due to Pef stimulation which is responsible for wakefulness (Mallick *et al.*, 2008) possibly by giving excitatory inputs to LC neurons. There was significant decrease in REM sleep duration/hr in glutamate treatment in Pef groups as compared to control, but no significant changes in REM frequency were observed. Thus it may be stated that the decrease in REM sleep after glutamate microinjection in Pef may be due to the decrease in REM duration /hr. Along with that a significant increase in waking was witnessed when both active wake and quite wake were clubbed, and simultaneously, a significant decrease in NREM-sleep (SWS1+SWS2) was observed after glutamate microinjection in Pef. Since REM sleep duration was affected, we can hypothesize that orexin is involved in maintenance of REM than of its occurrence. If each state was considered separately, effect was significant for only REM sleep.

We further hypothesized that blocking LC through antagonist of orexinergic receptor (OXR1) will reduce the modulatory effect of orexin (excitatory or inhibitory) which will consequently should have an impact on normal sleep and wakefulness cycle of rat. After OXR1 antagonist microinjection in LC and simultaneously, glutamate in Pef; effects of glutamate alone in Pef were modulated up to controls level. We observed that there was no significant difference for any stages of sleep and wakefulness, between both treatments (glutamate in Pef and antagonist in LC) compared to controls. A significant increase in wakefulness was observed in glutamate microinjection compared with glutamate in Pef and OXR1 antagonist in LC microinjection. Simultaneously as compared to glutamate in Pef and OXR1 antagonist microinjection, a significant decrease in NREM-sleep was also observed. There was no significant change in REM frequency observed as comparison to controls. But as compared to glutamate microinjection, an increase in frequency was observed but that was not up to significant level. REM sleep duration/hr was also similar to that of controls but again as compared to glutamate microinjection, it was not statistically significant. This study shows Pef stimulation induced decrease in REM sleep, therefore Pef has role in LC mediated regulation of sleep wakefulness and need further elaborate study.

Among nine rats, five rats posses microinjection at specific site but in those animals (n=2) where microinjection site were not confined within limit of LC structure, showed the similar result which were observed in glutamate microinjection; and in those animals (n=2) where microinjection site were not confined within limit of Pef, no significant effect of glutamate microinjection were seen as compared to controls. Therefore our study confirms that orexin induced effects on REM sleep are mediated through LC NA-ergic neurons, but the possibility of other targets for orexin could not be over ruled and so further similar studies with different neuronal targets are advisable.

Thus, this study showed stimulating Pef neurons was significantly reduced by its antagonist into LC. After micoroinjecting the glutamate in Pef and orexin receptor antagonist (SB-408124) in LC, the effect of glutamate stimulation in Pef is minimized. The result of this study showed that the Pef stimulation-induced decrease in REM sleep could be prevented by microinjection of OXR1 antagonist into LC. This study suggests the role of orexin in LC mediated regulation of REM sleep.

Bibliography:

Alam MA, Mallick BN. Glutamic acid stimulation of the perifornical-lateral hypothalamic area promotes arousal and inhibits non-REM/REM sleep. Neurosci Lett. 2008 Jul 18;439 (3):281-6.

Ali M, Jha SK, Kaur S, Mallick BN. Role of GABA-A receptor in the preoptic area in the regulation of sleep-wakefulness and rapid eye movement sleep. Neurosci Res. 1999 Mar;33 (3):245-50.

Allison T., Van Twyer H. and Goff W.R. Electrophysiological studies of the echidna, Tachyglossus aculeatus. I. waking and sleep. Arch. Ital. Biol. 110; 1972; 145-184.

Amici R, Sanford LD, Kearney K., McInerney B, Ross RJ, Horner RL, Morrison AR. A serotonergic (5-HT2) receptor mechanism in the laterodorsal tegmental nucleus participates in regulating the pattern of rapid-eye-movement sleep occurrence in the rat. Brain Res. 2004 Jan 16;996 (1):9-18.

Arrigoni E, Chamberlin NL, Saper CB, McCarley RW. Adenosine inhibits basal forebrain cholinergic and noncholinergic neurons in vitro. Neuroscience. 2006 Jun 30;140 (2):403-13.

Aserinsky E, Kleitman N. Regularly occurring periods of eye motility, and concomitant phenomena, during sleep. Science. 1953 Sep 4;118 (3062):273-4.

Aston-Jones G, Bloom FE. Activity of norepinephrine-containing locus coeruleus neurons in behaving rats anticipates fluctuations in the sleep-waking cycle. J Neurosci. 1981 Aug;1 (8):876-86.

Ayala-Guerrero F. and Huitron-Resendiz S. Behavioral and electrophysiological patterns of wakefulness-sleep states in a lizard. Bol. Estud. Med. Biol. Mex., 39 (1991)9-14.

Ayala-Guerrero F. Huitron-Resendiz S. and Mexicano G. Effect of arachlorophenylalanine on sleep spikes in the inguanid lizard *Ctenosaura pectinata*, Proc.West. Pharmacol. Soc. 37, 1994,149-152.

Baghdoyan HA, Rodrigo-Angulo ML, McCarley RW, Hobson JA. Site-specific enhancement and suppression of desynchronized sleep signs following cholinergic stimulation of three brainstem regions. Brain Res. 1984 Jul 23;306 (1-2):39-52.

Batini C, Moruzzi G, Palestini M, Rossi GF, Zanchetti A. Effects of complete pontine transactions on the sleep-wakefulness rhythm: the midpontine pretrigeminal preparation. Arch Ital Bio, 1959;1 97: 1-12.

Berridge CW, Bolen SJ, Manley MS, Foote SL. Modulation of forebrain electroencephalographic activity in halothane-anesthetized rat via actions of noradrenergic beta-receptors within the medial septal region. J Neurosci. 1996 Nov 1;16 (21):7010-20.

Berridge CW, Foote SL. Enhancement of behavioral and electroencephalographic indices of waking following stimulation of noradrenergic beta-receptors within the medial septal region of the basal forebrain. J Neurosci. 1996 Nov 1;16 (21):6999-7009.

Berridge CW, Stellick RL, Schmeichel BE. Wake-promoting actions of medial basal forebrain beta2 receptor stimulation. Behav Neurosci. 2005 Jun; 119(3):743-51.

Bert J, Vuillon-Cacciuttolo G, Balzamo E, De Mico P, Gambarelli D, Tamalet J. Precocious sleep modifications in the evolution of experimental Kuru in rhesus monkeys. Neurosci Lett. 1977, Dec;6 (4):333-8.

Blouin AM, Thannickal TC, Worley PF, Baraban JM, Reti IM, Siegel JM. Narp immunostaining of human hypocretin (orexin) neurons: loss in narcolepsy. Neurology. 2005 Oct 25;65 (8):1189-92.

Bockstaele EJ, Biswas A, Pickel VM. Topography of serotonin neurons in the dorsal raphe nucleus that send axon collaterals to the rat prefrontal cortex and nucleus accumbens. Brain Res. 1993 Oct 8; 624(1-2):188-98.

Bourgin P, Huitrón-Résendiz S, Spier AD, Fabre V, Morte B, Criado JR, Sutcliffe JG, Henriksen SJ, de Lecea L. Hypocretin-1 modulates rapid eye movement sleep through activation of locus coeruleus neurons. J Neurosci. 2000 Oct 15;20 (20):7760-5.

Bremer F: Cerveau isole et physiologie du sommeil. C R Soc Biol 1935; 118: 1235-1242.

Bremer F: Intrathalamic recurrent inhibition and the physiology of sleep. EEG clin neurophysiol 1936; 421-430.

Broberger C, De Lecea L, Sutcliffe JG, Hökfelt T. Hypocretin/orexin- and melanin-concentrating hormone-expressing cells form distinct populations in the rodent lateral hypothalamus: relationship to the neuropeptide Y and agouti gene-related protein systems. J Comp Neurol. 1998 Dec 28;402 (4):460-74.

Carli G, Zanchetti A. A study of pontine lesions suppressing deep sleep in the cat. Arch Ital Biol. 1965 Dec 10; 103(4):751-88.

Carrol R.L. Vertebrate Paleontology and Evolution. W.H. Freeman and Company, New York, 1988.

Chase MH, Soja PJ, Morales FR. Evidence that glycine mediates the postsynaptic potentials that inhibit lumbar motor neurons during the atonia of active sleep. J Neurosci. 1989 Mar; 9(3):743-51.

Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, Richardson JA, Williams SC, Xiong Y. Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell. 1999 Aug 20;98 (4):437-51.

Chu N, Bloom FE. Norepinephrine-containing neurons: changes in spontaneous discharge patterns during sleeping and waking. Science. 1973 Mar 2; 179(76):908-10.

Chu NS, Bloom FE. Activity patterns of catecholamine-containing pontine neurons in the dorso-lateral tegmentum of unrestrained cats. J Neurobiol. 1974;5 (6):527-44.

Ciriello J, McMurray JC, Babic T, de Oliveira CV. Collateral axonal projections from hypothalamic hypocretin neurons to cardiovascular sites in nucleus ambiguus and nucleus tractus solitarius. Brain Res. 2003 Nov 21; 991 (1-2):133-41.

Date Y, Mondal MS, Matsukura S, Nakazato M. Distribution of orexin-A and orexin-B (hypocretins) in the rat spinal cord. Neurosci Lett. 2000 Jul 14; 288 (2):87-90.

de Lecea L, Bartfai T. Transgenic mice with a reduced core body temperature have an increased life span. Science. 2006 Nov 3; 314 (5800):825-8.

de Lecea L, Bloom FE. The type 3 serotonin receptor is expressed in a subpopulation of GABAergic neurons in the rat neocortex and hippocampus. Brain Res. 1996 Aug 26;731 (1-2):199-202.

de Lecea L, Criado JR, Rivera S, Wen W, Soriano E, Henriksen SJ, Taylor SS, Gall CM, Sutcliffe JG. Endogenous protein kinase A inhibitor (PKIalpha) modulates synaptic activity. J Neurosci Res. 1998 Aug 1; 53 (3):269-78.

Deboer T, Sanford LD, Ross RJ, Morrison AR. Effects of electrical stimulation in the amygdala on ponto-geniculo-occipital waves in rats. Brain Res. 1998 May 18;793 (1-2):305-10.

Dement W. The occurrence of low voltage, fast, electroencephalogram patterns during behavioral sleep in the cat. EEG Clin Neurophysiol. 1958 May;10 (2):291-6.

Dement W.C. and Kleitman N. Cyclic variations in EEG during sleep and their relation to eye movements, body motility, and dreaming, Electroenceph. Clin. Neurophysiol., 1957; 9: 673-690.

Derbyshire et al. The effects of anesthetics on action potentials in the cerebral cortex of the cat. *Am j physiol.* 1936; 116: 577-596.

Egan TM, North RA. Actions of acetylcholine and nicotine on rat locus coeruleus neurons in vitro. Neuroscience. 1986 Oct;19 (2):565-71.

Eggermann E, Serafin M, Bayer L, Machard D, Saint-Mleux B, Jones BE, Mühlethaler M. Orexins/hypocretins excite basal forebrain cholinergic neurons. Neuroscience. 2001;108 (2):177-81.

Ennis M, Aston-Jones G. GABA-mediated inhibition of locus coeruleus from the dorsomedial rostral medulla. J Neurosci. 1989 Aug;9 (8):2973-81.

Eriksson KS, Sergeeva OA, Selbach O, Haas HL. Orexin (hypocretin)/dynorphin neurons control GABAergic inputs to tuberomammillary neurons. Eur J Neurosci. 2004 Mar;19 (5):1278-84.

Facciolo RM, Crudo M, Giusi G, Alò R, Canonaco M. Light- and dark-dependent orexinergic neuronal signals promote neurodegenerative phenomena accounting for distinct behavioral responses in the teleost Thalassoma pavo. J Neurosci Res. 2009 Feb 15;87 (3):748-57.

Fritschy JM, Grzanna R. Distribution of locus coeruleus axons within the rat brainstem demonstrated by Phaseolus vulgaris leucoagglutinin anterograde tracing in combination with dopamine-beta-hydroxylase immunofluorescence. J Comp Neurol. 1990 Mar 22;293 (4):616-31.

Gerashchenko D, Salin-Pascual R, Shiromani PJ. Effects of hypocretin-saporin injections into the medial septum on sleep and hippocampal theta. Brain Res. 2001 Sep 14;913 (1):106-15.

Gonzalo-Ruiz A, Lieberman AR, Sanz-Anquela JM. Organization of serotoninergic projections from the raphé nuclei to the anterior thalamic nuclei in the rat: a combined

retrograde tracing and 5-HT immunohistochemical study. J Chem Neuroanat. 1995 Feb;8 (2):103-15.

Hagan JJ, Leslie RA, Patel S, Evans ML, Wattam TA, Holmes S *etal.* Orexin A activates locus coeruleus cell firing and increases arousal in the rat. Proc Natl Acad Sci U S A. 1999 Sep 14; 96 (19):10911-6.

Harrison DC, Roberts JC, Leslie RA. Gene expression and protein distribution of the orexin-1 receptor in the rat brain and spinal cord. Neuroscience. 2001;103 (3):777-97.

Hashimoto J, Shrestha GR, Tamura O. Comparative anatomy of the locus coeruleus. I. Organization and ascending projections of the catecholamine containing neurons in the pontine region of the bird, Melopsittacus undulatus. J Hirnforsch. 1974;15 (4):319-30.

Henley K, Morrison AR. A re-evaluation of the effects of lesions of the pontine tegmentum and locus coeruleus on phenomena of paradoxical sleep in the cat. Acta Neurobiol Exp (Wars). 1974;34 (2):215-32.

Higashi A. Identification of r.e.m. sleep in the mouse by measuring the body movements. Med Biol Eng Comput. 1983 Sep;21 (5):644-6.

Hobson J.A., Electrographic correlates of behavior in the tree frog with special reference to sleep. Electrcencephalography and Clinical Neurophysiology, 1967; 22: 113-121.

Hobson J.A., Electrographic correlates of behavior in the tree frogs. Nature, 1968; 220: 386-387.

Hobson JA, McCarley RW, Wyzinski PW. Sleep cycle oscillation: reciprocal discharge by two brainstem neuronal groups. Science. 1975 Jun 4; 189 (4196):55-8.

Hobson JA, Sleep: physiologic aspects. N Engl Med 1969; 281: 1343-1345.

Honda T, Semba K. Serotonergic synaptic input to cholinergic neurons in the rat mesopontine tegmentum. Brain Res. 1994 Jun 6; 647 (2):299-306.

Huang ZL, Qu WM, Li WD, Mochizuki T, Eguchi N, Watanabe T, Urade Y, Hayaishi O. Arousal effect of orexin A depends on activation of the histaminergic system. Proc Natl Acad Sci U S A. 2001 Aug 14;98 (17):9965-70. Epub 2001 Aug 7.

Huntley A.C., Friedman J.K. and Cohen H.B. Sleep in an Iguanid lizard *Dipsosaurus dorsalis*, Sleep Research, 6, 1977, 104.

Iijima K, Ohtomo K. Immunocytochemical study using a GABA antiserum for the demonstration of inhibitory neurons in the rat locus ceruleus. Am J Anat. 1988 Jan; 181 (1):43-52.

Ivanov A, Aston-Jones G. Hypocretin/orexin depolarizes and decreases potassium conductance in locus coeruleus neurons. Neuroreport. 2000 Jun 5;11 (8):1755-8.

Iwakiri H, Matsuyama K, Mori S. Extracellular levels of serotonin in the medial pontine reticular formation in relation to sleep-wake cycle in cats: a microdialysis study. Neurosci Res. 1993 Nov;18 (2):157-70.

Jacobs, B. L. Single unit activity of locus coeruleus neurons in behaving animals. Prog. Neurobiol. 1986; 27: 183-194.

John J, Wu MF, Boehmer LN, Siegel JM. Cataplexy-active neurons in the hypothalamus: implications for the role of histamine in sleep and waking behavior. Neuron. 2004 May 27;42 (4):619-34.

Jones BE, Harper ST, Halaris AE. Effects of locus coeruleus lesions upon cerebral monoamine content, sleep-wakefulness states and the response to amphetamine in the cat. Brain Res. 1977 Apr 1;124 (3):473-96.

Jouvet M, Michel F. Corrélations électromyographiques du sommeil chez le chat décortiqué mésencéphalique chronique. C R Soc Biol, 1959; 53: 422-425.

Jouvet M. and Delorme F. Locus coeruleus et sommeil paradoxal, C.R. Soc. Biol., 159 (1965) 895-899.

Jouvet M. Biogenic amines and the states of sleep. Science. 1969 Jan 3;163 (862):32-41.

Jouvet M. Recherche sur les structures nerveuses et les mécanismes responsables des différentes phases du sommeil physiologique. Arch Ital Biol 1962, 100: 125-206.

Jouvet M. Research on the neural structures and responsible mechanisms in different phases of physiological sleep. Arch ital biol. 1962; 100:125-206.

Jouvet M. The role of monoamines and acetylcholine-containing neurons in the regulation of the sleep-waking cycle. Ergeb Physiol. 1972; 64:166-307.

Kaur S, Saxena RN, Mallick BN. GABA in locus coeruleus regulates spontaneous rapid eye movement sleep by acting on GABAA receptors in freely moving rats. Neurosci Lett. 1997 Feb 21;223 (2):105-8.

Klaue R. Die bioelektrische Tätigkeit der Grosshirnrinde im normalen Schlaf und in der Narkose durch Schlafmittel. J Psychol Neurol. 1937; 47:510–31.

Kodama T, Takahashi Y, Honda Y. Enhancement of acetylcholine release during paradoxical sleep in the dorsal tegmental field of the cat brain stem. Neurosci Lett. 1990 Jul 13;114 (3):277-82.

Koyama Y, Takahashi K, Kodama T, Kayama Y. State-dependent activity of neurons in the perifornical hypothalamic area during sleep and waking. Neuroscience. 2003;119 (4):1209-19.

Królicki L, Chodobski A, Skolasińska K. The effect of stimulation of the reticulo-hypothalamic-hippocampal systems on the cerebral blood flow and neocortical and hippocampal electrical activity in cats. Exp Brain Res. 1985;60 (3):551-8.

Lavoie B, Parent A. Pedunculopontine nucleus in the squirrel monkey: projections to the basal ganglia as revealed by anterograde tract-tracing methods. J Comp Neurol. 1994 Jun 8;344 (2):210-31.

Lee HS, Lee BY, Waterhouse BD. Retrograde study of projections from the tuberomammillary nucleus to the dorsal raphe and the locus coeruleus in the rat. Brain Res. 2005 May 10;1043 (1-2):65-75.

Lin SH, Miyata S, Weng W, Matsunaga W, Ichikawa J, Furuya K, Nakashima T, Kiyohara T. Comparison of the expression of two immediate early gene proteins, FosB and Fos in the rat preoptic area, hypothalamus and brainstem during pregnancy, parturition and lactation. Neurosci Res. 1998 Dec;32 (4):333-41.

Losier BJ, Semba K. Dual projections of single cholinergic and aminergic brainstem neurons to the thalamus and basal forebrain in the rat. Brain Res. 1993 Feb 26;604 (1-2):41-52.

Lyons WE, Fritschy JM, Grzanna R. The noradrenergic neurotoxin DSP-4 eliminates the coeruleospinal projection but spares projections of the A5 and A7 groups to the ventral horn of the rat spinal cord. J Neurosci. 1989 May;9 (5):1481-9.

Mallick BN, Fahringer HM, Wu MF, Siegel JM. REM sleep deprivation reduces auditory evoked inhibition of dorsolateral pontine neurons. Brain Res. 1991 Jun 28;552(2):333-7.

Mallick BN, Thankachan S, Islam F. Differential responses of brain stem neurons during spontaneous and stimulation-induced desynchronization of the cortical eeg in freely moving cats. Sleep Res Online. 1998;1 (4):132-46.

Mallick BN, Thankachan S, Islam F. Influence of hypnogenic brain areas on wakefulness- and rapid-eye-movement sleep-related neurons in the brainstem of freely moving cats. J Neurosci Res. 2004 Jan 1; 75 (1):133-42.

Mallick,B.N.; Gulyani,S. Mild electrical stimulation of pontine tegmentum around locus coeruleus reduces rapid eye movement sleep in rats. Neurosci Res. 1996 Feb; 24 (3):227-35.

Marcus D, Kotani M, Mollereau C, Roumy M, Zajac JM. Structure-activity relationships of neuropeptide FF: role of C-terminal regions. Peptides. 2001 Sep; 22 (9):1471-8.

Marcus JN, Aschkenasi CJ, Lee CE, Chemelli RM, Saper CB, Yanagisawa M, Elmquist JK. Differential expression of orexin receptors 1 and 2 in the rat brain. J Comp Neurol. 2001 Jun 18; 435 (1):6-25.

McCarley RW, Hobson JA. Discharge patterns of cat pontine brain stem neurons during desynchronized sleep. J Neurophysiol. 1975 Jul; 38 (4):751-66.

McGinty D, Szymusiak R. Brain structures and mechanisms involved in the generation of NREM sleep: focus on the preoptic hypothalamus. Sleep Med Rev. 2001 Aug;5 (4):323-342.

McGinty DJ, Harper RM. Dorsal raphe neurons: depression of firing during sleep in cats. Brain Res. 1976 Jan 23; 101 (3):569-75.

Michel F. Neurophysiological interpration of the EEG aspects of sleep as a function of the existence of 2 different inhiditory systems. Rev Neurol (Paris). 1961; 102:310-11.

Mignot E. Perspectives in narcolepsy and hypocretin (orexin) research. Sleep Med. 2000 Apr 1;1 (2):87-90.

Monti JM, Jantos H, Monti D, Alvariño F. Dorsal raphe nucleus administration of 5-HT1A receptor agonist and antagonists: effect on rapid eye movement sleep in the rat. Sleep Res Online. 2000;3 (1):29-34.

Monti JM, Monti D. Role of dorsal raphe nucleus serotonin 5-HT1A receptor in the regulation of REM sleep. Life Sci. 2000 Apr 14;66 (21):1999-2012.

Monti JM. Catecholamines and the sleep-wake cycle. II. REM sleep. Life Sci. 1983 Mar 28;32 (13):1401-15.

Nakamachi, T., Matsuda, K., Maruyama K., Miura, T.,Uchiyama, M., Funahashi, H., Sakurai, T. & Shioda, S. 2006. Regulation by orexin of feeding behaviour and locomotor activity in the goldfish. J Neuroendocrinol 18, 290–297.

Nishino S, Ripley B, Overeem S, Nevsimalova S, Lammers GJ, Vankova J, Okun M, Rogers W, Brooks S, Mignot E. Low cerebrospinal fluid hypocretin (Orexin) and altered energy homeostasis in human narcolepsy. Ann Neurol. 2001 Sep;50 (3):381-8.

Pal D, Madan V, Mallick BN. Neural mechanism of rapid eye movement sleep generation: Cessation of locus coeruleus neurons is a necessity. Sheng Li Xue Bao. 2005 Aug 25; 57 (4):401-13.

Panula P, Yang HY, Costa E. Histamine-containing neurons in the rat hypothalamus. Proc Natl Acad Sci U S A. 1984 Apr; 81 (8):2572-6.

Parent A, Poitras D. The origin and distribution of catecholaminergic axon terminals in the cerebral cortex of the turtle (Chrysemys picta). Brain Res. 1974 Oct 4;78 (3):345-58.

Paxinos G, Watson C. The rat brain in stereotaxic co-ordinates. Academic press Australia.1997.

Peyron C, Faraco J, Rogers W, Ripley B, Overeem S, Charnay Y, Nevsimalova S, Aldrich M, Reynolds D. A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. Nat Med. 2000 Sep; 6 (9):991-7.

Peyron C, Luppi PH. Anatomical demonstration of a medullary enkephalinergic pathway potentially implicated in the oro-facial muscle atonia of paradoxical sleep in the cat. Sleep Res Online. 1998;1 (3):102-8.

Peyron C, Tighe DK, van den Pol AN, de Lecea L, Heller HC, Sutcliffe JG, Kilduff TS. Neurons containing hypocretin (orexin) project to multiple neuronal systems. J Neurosci. 1998 Dec 1;18 (23):9996-10015.

Quattrochi J, Datta S, Hobson JA. Cholinergic and non-cholinergic afferents of the caudolateral parabrachial nucleus: a role in the long-term enhancement of rapid eye movement sleep. Neuroscience. 1998 Apr;83 (4):1123-36.

Rechtschaffen A, Kales A: *A manual of standardized terminology , techniques and scoring system for sleep stages of human subjects*, Los Angles, Brain Research service/ Brain Information service; 1968.

Reiner PB. Correlational analysis of central noradrenergic neuronal activity and sympathetic tone in behaving cats. Brain Res. 1986 Jul 16;378 (1):86-96.

Reite ML, Rhodes JM, Kavan E, Adey WR. Normal Sleep Patterns in Macaque Monkey. Arch Neurol. 1965 Feb; 12:133-44.

Sakai K, Jouvet M. Are the gigantocellular tegmental field neurons responsible for paradoxical sleep? Brain Res. 1981 Dec 14;229 (1):147-61.

Sakai K. Executive mechanisms of paradoxical sleep. Arch Ital Biol. 1988 Oct;126 (4):239-57.

Sakurai T, Shioda S. Regulation by orexin of feeding behaviour and locomotor activity in the goldfish. J Neuroendocrinol. 2006 Apr;18 (4):290-7.

Sakurai T. Orexins and orexin receptors: implication in feeding behavior. Regul Pept. 1999 Nov 30;85 (1):25-30.

Sakurai T. Roles of orexin/hypocretin in regulation of sleep/wakefulness and energy homeostasis. Sleep Med Rev. 2005 Aug;9 (4):231-41.

Sakurai T. The orexin/hypocretin system. *Physiology and path physiology.* 2006.

Saper CB, Boulant JA. Synaptic and morphological characteristics of temperature-sensitive and -insensitive rat hypothalamic neurones. J Physiol. 2001 Dec 1;537 (Pt 2):521-35.

Saper CB, Maratos-Flier E, Tritos NA, Lee C, Kelly J, Tatro JB, Hoffman GE, Ollmann MM, Barsh GS, Sakurai T, Yanagisawa M, Elmquist JK. Chemically defined projections linking the mediobasal hypothalamus and the lateral hypothalamic area. J Comp Neurol. 1998 Dec 28;402 (4):442-59.

Sarnat H.B. and Netsky M.B. Evolution of the nervous system, Oxford University Press, New York, 1981.

Scammell TE. The neurobiology, diagnosis, and treatment of narcolepsy. Ann Neurol. 2003 Feb;53 (2):154-66.

Semba K, Fibiger HC. Afferent connections of the laterodorsal and the pedunculopontine tegmental nuclei in the rat: a retro- and antero-grade transport and immunohistochemical study. J Comp Neurol. 1992 Sep 15;323 (3):387-410.

Sherin JE, Elmquist JK, Torrealba F, Saper CB. Innervation of histaminergic tuberomammillary neurons by GABAergic and galaninergic neurons in the ventrolateral preoptic nucleus of the rat. J Neurosci. 1998 Jun 15;18 (12):4705-21.

Sherin JE, Shiromani PJ, McCarley RW, Saper CB. Activation of ventrolateral preoptic neurons during sleep. Science. 1996 Jan 12;271 (5246):216-9.

Shigemoto Y, Fujii Y, Shinomiya K, Kamei C. Participation of histaminergic H1 and noradrenergic alpha 1 receptors in orexin A-induced wakefulness in rats. Brain Res. 2004 Oct 8;1023 (1):121-5.

Shiuchi T, Haque MS, Okamoto S, Inoue T, Kageyama H, Lee S, Toda C. Hypothalamic orexin stimulates feeding-associated glucose utilization in skeletal muscle via sympathetic nervous system. Cell Metab. 2009 Dec;10 (6):466-80.

Shouse MN, Farber PR, Staba RJ. Physiological basis: how NREM sleep components can promote and REM sleep components can suppress seizure discharge propagation. Clin Neurophysiol. 2000 Sep; 111 Suppl 2:S9-S18.

Siegel J.M., Manger P.R. Nienhuis R., Fahringer H.M. and Pettigrew J.D. The echidna Tachyglossus aculeatus combines REM and non-REM aspects in asingle sleep stste: implications for the evolution of sleep, Neuroscience, 1996; 16: 3500-3506.

Siegel JM. Hypocretin (orexin): role in normal behavior and neuropathology. Annu Rev Psychol. 2004; 55:125-48.

Singewald N, Philippu A. Release of neurotransmitters in the locus coeruleus. Prog Neurobiol. 1998 Oct;56 (2):237-67.

Singh S, Mallick BN. Mild electrical stimulation of pontine tegmentum around locus coeruleus reduces rapid eye movement sleep in rats. Neurosci Res. 1996 Feb;24 (3):227-35.

Skagerberg G, Björklund A, Lindvall O, Schmidt RH. Origin and termination of the diencephalo-spinal dopamine system in the rat. Brain Res Bull. 1982 Jul-Dec;9 (1-6):237-44.

Starzl TE, Taylor CW, Magoun HW. Collateral afferent excitation of reticular formation of brain stem. J neurophysiol. 1951 Nov; 14 (6):479-96.

Steriade M, Datta S, Paré D, Oakson G, Curró Dossi RC. Neuronal activities in brain-stem cholinergic nuclei related to tonic activation processes in thalamocortical systems. J Neurosci. 1990 Aug; 10 (8):2541-59.

Strecker RE, Moneta ME. Electrical stimulation of the kindled hippocampus briefly increases extracellular dopamine in the nucleus accumbens. Neurosci Lett. 1994 Aug 1; 176 (2):173-7.

Takeda N, Inagaki S, Taguchi Y, Tohyama M, Watanabe T, Wada H. Origins of histamine-containing fibers in the cerebral cortex of rats studied by immunohistochemistry with histidine decarboxylase as a marker and transection. Brain Res. 1984 Dec 3;323 (1):55-63.

Tauber E.S., Roffwarg H.P. and Weitzman E.D. Eye movements and electroencephalogram activity during sleep in diurnal lizards, nature.1966; 212: 1612-1613.

Tauber E.S., Rojas-Ramirez J. and Peon R.H. Electrophysiclogical and behavioral correlates of wakefulness and sleep in the lizards *Ctenosaura pectinata*, Electroencephalography and Clinical Neurophysiology, 1968;24: 424-433.

Thannickal T, Nienhuis R. A brief history of hypocretin/orexin and narcolepsy. Neuropsychopharmacology. 2001 Nov; 25 (5 Suppl):S14-20.

Thannickal TC, Moore RY, Nienhuis R, Ramanathan L, Gulyani S, Aldrich M, Cornford M, Siegel JM. Reduced number of hypocretin neurons in human narcolepsy. Neuron. 2000 Sep; 27 3):469-74.

Timo-Iaria C, Negrão N, Schmidek WR, Hoshino K, Lobato de Menezes CE, Leme da Rocha T. Phases and states of sleep in the rat. Physiol Behav. 1970 Sep;5 (9):1057-62.

Tohyama M, Maeda T, Shimizu N. Comparative anatomy of the locus coeruleus. II. Organization and projection of the catecholamine containing neurons in the upper rhombencephalon of the frog, Rana catesbiana. J Hirnforsch. 1976; 17 (1):81-9.

Trivedi P, Yu H, MacNeil DJ, Van der Ploeg LH, Guan XM. Distribution of orexin receptor mRNA in the rat brain. FEBS Lett. 1998 Oct 30; 438 (1-2):71-5.

Trulson ME, Jacobs BL. Chronic amphetamine administration to cats: behavioral and neurochemical evidence for decreased central serotonergic function. J Pharmacol Exp Ther. 1979 Nov; 211(2):375-84.

Ursine R, Sterman MB: *A manual for standardized scoring of sleep and waking states in the adult cats.* Brain Information Service/Brain Research Institute, Los Angles, University of California; 1981.

Van den Pol AN. Hypothalamic hypocretin (orexin): robust innervation of the spinal cord. J Neurosci. 1999 Apr 15; 19 (8):3171-82.

Vanni-Mercier G, Sakai K, Lin JS, Jouvet M. Mapping of cholinoceptive brainstem structures responsible for the generation of paradoxical sleep in the cat. Arch Ital Biol. 1989 Jun; 127 (3):133-64.

Vertes RP, Kinney GG, Kocsis B, Fortin WJ. Pharmacological suppression of the median raphe nucleus with serotonin1A agonists, 8-OH-DPAT and buspirone, produces hippocampal theta rhythm in the rat. Neuroscience. 1994 May; 60 (2):441-51.

Vertes RP. Brain stem generation of the hippocampal EEG. Prog Neurobiol. 1982;19 (3):159-86.

Volkoff H, Bjorklund JM, Peter RE. Stimulation of feeding behavior and food consumption in the goldfish, Carassius auratus, by orexin-A and orexin-B. Brain Res. 1999 Nov 6;846 (2):204-9.

Watanabe T, Taguchi Y, Shiosaka S, Tanaka J, Kubota H, Terano Y, Tohyama M, Wada H. Distribution of the histaminergic neuron system in the central nervous system of rats; a fluorescent immunohistochemical analysis with histidine decarboxylase as a marker. Brain Res. 1984 Mar 12;295 (1):13-25.

Watson CJ, Soto-Calderon H, Lydic R, Baghdoyan HA. Pontine reticular formation (PnO) administration of hypocretin-1 increases PnO GABA levels and wakefulness. Sleep. 2008 Apr 1;31 (4):453-64.

Yamanaka A, Beuckmann CT, Willie JT, Hara J, Tsujino N, Mieda M. Hypothalamic orexin neurons regulate arousal according to energy balance in mice. Neuron. 2003 Jun 5;38 (5):701-13.

Yanagisawa M. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell. 1998 Mar 6;92 (5):1 page following 696.

Yi CX, Serlie MJ, Ackermans MT, Foppen E, Buijs RM, Sauerwein HP, Fliers E, Kalsbeek A. A major role for perifornical orexin neurons in the control of glucose metabolism in rats. Diabetes. 2009 Sep; 58 (9):1998-2005. Epub 2009 Jul 10.

Zepelin H: Mammalian sleep in Kryger MH, Roth T, Dement WC (eds): *Principle and Practice of Sleep Medicine.* Philadelphia, W.B. Saunders Co; 1994. 69-80.

Zhu Y, Miwa Y, Yamanaka A, Yada T, Shibahara M, Abe Y, Sakurai T, Goto K. Orexin receptor type-1 couples exclusively to pertussis toxin-insensitive G-proteins, while orexin receptor type-2 couples to both pertussis toxin-sensitive and -insensitive G-proteins. J Pharmacol Sci. 2003 Jul;92 (3):259-66.

***