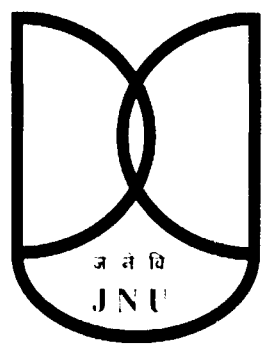
# *Role of Proton Pump Inhibitor/Exchanger in the Modulation of Sleep-Wake Architecture in Rat*

THESIS SUBMITTED TO
**JAWAHARLAL NEHRU UNIVERSITY**

FOR THE DEGREE OF
**MASTER OF PHILOSOPHY IN LIFE SCIENCES**

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**NEW DELHI**

**JULY 2012**

# JAWAHARLAL NEHRU UNIVERSITY

## SCHOOL OF LIFE SCIENCES

## CERTIFICATE

This is to certify the work contained in this thesis entitled, "***Role of proton pump inhibitor/exchanger in the modulation of sleep-wake architecture in rat***", is a bonafide record of independent research work of **Ms. Munazah Fazal Qureshi,** Enr. No.: 10/30/ML/20 and is a worthy of consideration for the award of **MASTER OF PHILOSOPHY IN LIFE SCIENCES.**

Dr. N.B. Sareen
(Dean, School of Life Sciences)

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(Supervisor)

# DECLARATION

The research work entitled: ***"Role of Proton Pump Inhibitor/Exchanger in the Modulation of Sleep-Wake Architecture in Rat"***, presented in this thesis embody the original research work done by me for the **Master of Philosophy** (M. Phil.) degree in Life Sciences at Neurobiology Division, School of Life Sciences, Jawaharlal Nehru University, New Delhi. This work has not been submitted in part or in full for any other degree or diploma elsewhere till date.

Munazah .

***Munazah Fazal Qureshi***

Date: 27.07.12

Place: New Delhi

*Dedicated to My Parents:*

*My Father's Belief*

*and*

*My Mother's Perseverance!!!*

# *Acknowledgements*

***In The Name Of Allah, The Most Gracious And Merciful.***

*Well, to say this is "My M.Phil thesis" and that I have worked solely on it would be totally untrue. At best, this was a rung to me and my father's dream. There are people in this world, some of them so wonderful who made this dream become a 'reality', that you are holding in your hand. I would like to thank all of them and in particular:*

***Dr N.B. Sareen,*** *Dean, School of Life Sciences, for her affection, timely help, guidance and encouragement.*

***Dr. Sushil K Jha,*** *my supervisor, for boosting my morale and believing in my dream. He taught me the very basics of the project and stayed with me right till the end. If he hadn't encouraged me all the way, I would have given this up a long time ago. It is his belief that kept my nerves on the move right till the end. Thank you Sir.*

***My Labmates,*** *Gunjan Singh, Tankesh Kumar, Shweta Tripathi (Ashu), Swati Panday, Govinda Yadav, Shweta Tripathi, Fayaz Ahmad and Miriam Rojy for their timely help. Of special mention is Shweta Tripathi (Ashu), who was with me through thick and thin. She was a helping hand that was always on my shoulder. She always cared and supported me. I am indebted to her.*

***Friends,*** *Haseeb Shah, Meghna Gupta, Nilofer Naqvi, Nikhat Saleem, Nitesh Kumar Khandelwal, Tapan Sharma, Manjiri Mishra, Shweta Pandey and Baby Santosh who gave honest comments to make my report better. They also stayed with me in the process and handled me and my sometimes out of control emotions so well. Thank you so much, friends. At a distance from my parents, you make my world here.*

***Dr. Ishfaq Ahmad,*** *a helping hand just a click away. Thank you for responding to my buzz and sending me all the necessary articles as and when required. I am highly indebted to you.*

***Taseem Ahmad Mokhdomi,*** *brother and friend who always helped and cared for me. He would keep me on the toes to accomplish the task but at the same time he was always there helping with even the slightest of difficulty I ever faced. Thank you, brother.*

***My Friends back home,*** *Beenish Iqbal, Durafshan Sakeena, Fakhra Nazki, Farrukh Rana, Misba Khan, Sanam Mansoor and Sobia Zaffar. Thank you for being there and encouraging me all the way down. Thank you for being patient listeners when I emptied my frustrations into you.*

***Adeena,*** *my niece, my little bunch of joy; her broken words would always soothe my rusted nerves and get me going afresh. Thank you my love, you always made my day.*

***My Rats,*** *The whole thesis lies on the tiny backs of my rats. They sacrificed their lives for the completion of this thesis. Thank you so much for carrying the entire burden yourselves and rewarding me in the end.*

***SLS, JNU,*** *Thank you for making everything available from the ease of internet access to the ease of instrument access. Special thanks, to Meenu ma'am for patiently paying heed to our confusions during the submission process.*

***My Parents, my sisters and my brother,*** *People with an irrational and unbreakable belief in me that bordered on craziness at times. My relationship with them goes beyond the common genes, we share, and I needed their irrational support for me. Thank you for everything.*

*And lastly, it's only when one compiles a report, that one realizes the true power of MS Word, from grammar checks to replace all. It is simple that without this software; this report would not be written. Thank you, Mr.* ***Bill Gates*** *and* ***Microsoft Corp.***

***Munazah Fazal Qureshi***

# CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| 5-HT | Serotonin |
| Ach | Acetylcholine |
| ANOVA | Analysis of variance |
| ARAS | Ascending reticular activating system |
| CBT | Core body temperature |
| DA | Dopamine |
| DMSO | Dimethyl sulfoxide |
| DRN | Dorsal raphe nucleus |
| EEG | Electroencephalogram |
| EMG | Electromyogram |
| eVLPO | Extended ventrolateral preoptic area |
| FTG | Gigantocellular tegmental field |
| GABA | Gamma amino butyric acid |
| i.p | Intraperitoneal |
| ICV | Intracerebroventricular |
| LC | Locus coeruleus |
| LDT | Laterodorsal tegmentum |
| LNREM | Latency non-rapid eye movement sleep |
| LPT | Lateropontine tegmentum |

| | |
|---|---|
| LREM | Latency rapid eye movement sleep |
| MPB | Median parabrachial nucleus |
| mPRF | Median pontine reticular formation |
| NA | Noradrenaline |
| NE | Norepinephrine |
| NHE | Sodium/hydrogen exchanger |
| NREMS | Non-rapid eye movement sleep |
| NTS | Nucleus tractus solitarii |
| $PaCO_2$ | Partial pressure of carbon dioxide |
| $PaO_2$ | Partial pressure of oxygen |
| PC | Precoeruleus |
| peri- LC | Peri locus coeruleus |
| pH | Power of hydrogen |
| PPI | Proton pump inhibitor |
| PPT | Pedunculopontine tegmentum |
| REMS | Rapid eye movement sleep |
| RTN | Retrotrapezoid nucleus |
| SC | Subcoeruleus |
| SLD | Sublaterodorsal nucleus |
| S-W | Sleep/wake cycle |
| Tbr | Brain temperature |

| | |
|---|---|
| TMN | Tuberomammillary nucleus |
| vlPAG | Ventrolateral periaqueductal gray |
| VLPO | Ventrolateral preoptic area |
| vSLD | Ventral sublaterodorsal nucleus |

# LIST OF FIGURES

# *Abstract*

## Abstract

The proton pump plays an important role in the homeostatic regulation of cellular pH and acidification of intracellular organelles. Increased $CO_2$ concentration contributes in decreasing cellular pH and studies suggest that a slight increase in body $CO_2$ significantly increases sleep with concomitant increase in number of REM sleep episodes. Therefore, we reasoned that the proton pump may also modulate REM sleep. We investigated the effects of proton pump inhibitor '*Lansoprazole*' on REM sleep in the rat. Male Wistar rats were surgically prepared for chronic polysomnographic recordings. Two different doses of *Lansoprazole* (low dose: 1mg/kg; high dose: 10 mg/kg) were injected intraperitoneally (n = 7) and thereafter, sleep-wakefulness was recorded for 6 hrs (11:30 AM – 5: 30 PM). The changes in sleep-wakefulness were scored and compared statistically (one-way repeated measure ANOVA) with vehicle and low dose. REM sleep amount in animals of vehicle control and *Lansoprazole* low dose groups were 9.26 $\pm$ 1.03 and 9.09 $\pm$ 0.54, which increased significantly by 31.53 % and 33.99 % respectively, in animals treated with high dose ($p < 0.01$; $F_{(3,27)} = 6.53$). *Lansoprazole* significantly increased REM sleep frequency ($p < 0.05$; $F_{(2,20)} = 4.82$), while REM sleep episode duration length and REM sleep latency remained unaltered. Further, we injected sodium-hydrogen exchanger blocker '*Amiloride*', (10 mg/kg) (n = 5) which plays an important role in intracellular pH recovery. We observed that *Amiloride* did not alter sleep-wake architecture. Our results suggest that proton pump plays a role in the modulation of REM sleep and supports our view that REM sleep might act as a sentinel to help maintain normal $CO_2$ level for unperturbed sleep.

# *Introduction*

## Introduction

Proton pump helps in performing several important cellular function including homeostatic regulation of cellular pH and acidification of intracellular organelles (Breton and Brown 2007). In the kidney, plasma membrane bound proton pump plays a critical role in the regulation of systemic acid-base balance (Gluck et al. 1996; Hashioka et al. 2009). Further, its altered activity is associated with development of several pathophysiological conditions such as gastroesophageal reflex disease (GERD), increased oesteoclast activity, sleep apnea etc. (Hashioka et al. 2009; Insogna 2009; Shaheen et al. 2008; Tabares and Betz 2010; Wasilewska et al. 2012). In addition, it is related intricately to the release of neurotransmitters like glutamate, GABA, serotonin and acetylcholine (Nakamura et al. 1995) (Tabares and Betz 2010). It is known that the proton pump drives protons into synaptic vesicles, which could further be associated with neurotransmitter re-uptake mechanisms (Tabares and Betz 2010). These studies suggest that the proton pump plays an important role in the modulation of various physiological functions.

Patients with medical conditions such as GERD, sleep apnea etc., also suffer from sleep impairments and if treated with proton pump inhibitors (PPIs), their sleep efficiency improves (Shaheen et al. 2008). Non-Rapid Eye Movement (NREM) and Rapid Eye Movement (REM) sleep are regulated by the hypothalamic and brainstem neural circuitries of the brain, respectively (Jha and Mallick 2011). Brainstem locus coeruleus (LC) area has one distinctive group of neurons called 'REM-OFF' neurons, which do not fire during REM sleep (Aston-Jones and Bloom 1981; Jha and Mallick 2011). It is believed that complete cessation of these neurons plays a permissive role for REM sleep generation (Jha and Mallick 2011). LC neurons also act as chemosensors and play an important role in regulating proton ion concentration by modulating the cardio-respiratory system (Dean et al. 2001; Madan and Jha 2012). Interestingly, it has been observed that a slight increase in body $CO_2$ concentration increases sleep significantly with concomitant increase in number of REM sleep episodes (Fraigne et al. 2008; Madan and Jha 2012). On the other hand, REM sleep amount is decreased with a decrease in body $CO_2$ level (Ryan et al. 1983). These studies suggest that sleep can be affected by the changes in $CO_2/H^+$ ion concentration during hypo-or hypercapnia. The proton

pump helps in maintaining this normal $CO_2/H^+$ ion concentration and its altered activity perturbs the homeostatic balance which in-turn may probably affect sleep (Madan and Jha 2012). Thus, it is possible that in addition to being used for the treatment of gastro-intestinal complications and sleep related breathing problems; PPIs may also play a role in improving sleep efficiency. Its role in sleep modulation is, however, not yet known.

The change in intracellular pH is a major part of the intracellular signaling pathway which alters the firing rate of LC neurons (Filosa et al. 2002). Few studies have demonstrated an activity dependent acidification of neuronal cytoplasm, which is soon followed by the spontaneous process of alkalization (Tabares and Betz 2010; Zhang et al. 2010). This biphasic pH change is achieved through the proton pump by extruding hydrogen ions (Zhang et al. 2010), and if this biphasic pH change is perturbed by using PPIs, neuronal firing is inhibited (Tabares and Betz 2010). The sodium-hydrogen exchanger (NHE), on the other hand, plays a role in intracellular pH recovery (Nattie 1995). Neurons maintain homeostatic balance of their intracellular pH by means of both, proton pump and NHE but through different mechanisms (Dean 2010; Kersh et al. 2009; Nattie 1995; Tabares and Betz 2010; Zhang et al. 2010). It has been observed that the brainstem NHE is up-regulated by chronic acidosis but not by prolonged hypercapnia (Kiwull-Schone et al. 2007), whereas LC neurons are hyperpolarized during sustained hypercapnia (Dean et al. 2001). As it is widely known that LC neurons also play an important role in the modulation of sleep-wakefulness (S-W), therefore, we reason that the proton pump would also modulate S-W, but NHE would not have any role in its modulation. Hence, we aimed to investigate the effect of PPI '*Lansoprazole*' and NHE blocker '*Amiloride*' on S-W in the rat.

# Chapter 1
## *Review of Literature*

## 1. REVIEW OF LITERATURE

Phylogenetically more evolved animals for example; birds and mammals demonstrate two major distinct sleep states; non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep, but the neural mechanism associated with the generation of specific vigilant state is still unclear. In human, however, these sleep states progress in five stages (stage I – V), while it comprises two to three stages in the rat, cat, dog, ferrets and other mammals (Jha et al. 2006; Zeplin et al. 2005) **(Fig. 1.1.)**. On the other hand, lower vertebrates such as fishes, amphibians and reptiles show a single sleep state (analogue to mammalian NREM sleep) and REM sleep has not been noted in these animals till date (Nicolau et al. 2000), suggesting that REM sleep has been evolved in the birds and mammals from the stem reptiles. Interestingly, REM sleep is also absent in some of the aquatic mammals exhibiting convergent evolution such as dolphin, whales, manatee, etc., suggesting that the natural forces have facilitated the evolution of sleep states only in more advanced terrestrial animals, the birds and mammals. But the intriguing question "why nature has evolved complex sleep states; NREM and REM sleep in birds and terrestrial mammals" still remains elusive.

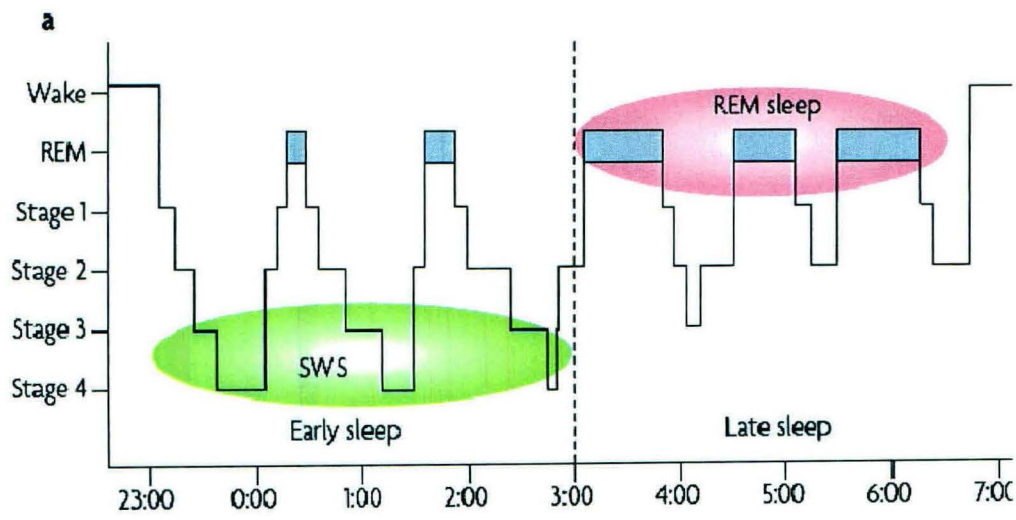


**Figure 1.1. Cyclic occurrence of different sleep stages as a function of time.**
(*Source: Nature Reviews Neuroscience* 11, 2010)

## 1.1. Historical background of sleep research:

During the World War-I, there was a pandemic of a disease called "*Encephalitis lethargic*", a presumed viral disease that caused excessive sleepiness in the afflicted individuals. These patients could be briefly awakened by the sufficient amount of stimulus but they tended to sleep more often. A Viennese neurologist, Baron Constantin von Economo, reported that this state of prolonged sleepiness could be due to injury to the posterior hypothalamus and rostral midbrain (von Economo 1930). At the same time, another group of individuals, infected with the same virus, complained of insomnia which is believed to have occurred with lesions of the anterior hypothalamus. Based on his observations, von Economo predicted that the hypothalamic region near the optic chiasma contains sleep-promoting neurons, whereas the posterior hypothalamus contains neurons that promote wakefulness (von Economo 1930)

Even after *von Economo's* findings, it was still debatable during 1930s', whether sleep is an active or a passive process. Bremer in 1935, reported that sleep was a passive process and that wakefulness required a high level of continuous sensory input from the periphery to maintain activity within the cerebral hemispheres (Bremer 1935). He found that the *cerveau isole* animals maintained a continuous sleep-like state with synchronous slow wave activity, whereas the *encephala isole* cats were awake and their electroencephalograms (EEGs) contained synchronous and desynchronized activity resembling sleep-alert states. However, W.R. Hess and W. Nauta viewed that sleep was an active process as they were able to induce sleep by stimulating diencephalic brain areas (Hess 1932; Nauta 1946). Further, in 1949, Moruzzi and Magoun identified the *brainstem ascending reticular activating pathway* (ARAS), which modulates cortical arousal and alertness (Moruzzi and Magoun 1949). Based on these findings, it was determined that sleep is an active process and the anterior hypothalamus presumably plays an important role in sleep genesis, while the ARAS complex regulates cortical arousal and alertness. Later, Eugene Aserinsky and Nathaniel Klietman in 1953, discovered a new sleep state, during which eyes showed rapid movements and they named this sleep state as Rapid Eye Movement (REM) sleep (Aserinsky 1953). Further, in 1959, Michel Jouvet and Francois Michel observed a sleep

phase that accompanied with a complete disappearance of the muscle tone and was paradoxically associated with a cortical activation and rapid eye movements (REM) in cats. They called this rapid eye movement state as paradoxical sleep (PS) (Jouvet 1959). Later, it was determined that REM sleep executive circuitries are located within brainstem cholinergic and aminergic areas (Hobson et al. 1975). Thus from 1950's onward, sleep was unequivocally considered as an active process, which is generated and terminated by activation and deactivation of specific neuronal circuitries, those are located in the anterior and posterior hypothalamic areas and also within quite a few brainstem nuclei (Aserinsky and Kleitman 1955; Aserinsky 1953; Dement and Kleitman 1957).

## 1.2. Theories of sleep genesis.

In conjuncture with the findings of *von Economo*, it was shown later by several studies, for example; lesion studies, pharmacological studies as well as through neuronal studies that wakefulness is controlled by the histaminergic group of neurons in posterior lateral hypothalamus, the basal forebrain cholinergic neurons, and the brainstem aminergic groups of neurons, while sleep is regulated by the preoptic area of anterior hypothalamus neurons (Gvilia et al. 2006; Lin et al. 1989; Suntsova and Dergacheva 2004). The various models that help us understand the neural mechanisms of sleep generation can be listed as follows:

1. Sleep generation: Hypothalamic Switch Model
2. Reciprocal interaction between brainstem neuronal populations for REM sleep modulation.
3. Role of Dopaminergic Systems in Sleep Regulation.

### 1.2.1. Sleep generation: Hypothalamic Switch Model.

With the discovery of ARAS by *Moruzzi* and *Magoun*, it was proposed that ARAS regulates alertness and wakefulness. Tracing the ARAS, it was shown that the brainstem transection at the midpons or below did not reduce arousal while more rostral transections at mid collicular level caused an acute loss of wakefulness. This crucial slab of tissue at the pons and mid brain junction was called the mesopontine tegmentum, a central player of ARAS. With the help of anatomical

and physiological techniques, the neuronal connections from mesopontine tegmentum were traced to diencephalon, where it divides into two branches, one that innervates the thalamus, while the other to the hypothalamus (Moruzzi and Magoun 1949) **(Fig. 1.2.)**.

The origin of thalamic projection from the mesopontine tegmentum was traced to laterodorsal tegmental and pedunculopontine tegmental (LDT/PPT) nuclei (Edley and Graybiel 1983; Rye et al. 1987). These nuclei were found to be cholinergic in nature and the activity of these neurons varied with the different vigilant states (Berendse and Groenewegen 1990; Herkenham 1980). During wakefulness, when the cortical electroencephalogram (EEG) shows low-voltage fast activity, many PPT/LDT neurons fire rapidly. As the individual goes to sleep, the EEG waves become slower and larger; during this period, few PPT/LDT neurons are active. Periodically during the night, the individual enters a very different state of active sleep in which there are rapid eye movements (REM sleep), a loss of muscle tone, except for the muscles involved in respiration, and a low-voltage fast EEG, which resembles a waking state. The PPT/LDT are released from tonic monoamine-mediated inhibition and hence fire rapidly during REM sleep (Massaquoi and McCarley 1992; Strecker et al. 2000).

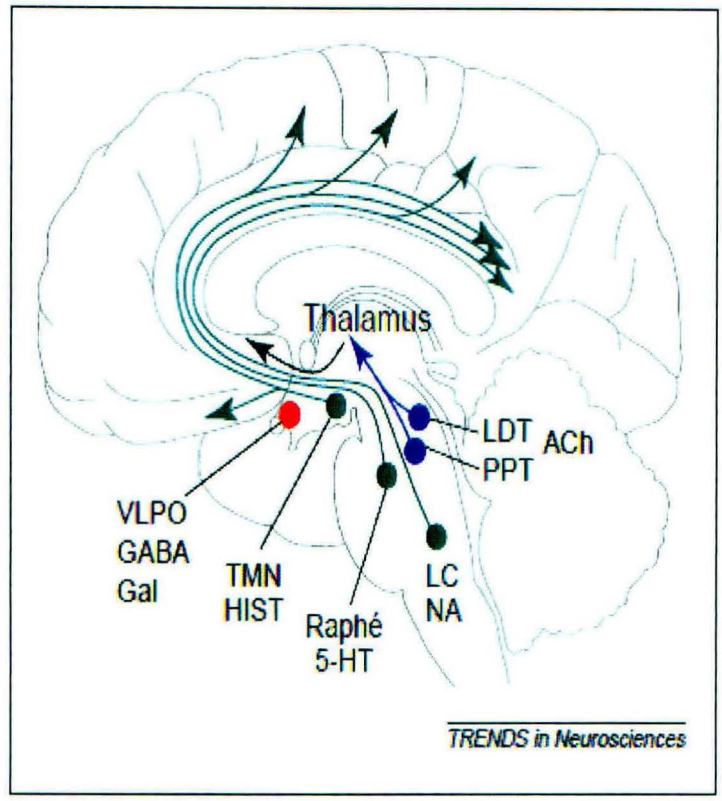


**Figure 1.2. ARAS and its projection system.** *The ascending arousal system sends projections from the brainstem and posterior hypothalamus throughout the forebrain. Neurons of the laterodorsal tegmental nuclei and pedunculopontine tegmental nuclei (LDT and PPT) (blue circles) send cholinergic fibers (Ach) to many forebrain targets, including the thalamus, which then regulate cortical activity. Aminergic nuclei (green circles) diffusely project throughout much of the forebrain, regulating the activity of cortical and hypothalamic targets directly. Neurons of the tuberomammillary nucleus (TMN) contain histamine (HIST), neurons of the raphé nuclei contain 5-HT and neurons of the locus coeruleus (LC) contain noradrenaline (NA). Sleep-promoting neurons of the ventrolateral preoptic nucleus (VLPO, red circle) contain GABA and galanin (Gal).* (*Source*: *TRENDS in Neurosciences* 24, 2001)

Since the thalamocortical branch of ARAS is active during wake as well as during sleep, the hypothalamic branch dictates the behavioral output of the subject. The hypothalamic branch originating from mesopontine tegmentum consists of noradrenergic locus coeruleus (LC), serotonergic raphe nucleus (DRN) (Saper 1985), the histaminergic tuberomammillary nucleus (TMN), orexinergic neurons of lateral hypothalamic area (Peyron et al. 1998) as well as the cholinergic neurons of basal forebrain (Fuller et al. 2007). The activity of these neuronal

groups is highest during wake, slows down during NREM and nearly stops during REM. Hence, the difference in the activity of thalamic and the hypothalamic branches of the ARAS determine the occurrence of different behavioral states (Aston-Jones et al. 1991; McGinty and Harper 1976; Vanni-Mercier et al. 1984).

Tracing the inputs to the TMN, DRN and LDT/PPT, it was found that these get projections from lateral hypothalamic area and a dense cluster of neurons in ventrolateral preoptic area (VLPO), and extended ventrolateral preoptic area (eVLPO) (Sherin et al. 1998; Sherin et al. 1996). These projections were found to be GABAergic indicating that the inputs from VLPO are inhibitory in nature, thereby promoting sleep (Sherin et al., 1998). Lu and colleagues produced excitotoxic lesions in VLPO and eVLPO to see the effect and they found that the lesion to VLPO causes loss of NREM sleep and the lesions to eVLPO cause loss of REM sleep (Lu et al. 2000).

The relationship between the VLPO and major monoamine groups appears to be reciprocal with VLPO being inhibited by the inputs from LC, DRN and TMN and these three in turn get inhibited by VLPO. This model of mutual inhibition between the VLPO and the arousal system states that during sleep the VLPO neurons fire rapidly thereby inhibiting the monoaminergic neurons and thus disinhibiting and reinforcing their own firing. Similarly when monoaminergic cell groups fire during wake, they inhibit VLPO, disinhibit and reinforce their own firing (Saper et al. 2005).

Another group of excitatory neurons called orexinergic neurons were found to innervate the components of ascending arousal system like that of VLPO (Chemelli et al. 1999) and were believed to be wake active (Kilduff and Peyron 2000). Recent studies have shown that the orexinergic neurons influence both sides of the flip–flop by sending projections to both the monoaminergic and cholinergic cell groups and to the VLPO region. These neurons increase the firing rate of LC, DRN and TMN and inhibit VLPO and hence act like a "finger" pressing the flip-flop switch into "wakeful" position and thus preventing the inappropriate switching into "sleep" position (Saper et al. 2001).

This model thus explains the stable transitions between different behavioral states. The model is shown in **Fig. 1.3.**

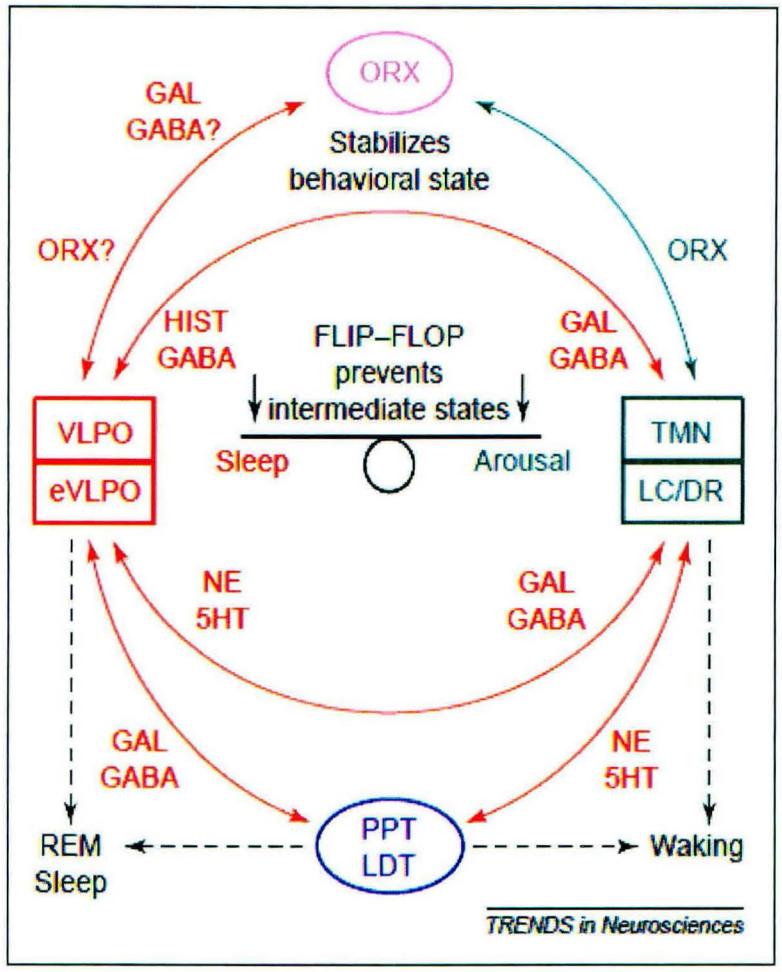


**Figure 1.3. Flip–Flop Model of sleep-wake generation.** *A model for reciprocal interactions between sleep and wake promoting brain regions, which produces a flip–flop switch. Inhibitory pathways are shown in red, and the excitatory pathways in green. The blue circle indicates neurons of the LDT and PPT; green boxes indicate aminergic nuclei; and the red box indicates the VLPO. Aminergic regions such as the TMN, LC and DR promote wakefulness by direct excitatory effects on the cortex and by inhibition of sleep-promoting neurons of the VLPO. Unbroken lines represent neuronal pathways. (Source: TRENDS in Neurosciences 24, 2001)*

### 1.2.2. Reciprocal interaction between brainstem neuronal populations for REM sleep modulation.

*Hobson* documented the presence of cell groups in LC with discharge activity curves opposite to those of the cells present in gigantocellular tegmental field

(FTG) or paramedian pontine reticular formation (mPRF) (Hobson et al. 1975). These cell groups were called as REM-OFF and REM-ON neurons respectively. In this model, Lotka-Volterra equations were used to mathematically describe the possible interactions between the REM-on and REM-off cells. This structural and mathematical model, for the first time proposed that the aminergic and cholinergic neurons of the brainstem reciprocally interact to cause the changes in the ultradian rhythm of REM-NREM sleep (McCarley and Hobson 1975). According to this model, REM-on cells of the medial pontine reticular formation (mPRF) are cholinergic and are post-synaptically excited by the activation of cholinergic receptors. Conversely, REM-off cells are aminergic (noradrenergic cells in the LC and serotonergic cells in the DRN) and are postsynaptically inhibited by the activation of noradrenergic and serotonergic receptors. During wakefulness, the aminergic REM-off system is tonically activated, thus inhibiting the cholinergic REM-on system. Throughout NREM sleep, the aminergic inhibition wanes and cholinergic excitation waxes as a result of the gradual withdrawal of aminergic inhibitory influences from cholinergic REM-on system. At REM sleep onset, aminergic inhibition is turned off and cholinergic excitability peaks, while other outputs are inhibited **(Fig. 1.4)**. The behavioral predictions of this reciprocal-interaction model might have been influenced by the contemporary anatomical, single cell recordings, and local microinjection studies of that time (Chu and Bloom 1973; McCarley and Hobson 1975).

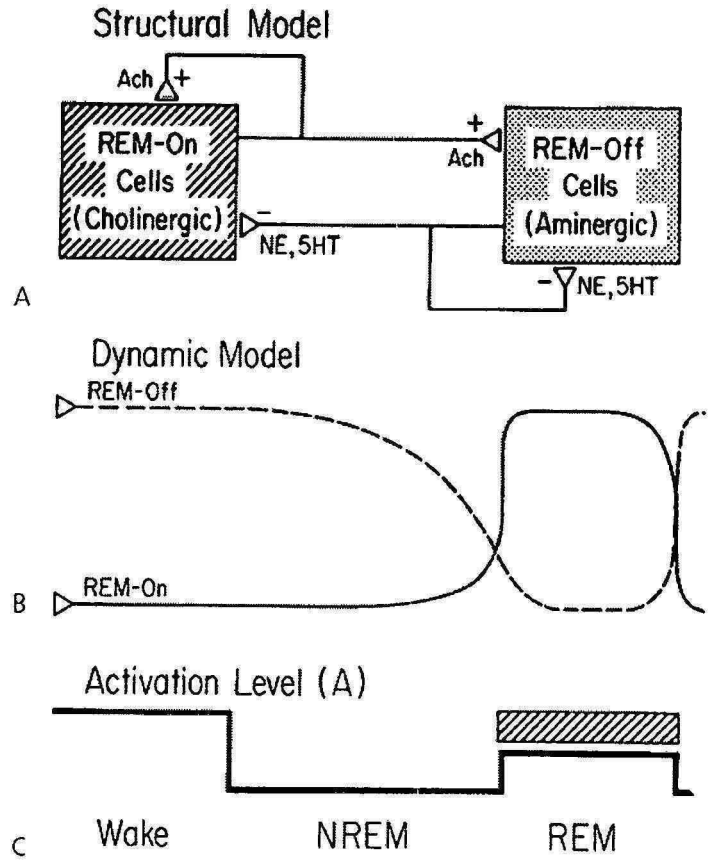


**Figure 1.4. Reciprocal interaction model of sleep generation.** *The original reciprocal interaction model of physiologic mechanisms determining alterations in activation level.* ***(A) Structural model of reciprocal interaction.*** *REM-on cells of the pontine reticular formation are cholinoceptively excited or cholinergically excitatory (ACH +) at their synaptic endings. Pontine REM-off cells are noradrenergically (NE) or serotonergically (5- HT) inhibitory (-) at their synapses.* ***(B) Dynamic model.*** *During waking, the pontine aminergic system is tonically activated and inhibits the pontine cholinergic system. During NREM sleep, aminergic inhibition gradually wanes, and cholinergic excitation reciprocally waxes. At REM sleep onset, aminergic inhibition is shut off, and cholinergic excitation reaches its high point.* ***(C) Activation level.*** *As a consequence of the interplay of the neuronal systems shown in* ***A*** *and* ***B,*** *the net activation level of the brain* ***(A)*** *is at equally high levels in waking and REM sleep and at about half this peak level in NREM sleep.(Source: Neuropsychopharmacology: The Fifth Generation of Progress, 2002)*

Although this original reciprocal-interaction model initiated a new era of research on the neurobiological mechanisms of REM sleep regulation, subsequent studies utilizing specific monoclonal antibodies of choline acetyltransferase (Bernard et al. 1999) on multiple brain regions, identified brainstem cholinergic cell groups in

the PPT and LDT but not the mPRF (Armstrong et al. 1983; Mesulam et al. 1983). Accordingly, single cell recording studies have shown that a subset of cholinergic cells in the PPT and LDT are the principal REM-on neurons in the brainstem (Datta 1995; Datta and Siwek 2002). To accommodate these new findings, a modified reciprocal-interaction model shifted the location for the major locus of cholinergic REM-on neurons to the LDT/PPT from its original postulated location in the mPRF (Pace-Schott and Hobson 2002) **(Fig. 1.5)**.

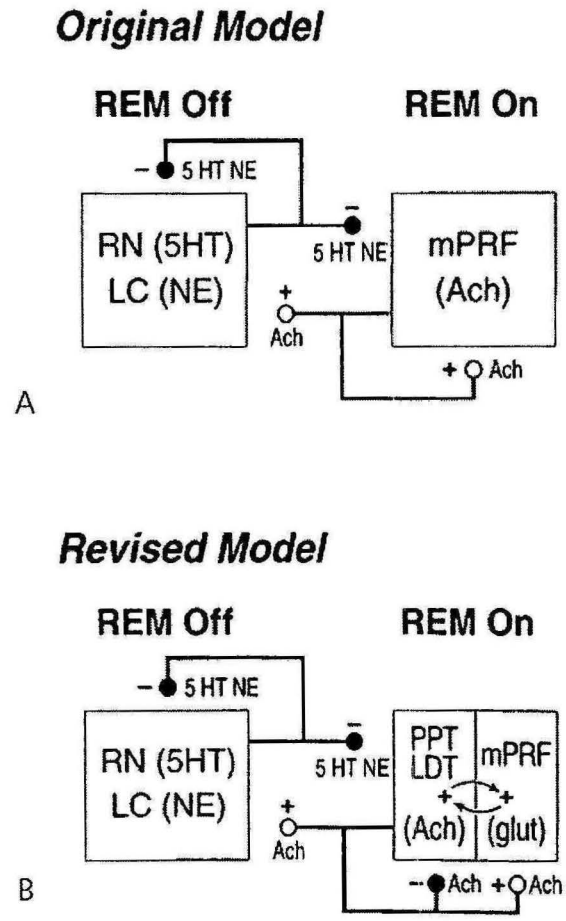


**Figure 1.5. Modified Reciprocal interaction model of sleep genesis.** *Synaptic modifications of the original reciprocal interaction model based on recent findings.* ***(A)*** *The original model proposed by McCarley and Hobson.* ***(B)*** *Synaptic modifications of the original reciprocal interaction model based on recent findings of self-inhibitory cholinergic autoreceptors in mesopontine cholinergic nuclei and excitatory interactions between mesopontine cholinergic and noncholinergicneurons Open circles, excitatory postsynaptic potentials; closed circles, inhibitory postsynaptic potentials; Ach, acetylcholine; glut, glutamate; 5-HT, serotonin; LC, locus coeruleus; mPRF, medial pontine reticular formation; NE, norepinephrine; RN, dorsal raphe nucleus. (Source: Neuropsychopharmacology: The Fifth Generation of Progress, 2002)*

### 1.2.3. Role of Dopaminergic Systems in Sleep Regulation.

Having identified the role of different monoamines in the regulation of sleep wake cycle, the effects of dopamine, that causes alertness, on sleep wake alteration and REM-NREM cyclicity have also been studied. It has been found that the levels of dopamine do not get altered with the different behavioral states to the extent as with 5-HT, NE, ACh but the REM sleep deprivation causes a substantial increase in DA levels as well as enhanced DA receptor sensitivity.

Experimental manipulation of dopaminergic systems also gives varying results. For example, a DA agonist reduced REM sleep at low doses but enhanced it at higher ones (Python et al. 1996), whereas a DA reuptake inhibitor had the opposite effect (de Saint Hilaire et al. 1995). In addition, although many human studies report REM suppression by DA reuptake inhibitors and indirect agonists (Gillin et al. 1973; Nicholson et al. 1989), a DA-enhancing agent, bupropion, has been shown to enhance REM sleep in humans (Nofzinger et al. 1995). Moreover, studies on the administration of dopaminergic drugs have suggested that DA may play a role in the induction or intensification of nightmares. Therefore, the effects of DA on sleep appear to be variable and are in much need of further study.

As is the case with many other neuromodulators, the sleep effects of DA may be mediated by dopaminergic effects on the aminergic and cholinergic systems involved in the executive control of the REM-NREM sleep cycle. For example, DA has been shown to enhance cortical ACh release (Moore et al. 1999), whereas cholinergic mesopontine neurons have been shown to enhance mesolimbic DA release (Oakman et al. 1999). Such mutual facilitation between cholinergic and dopaminergic systems may serve to maintain or intensify REM sleep, especially given DA neurons' continued activity during REM (Miller et al. 1983; Trulson et al. 1981).

## 1.3. Modifications and current concept of reciprocal interaction model

Neuropharmacological and electrophysiological experiments strongly support the pontine reciprocal interaction model of REM sleep genesis and the critical role of

LDT/PPT as REM-on groups. However, the accuracy of the model has been questioned on the basis of few experimental findings like limited alterations to the REM sleep following lesions to LDT/PPT nuclei (Jones et al. 1977) and limited c-Fos expression in LDT/PPT neurons during REM sleep (Lu et al. 2006; Verret et al. 2005).

To account for these findings and to accommodate them in reciprocal interaction model, Lu *et al.,* traced the convergence of two descending pathways from hypothalamus to mesopontine tegmentum, previously established to be involved in the control of REM sleep in rats, the eVLPO , which contains REM- active neurons (Lu et al. 2002) that are inhibitory but promote REM sleep and the orexinergic neurons of lateral hypothalamus, which cease firing during REM sleep (Lee et al. 2005) but are excitatory and presumably inhibit REM sleep. Following the tracer injections, the convergence of the two pathways was found in several areas of the mesopontine tegmentum, including the ventrolateral periaqueductal grey (vlPAG), lateral pontine tegmentum (LPT), dopaminergic neurons of ventral PAG, DRN and LC. The vlPAG and the LPT were found to contain REM-off population of neurons as the injection of GABA agonist, muscimol in these regions triggers REM sleep (Sastre et al. 1996). Also, the control lesions of DRN sparing vlPAG, and mPRF sparing LPT, did not produce any effects on REM sleep. Thus, the REM-off region was identified as the vlPAG and LPT.

Anterogradely tracing the connections of vlPAG and LPT helped identify the putative REM-on regions as sublaterodorsal nucleus (SLD) in rats' equivalent to subcoerulues (SC) or peri-locus coeruleus alpha (peri-LC) in cats, the adjacent regions of precoeruleus area (PC) of the periventricular grey matter and the median parabrachial nucleus (MPB). To substantiate the finding it was shown that stimulation of SLD with bicuculline (GABA antagonist) induced REM sleep in cats and rats whereas injection of muscimol in the same region decreased REM sleep (Xi et al. 1999). In addition, c-Fos expression profile showed the presence of REM active cells in this region, as opposed to cholinergic mesopontine neurons which, despite, their REM activity profile do not show c-Fos expression during REM sleep (Lu et al. 2006; Verret et al. 2005).Thus, the REM-off regions viz., vlPAG and LPT and the REM-on regions viz., SLD-PC-PB, stand deciphered (Lu et al., 2006). To add to the modifications of the reciprocal interaction model, it was shown that the cells in vlPAG and LPT and SLD that target each other are

GABAergic. This circuit arrangement of mutual inhibitory interactions between vlPAG-LPT REM-off neurons and SLD REM-on neurons suggested a flip–flop switch arrangement in which each side by (inhibiting the other) also disinhibits (and thus reinforces) its own firing.

In summary, recent work by Saper and Lu has uncovered three REM-on groups with distinct projections and neurotransmitters. The SLD contains glutamatergic neurons that project to the spinal cord and GABAergic neurons that project to REM-off neurons in the vlPAG and LPT. The PC and PB also contain glutamatergic neurons, but these neurons project to the basal forebrain and medial septum and regulate REM sleep EEG in the cerebral cortex and hippocampus. Thus, lesions of the SLD, vSLD and PC produce a specific loss of REM sleep components: REM sleep, atonia and theta EEG, respectively. The model for regulation of REM sleep, therefore, predicts a circuit arrangement similar to a 'flip flop' switch wherein REM-off neurons in the vlPAG–LPT inhibit all three REM-on groups and GABAergic REM-on SLD neurons feed back to the REM-off neurons. In contrast to the original reciprocal interaction model which emphasized interactions of cholinergic and monoaminergic neurons, this model hypothesizes that the switching circuitry for REM sleep involves reciprocal interactions between GABAergic REM-off and REM-on populations. This brings in to the picture the currently accepted model of REM sleep control called the 'flip-flop' model for REM sleep maintenance and generation **(Fig.1.6.)** (Fuller et al. 2007).

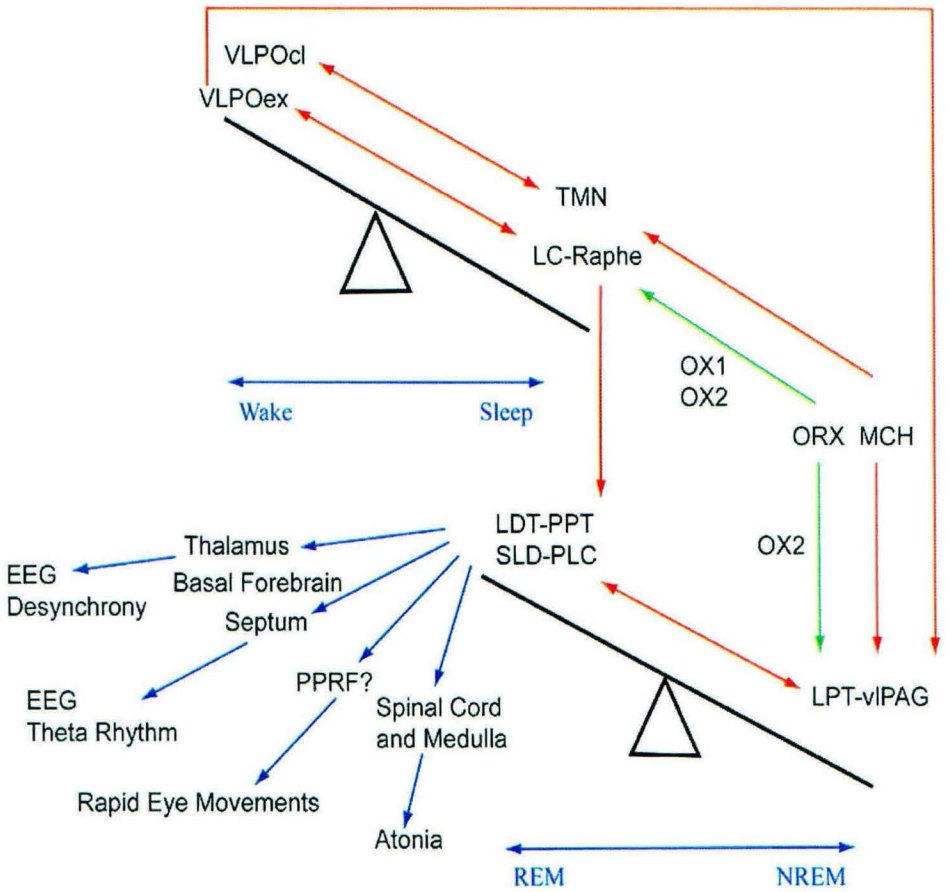


**Figure 1.6. REM flip-flop switch.** *The REM flip-flop switch is part of a cascading pair of switches that generate forebrain vs. brainstem-spinal manifestations of REM sleep. The VLPO and the ascending arousal systems, particularly the monoaminergic pathways (TMN, LC, Raphe), form a mutually inhibitory flip-flop switch that controls transitions into sleep. The orexin or hypocretin neurons (ORX) play the role of a "finger" that, when active presses the wake-sleep flip-flop switch into the wake position by reinforcing the activity of the arousal neurons. Similarly, the orexin inputs to the vlPAG-LPT normally prevent the emergence of REM phenomena except during sleep (when the orexin neurons are nearly silent). Absence of the orexin influence not only permits unwanted switching of the wake-sleep state, but also allows REM phenomena (atonia during cataplexy, dreams during hypnogogic hallucinations) to emerge during wakefulness (i.e., without tripping the wake-sleep flip-flop switch). The neurons that contain melanin-concentrating hormone (MCH) have the same targets as the orexin neurons, but the opposite influence (mainly inhibitory) and opposite activity pattern (mainly REM active). Hence, their net effect is to reinforce the influence of the orexin neurons. (Source: Nature 441, 2006)*

## 1.4. The potential role of LC in REM sleep genesis.

Apart from the mutual inhibition of mesopontine REM-off and REM-on regions, the role of LC REM-off neurons came into focus. It was proposed that the cessation of the firing of REM-off neurons in LC is a prerequisite for REM sleep initiation and therefore, if these neurons were stimulated, the REM sleep would be inhibited. It was found that continuous low frequency mild electrical stimulation of LC REM-off neurons resulted in the loss of REM sleep initiation and withdrawal of the stimulus resulted in the rebound increase in REM sleep as was seen after REM sleep deprivation (Singh and Mallick 1996). This confirmed the earlier reports of LC REM-off neuronal firing during REM sleep deprivation (Mallick et al. 1990). Simultaneous recording of these two neurons helped develop a temporal relation between these two neuronal groups and it was found that the increase in the firing rate of REM-on neurons inhibits the REM-off neurons to initiate the REM sleep. Thus, it was proposed that cessation of REM-off neurons is a prerequisite for the REM sleep initiation (Pal and Mallick 2007).

In conjuncture with the reciprocal inhibition model between LC/DRN and LDT/PPT, it was proposed and seen that microinjection of ACh agonist to LC augmented REM sleep (Vanni-Mercier et al. 1989) and also the ACh levels increased during spontaneous REM sleep (Kodama et al. 1990). Since ACh did not hyperpolarize the LC neurons (Egan and North 1986), it was proposed that actual inhibition of REM-off neurons in LC may be due to an inhibitory neurotransmitter, which might be triggered by ACh leading to the generation of REM sleep. The potential candidate considered for the inhibitory neurotransmitter was GABA because of the following:

(i) Presence of GABA-ergic interneurons and terminals in LC (Jones 1990, 1991);
(ii) Presence of GABA-ergic receptors (Luque et al. 1994) on the neurons in LC;
(iii) Increased GABA levels in LC during REM sleep (Nitz and Siegel 1997);

(iv) Reduction of REM sleep by microinjection of picrotoxin, a GABA-A receptor antagonist, in LC (Kaur et al. 1997);

(v) Activation of GABA-ergic neurons in LC during REM sleep (Maloney et al. 1999); and

(vi) Inhibition of NA-ergic neurons in LC by GABA (Gervasoni et al. 1998).

In a series of studies it was found that blocking of GABA-ergic as well as cholinergic transmission in LC by picrotoxin and scopolamine, respectively decreased REM sleep while microinjection of GABA (Mallick et al. 2001) and acetylcholine agonist (Quattrochi et al. 1998); Mallick et al., 2001) into the LC increased REM sleep. Based on these findings, a "GABA-ergic interneuron based model" was proposed (Alam et al. 1993; Mallick et al. 1999) that envisaged GABA mediating the neurotransmission between cholinergic LDT/PPT and the NA-ergic LC neurons. According to this model, the cholinergic inputs from REM-on neurons excite the GABA-ergic neurons in LC, which in turn inhibit the NA-ergic REM-off neurons in LC facilitating the generation of REM sleep (Mallick et al. 2001).

## 1.5. Alteration in physiological variables during Sleep.

### 1.5.1. Metabolism.

A study was conducted to see the metabolic rate and substrate oxidation during sleep in relation to time of sleep and sleep stage. It was found that the energy expenditure decreased during the first half of the night, reached a maximum decrease (a 35% decrease), and remained relatively stable until awakening. Similarly, fat oxidation decreased from the onset of sleep. On the other hand, it was found that the carbohydrate oxidation showed no remarkable changes from the onset of sleep but began to increase before awakening. It was also revealed that energy expenditure during REM sleep was significantly greater than that during sleep stages 2 and 3/4. However, carbohydrate oxidation during REM sleep was found to be significantly greater than that during sleep stage 3/4. The increase in energy expenditure and carbohydrate oxidation during REM sleep are consistent with a notion that changes in energy metabolism in brain are manifested as small

fluctuations in whole-body energy metabolism during sleep (Katayose et al. 2009). Brain also shows varied changes in metabolism during different sleep stages with the metabolic rate showing a decline in SWS and an increase in REM sleep which is almost similar to that of wakefulness (Maquet 1995).

### 1.5.2. Heart Rate.

Heart rate changes during sleep have also been studied in order to determine if this could provide a new basis for sleep staging. The majority of these studies show that heart rate decreases during NREM sleep and increases during the subsequent REM sleep. These changes however have not always been significant. Many studies compared heart rate during awake, stage 2 sleep, slow wave sleep 3 & 4 and REM sleep. These reports have shown a significant decrease in heart rate from REM sleep, to stage 2 sleep, to slow-waves sleep (Pivik et al. 1996) Further results demonstrated that heart rate decreased from awake to slow-waves and REM sleep to stage 2 (Vaughn et al. 1995), from wakefulness and REM sleep to stage 4 (Somers et al. 1993) Heart rate also increased rapidly over the NREM-REM transition periods and decreased more gradually at the REM-NREM transition periods. It increased several minutes before REM sleep and continues to rise into the REM sleep (Burgess et al. 2001; Cajochen et al. 1994). These observations reveal a tight link between changes in sleep stages and heart rate.

### 1.5.3. Blood pressure.

Numerous studies have recorded blood pressure in humans over a period of 24hrs and found a decrease in blood pressure during sleep (Bevan et al. 1969; Mancia et al. 1983; Parati et al. 1990). This falls in line with the changes in BP recorded from reptiles, birds and mammals studied so far (Coote 1982). Studies that have specifically investigated sleep in humans have confirmed that there is a fall in BP during NREM sleep as compared with relaxed wakefulness. Blood pressure may also be lower in slow wave sleep stage 3 & 4 than stage 2. During REM sleep BP returns to waking

levels. During phasic REM sleep, BP and HR both show pulsatile increases in activity (Van de Borne et al. 1994; Zemaityte et al. 1984). Sleep related changes in HR and BP appear to be mediated primarily by changes in autonomic circulatory control.

### 1.5.4. Respiration.

Respiratory rate decreases at NREM sleep onset compared to wakefulness, which becomes regular with definite expiratory pause. In man, cat and dog where more extensive respiratory measurements have been made there is, in addition, a small increase in tidal volume but even so, the decrease in frequency is sufficiently large to decrease minute ventilation (Bulow 1963; Bulow and Ingvar 1961; Orem et al. 1977; Phillipson 1978a). Accompanying the decrease in frequency there is also a reduction in the uptake of oxygen and production of carbon dioxide amounting to 10-20%, so indicating a decrease in metabolic rate during synchronized sleep. Periodic fluctuations during early stages of NREM sleep consist of successive reductions in the tidal volume with each inspiration and end in an expiratory apnea which is terminated by a large inspiration and the cycle repeats itself. On the other hand, breathing during REM sleep is characteristically more rapid than during NREM sleep and is often highly irregular. Periods of hyperventilation are interspersed with periods of more regular respiration and apneas of varying length. The degree to which these changes occur varies from one animal species to another. Arterial gas measurements have shown that partial pressure of $CO_2$, decreases to approach awake values, whereas partial pressure of $O_2$, is unchanged or slightly increased over the course of a REM sleep cycle (Bulow 1963; Phillipson 1978b, 1978a; Snyder 1967; Snyder et al. 1964).

### 1.5.5. Temperature.

The idea that temperature and sleep are interrelated is based on evolutionary history. Sleep, specifically the stage of rapid-eye movement (REM) sleep, developed in association with endothermy (Campbell and Tobler 1984; Zepelin 2000). All higher species, independent of temporal

niche, usually sleep during the circadian trough of their core body temperature (CBT) rhythm (Glotzbach and Heller 2000; Zepelin 2000). Early studies in humans revealed a close temporal relationship between sleep onset and the CBT rhythm (Aschoff 1970; Zulley et al. 1981). When the sleep–wake cycle is synchronized with the geophysical light–dark cycle, the maximum of CBT occurs in the early evening, and the minimum in the second half of the nocturnal sleep episode. Sleep is then typically initiated on the declining portion of the CBT curve when its rate of change, and body heat loss, is maximal (Murphy and Campbell 1997; Zulley et al. 1981). In the morning when heat production is dominant over heat loss, CBT increases, as does the propensity to wake-up. These preferred zones for falling asleep and for waking up have a profound effect on sleep duration — sleep length is maximal (circa 14 h) when sleep is initiated around the CBT maximum (Zulley et al. 1981). All these findings indicate that sleep propensity and sleep duration are tightly coupled with the thermoregulatory system (Krauchi 2007).There are changes in brain temperature (Tbr) as well that are very tightly coupled to sleep states. For example, during the transition from wakefulness to NREMS, brain temperature gradually decreases (1-2 °C). These changes are actively regulated and since they begin before the onset of NREM sleep, they can predict the occurrence of NREM sleep. In contrast, just after entry into REM sleep there is a relatively rapid rise in brain temperature occurring within 1-2 minutes. It is likely that these changes reflect the brain metabolic and/or blood flow changes associated with sleep states; NREM sleep is associated with reduced brain metabolism, while during REM sleep brain metabolism increases (Krueger and Takahashi 1997).

All these variations in the physiological variables during sleep (NREM) suggest the energy conservation hypothesis of sleep and at the same time placing REM sleep as a paradox between sleeping and waking states.

## 1.6. Functions of Sleep

Body needs rest and the desire to compensate for the lost sleep does not answer the question for the functional role of sleep. However, sleep, probably has an

important physiological function can be suggested considering the following arguments (1) sleep has persisted in animal kingdom across the evolution, despite the fact that it leads to decrease awareness of the surroundings and hence increase the chances of being harmed, implies that it must provide them with some advantage, (2) sleep deprivation in animals poses a threat to their lives as rats continually deprived of sleep for 12- 14 days die. However, it is still not clear whether the loss of sleep is the actual cause of death. Humans apparently show no such signs, other than the urge for sleep after deprivation for about 11 days (Madan and Jha 2008; Maquet 1995). Until the 1960s, sleep was mostly conceptualized within the framework of homeostatic principles. During sleep, energy or essential brain or bodily ingredients, depleted during waking, were thought to be restored. A complementary view posited the accumulation of toxic substances during wakefulness that are detoxified or removed from circulation during sleep. The immediate cause of sleep was sought in the production of these hypnotoxins that inhibit brain activities.

Many theories have now been put forward to entail functions of sleep. According to these theories, the sleep happens to save energy, keeps species inactive during inappropriate times and reverses the brain changes induced during waking. There are evidences that suggest that REM and NREM sleep have their own distinct roles. It is also clear that ecological factors shape the sleep expression and may differ qualitatively across species (Siegel 2005).

### 1.6.1. Theories of sleep function:

In general, by observing the sleep architecture and the intricacies of the various sleep stages of a few numbers of species of terrestrial mammal, most theories have assumed that sleep serves the same function in all animals.

**1.6.1.1 Neocortical Maintenance**: Sleep time is determined by the neuronal activity in the neocortex. However the EEG shows that the sleep changes the rates and the patterns of neuronal activity in nearly all the brain regions and not just in neocortex. The changes in the neocortical EEG are attributed to the calcium influx and the hyperpolarization of synchronous neocortical and thalamic neurons, producing high-voltage brain waves. However, the size of neocortex does not

correspond to the amount of sleep; alternately total brain weight and encephalization correlate poorly or negatively with total NREM and REM sleep amounts. Recent reports suggest that neocortical activity may be altered by prior waking activity and some such changes dissipate with continued waking, suggesting that localized renewal processes may take place either during waking or sleep in systems projecting to, or within the neocortex (Siegel 2005).

**1.6.1.2 Energy conservation:** In a period of 24 hrs, sleep tends to suppress our activities and conserve energy just as hibernation does across certain seasons. Large herbivores, owing to their vulnerability to the predators, have evolved reduced sleep amounts than small herbivores (Lima et al. 2005). Another hypothesis suggests that in small herbivores and other mammals, because of their high surface area to body mass ratio, may need to increase sleep amounts to conserve energy because for them it is difficult to maintain body temperature. Energy conservation may be particularly important in newborns, having high surface area to body mass ratio, for there is an adaptive advantage of conserving energy by means of sleep (Siegel 2005).

**1.6.1.3 Body mass, metabolism and sleep control**: There is an inverse relationship between body mass and mass-specific metabolic rate. Increase in the metabolic activity results in a number of biochemical changes, many of which have been linked to sleep control. Sleep time may also be important for defense against oxidative stress as high metabolic rate results in the formation of ROS known to have detrimental effects. Some of the manifested effects of ROS are ageing, wrinkling, arthritis and dementia in mouse by two years of age. It has also been seen that sleep deprivation in the rat is accompanied by increased oxidative stress and there is an evidence of membrane disruption in the hippocampus, subcortical brain regions and peripheral tissues (Everson et al. 2005).

**1.6.1.4. Role of sleep in neurogenesis:** Sleep may also have general role in allowing or facilitating neurogenesis as is evident from the fact that this proliferation is blocked in dentate gyrus after short–term (2-3days) total sleep deprivation (Guzman-Marin et al. 2003).

**1.6.1.5. Regulation of monoaminergic systems:** Noradrenaline, serotonin, hypocretin and histamine secretion is blocked during normal REM sleep and to some extent in NREM sleep. This suggests that sleep may re-sensitize these REM-off neurons, which remain active during wake, by increasing the quantity of their synthesizing enzymes, number of receptors and modify their transport mechanisms (Siegel 2005) . Since these neurotransmitters are involved in emotional regulation, REM sleep deprivation results in antidepressive effects.

**1.6.1.6. Role of sleep in brain development:** There is a positive correlation between REM sleep amount and the total sleep and a negative correlation with the body weight. However, it is more appropriate to say that REM sleep is positively correlated to the immaturity at birth. Mammals those are immature at birth (altricial) have more REM sleep than mammals those are mature at birth (precocial). This tendency is marked in the neonatal period. Remarkably, altricial mammals continue to have more REM sleep as adults as well. The change in REM sleep patterns through the stages of birth to maturity must be an important clue to its function. This time course, combined with the observation that brain is highly active during REM sleep, led to hypothesis that REM sleep is required for brain development. Monocular light deprivation during critical neonatal period leads to the loss of cells in the visual system and also to the shrinkage in lateral geniculate nucleus. REM sleep deprivation during the same period seems to accelerate this shrinkage, suggesting that REM sleep in which the visual activity is profound may be involved in the compensation of the asymmetrical, abnormal or absent inputs by preventing the processes that tend to wipe off the unused connections during development (Shaffery et al. 1999). It can thus be imagined

that REM sleep may serve this function for other sensory and motor systems.

**1.6.1.7. Memory Consolidation**: Recent evidence strengthens the hypothesis that sleep plays a role in learning and memory processing at several levels, including the REM-dependent developmental wiring of binocular cells in visual cortex (Maquet et al. 2003), procedural learning of a visual discrimination task (Stickgold et al. 2001), and the development of problem-solving skills. For memory consolidation or retaining memory for a long period of time, there is an information transfer between cortex and hippocampus during the sleep that realizes the fixation of memory traces.

**1.6.1.8. Role of REM sleep in maintaining normal body $CO_2$ level.** Our laboratory has proposed recently that one of the functions of REM sleep is to maintain normal body $CO_2$ level during sleep. As mentioned above that body $CO_2$ level increases during NREM sleep. Hypercapnia may have damaging effect to the cells therefore, its elimination would be mandatory. During REM sleep, the breathing rates increases, this helps eliminate excess of body $CO_2$. Interestingly, it has also been found that increased $CO_2$ level favors REM sleep genesis. Therefore, we have proposed that one of the functions of REM sleep is to maintain normal brain $CO_2$ level during sleep for a sustained and unperturbed slumber (Madan and Jha 2012).

But the intriguing question is how does it happen? LC neurons also act as chemosensors and play an important role in regulating proton ion concentration by modulating the cardio-respiratory system (Dean et al. 2001; Madan and Jha 2012). Interestingly, it has been observed that a slight increase in body $CO_2$ concentration increases sleep significantly with concomitant increase in number of REM sleep episodes (Fraigne et al. 2008; Madan and Jha 2012). On the other hand, REM sleep amount is decreased with a decrease in body $CO_2$ level (Ryan et al. 1983). These studies suggest that sleep can be affected by the changes in $CO_2/H^+$ ion concentration during hypo-or hypercapnia. The proton pump helps in maintaining

this normal $CO_2/H^+$ ion concentration and if its activity is perturbed, it may also alter REM sleep. The role of proton pump in sleep modulation is, however, not yet known. Here we studied the role of proton pump inhibitor "*Lansoprazole*" in the modulation of sleep-wakefulness in the rat.

# *Hypothesis/ Objectives*

## HYPOTHESIS

We reason that the proton pumps would also modulate S-W, but NHE would not have any role in its modulation.

## Objectives of the Study

1. The role of proton pump in the modulation of sleep –wake architecture in rat, by blocking the pumps by a PPI, *Lansoprazole*.
2. The role of sodium-hydrogen exchanger (NHE) in the modulation of sleep-wake architecture in rat, by blocking the exchanger with *Amiloride*.

# Chapter 2
## *Materials & Methods*

# 2. Materials and Methods

## 2.1. Materials:

### 2.1.1. Chemicals.

| | |
|---|---|
| *Lansoprazole* (*Cat No.L8533*) | Sigma, Aldrich |
| *Amiloride* (*Cat No. A7410*) | Sigma, Aldrich |
| DMSO (*Cat No. D5879*) | Sigma, Aldrich |

### 2.1.2. Formulations.

***Lansoprazole*:** *Lansoprazole* in a low and high dose of 1 mg/kg and 10 mg/kg respectively was dissolved in a 1:1 ratio of DMSO and 0.9% saline. The formulation was injected intraperitoneally to the animals in a volume of 400ul.

***Amiloride*:** *Amiloride* in a low and high dose of 10 mg/kg and 50 mg/kg respectively was dissolved in 1:1 ratio of DMSO and dist. water. The formulation was injected intraperitoneally to the animals in a volume of 400ul.

**Injection Schedule:** Injections were randomly made such that high and low doses fall on alternate days.

## 2.2. Methods.

In this study, we used male Wistar rats (300 – 350 gm). Rats were obtained from the University's animal house facility and brought to the school's in-house animal facility a week before commencement of experiments. Animals were maintained on 12:12 light–dark (L:D) cycle (lights on at 7:00 AM) at 23-24 $^{\circ}$C room temperature. They were given food and water *ad libitum.* All procedures used in this study were approved by the Institution's Animal Ethical Committee (IAEC) of Jawaharlal Nehru University, New Delhi, India.

### 2.2.1. Surgical procedures for polysomnographic recordings:

Animals were prepared for chronic S-W recordings. Animal surgery was performed in sterile conditions and using isoflurane (0.25%) inhalation anesthesia. The animal was anesthetized using facemask; head was shaved and was fixed in stereotaxic instrument. During surgery, the animal was maintained under gaseous anesthesia in the stereotaxic instrument. A midline incision was made and skin was cut aside to expose the skull for electrode implantations. Two pairs of small, stainless-steel screw electrodes were fixed bilaterally on the skull (2 mm lateral from the midline). These were fixed just above the frontal and parietal cortices to record fronto-frontal and parieto-parietal electroencephalogram (EEG). Three electrodes (flexible insulated wire except at the tip) were implanted in the dorsal neck muscles to record electromyogram (EMG) (third EMG was implanted as an extra safeguard). One screw electrode was fixed in the nasal bone as a reference electrode. Free ends of EEG, EMG and reference electrodes were connected to a 9-pin miniature connector, which was cemented onto the skull with dental acrylic and the neck skin was sutured. After surgery, the anaesthesia face mask was removed and the animal was taken out from the stereotaxic instrument.

Post-operatively, animals were treated with dexamethasone (1.5mg/kg, *i.p.*) and nebasulf powder (antibiotic) to control brain inflammation and infection. Dexamethasone and nebasulf were applied for 3-4 days. Soft food was given to animals during this period. The condition of animals was monitored regularly during the post-operative recovery period. They were allowed to recover from surgery for at least a week prior to the initiation of experiments.

### 2.2.2. Polysomnographic recording procedures:

After recovery from surgery, the animal was habituated in the recording cage (white plexiglass of 12" X 12" X 11" length, width and height). The recording cage was placed in a well ventilated, sound and light dampened (black color plexiglass) recording chamber (48" x 24" x 24") to minimize external disturbances during experiments. The recording chamber was illuminated with 20 Lux light. Food and water for the animal were placed in the food cup and water bottle attached to the recording cage. Animals were habituated daily for 6 hrs (11:30 AM

– 5:30 PM) for at least 2 days to the recording chamber and recording set-up. Also, during this period, animals were tethered to recording set-up through a commutator and EEG and EMG signals were examined in a computer through *spike-2 software* (Cambridge Electronic Design, UK). During baseline, EEG was recorded in two channels and EMG was recorded in a single channel. Electrophysiological signals were amplified using 15LT bipolar portable physiodata amplifier system (Astro-Med, USA). EEG signals were processed with a high-pass 0.1 Hz and low pass 40 Hz filters, while EMG was processed with high pass 10 Hz and a low-pass 90 Hz filter at 100 Hz sampling rate. Recordings were acquired in spike-2 and were saved for off-line analysis.

### 2.2.3. Experimental Design and drugs used.

We investigated the effect of '*Lansoprazole*' [a proton pump inhibitor (PPI)] and '*Amiloride*' [a sodium/hydrogen exchange blocker (NHE)] on S-W architecture. Animals were randomly divided into two groups: **[a]** *Lansoprazole* group and **[b]** *Amiloride* group. First, S-W was recorded for 6 hrs as baseline (11:30 AM – 5:30 PM) in both the groups. Thereafter, in *Lansoprazole* group, either low (1 mg/kg) (n = 7) or high dose (10 mg/kg) (n = 7) of *Lansoprazole* was injected intraperitoneally (*i.p.*) randomly on two different days with a gap of 1or 2 days. *Lansoprazole* was dissolved in 200µl dimethylsulfoxide (DMSO) and 200µl of sterile 0.9% normal saline. The same animal served as its own control, in which 400µl vehicle (DMSO and saline 200µl each) was injected *i.p.* However, in the *Amiloride* group, 10 mg/kg was used as low dose (n = 5), while 50 mg/kg as high dose (n = 3). Both the dosages of *Amiloride* were dissolved in 400µl of DMSO and water (with a ratio of 1:1) (water was used because adding saline in the solution precipitated *Amiloride*). Similar to earlier group, low and high dose of *Amiloride* was injected (*i.p.*) randomly into the animal with a gap of 1-2 days. In vehicle group, DMSO and water (400 µl) was injected (*i.p.*). All drugs and vehicle were injected few min prior to 11:30 AM and soon after S-W was recorded for 6 hrs with baseline time matched hours.

### 2.2.4. Data Analysis:

Offline, spike-2 polysomnographic records were converted into "european data format" and were scored using '*Somnologica Science software*' (Medcare Flaga, Iceland). Records were displayed in *Somnologica* and were manually scored using 4-sec epochs employing the standard criteria for rats. Low voltage and high frequency EEG waves associated with increased motor activity were analysed as wake; high voltage, low frequency EEG waves (0.5 - 4 Hz) and decreased motor activity were analysed as NREM sleep and low voltage, high frequency EEG waves with a prominent theta peak (5 - 9 Hz) and nuchal muscle atonia were analysed as REM sleep **(Fig. 2.1.)**.

The total time spent in wake, NREM and REM sleep was calculated. These values were expressed as hourly and total mean percent of the total recording time. Percent difference in the vigilant states amount between vehicle, low dose and high dose of *Lansoprazole* were compared statistically using one-way repeated measures ANOVA followed by tukey posthoc test. However, changes in the vigilant state in *Amiloride* treated groups were compared statistically using one way ANOVA followed by tukey-posthoc test. This was done because we could perform the high dose (50 mg/kg) experiments of *Amiloride* in only three animals, while the low dose and vehicle experiments were performed in a total of five animals. We terminated the experiment of high dose of *Amiloride* because it caused acute ionic stress and majority of the animals (n = 4) died within 24 – 36 hours after *Amiloride* injection. Hence, we were not sure, if the changes in the vigilant states in these animals were specific to the drug or due to acute stress. Since, we had only two measures in the *Amiloride* group; vehicle and low dose, hence in this group, we used one-way ANOVA for statistical comparisons. Animals were sacrificed at the end of the experiments with an over dose of cocktail anaesthesia (80 mg/kg ketamine and 40 mg/kg Xylazine).

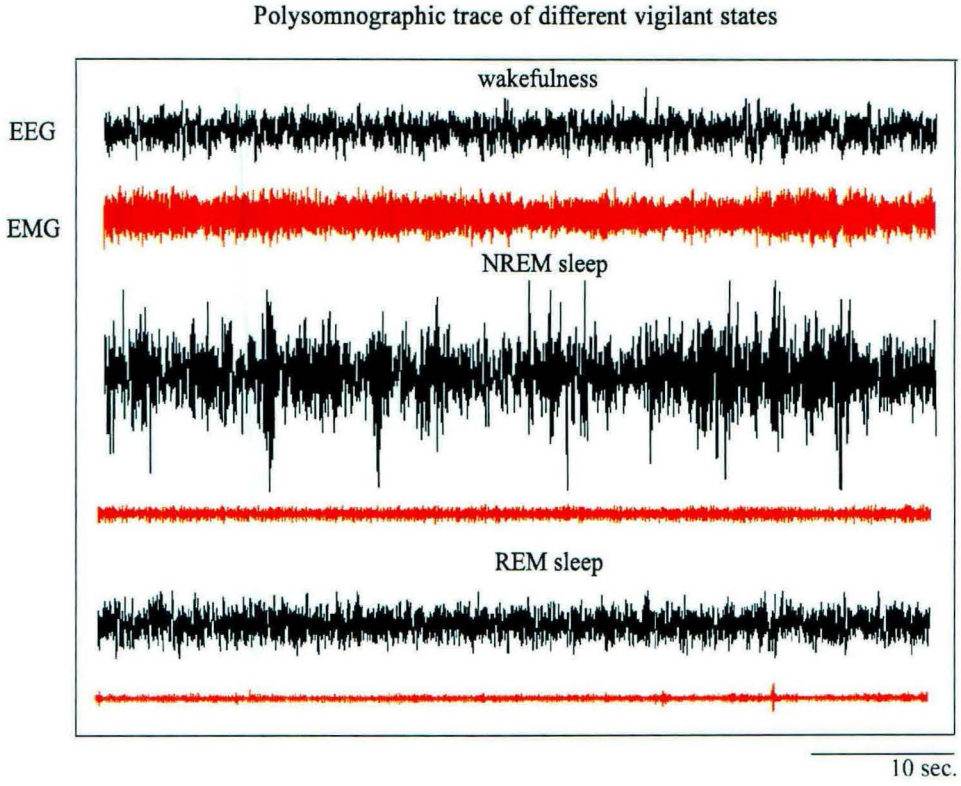


***Figure 2.1.*** *Polygraphic traces during wakefulness, NREM sleep and REM sleep show difference in the EEG frequency, voltage and the EMG tone.*

# Chapter 3
## *Results*

# 3. Results

## 3.1. Effect of proton pump inhibitor '*Lansoprazole*' on vigilant states:

High dose of *Lansoprazole* (10 mg/kg) significantly increased REM sleep amount, however amount of wakefulness and NREM sleep (% TRT) did not change **(Fig. 3.1.1, Fig. 3.1.2.)**. Percent REM sleep amount in animals of vehicle control and low dose (*Lansoprazole*: 1 mg/kg) groups were 9.26 ± 1.03 and 9.09 ± 0.54, respectively, which increased significantly by 31.53 % and 33.99 % respectively, in animals of high dose group ($p < 0.01$; $F_{(3,27)} = 6.53$) **(Fig. 3.1.3.)**. Animals in baseline group, however, exhibited 17.06 % more REM sleep compared to vehicle control animals, but it was not significant statistically. Hourly analysis of % REM sleep showed a significant increase in REM sleep at 4$^{th}$ hour in *Lansoprazole* high dose group ($p < 0.05$; $F_{(2, 20)} = 5.046$) compared to vehicle and low dose groups, although at other hourly time points, a trend towards increase in REM sleep persisted **(Fig. 3.1.4.)**. *Lansoprazole* significantly induced REM sleep frequency only ($p < 0.05$; $F_{(2,20)} = 4.82$) **(Fig. 3.1.5.)**, while REM sleep episode duration length and REM sleep latency remained unaltered **(Fig. 3.1.6, Fig. 3.1.7.)**. Injection of high dose of *Lansoprazole* did not induce any behavioral or physiological changes in animals. They exhibited normal cage behavior after injection and were alert, active and inquisitive. Animals did not show any signs of grogginess or stress on any day during and/or after experiments. They exhibited normal eating and drinking behavior and no alterations in their breathing rate (observed manually).

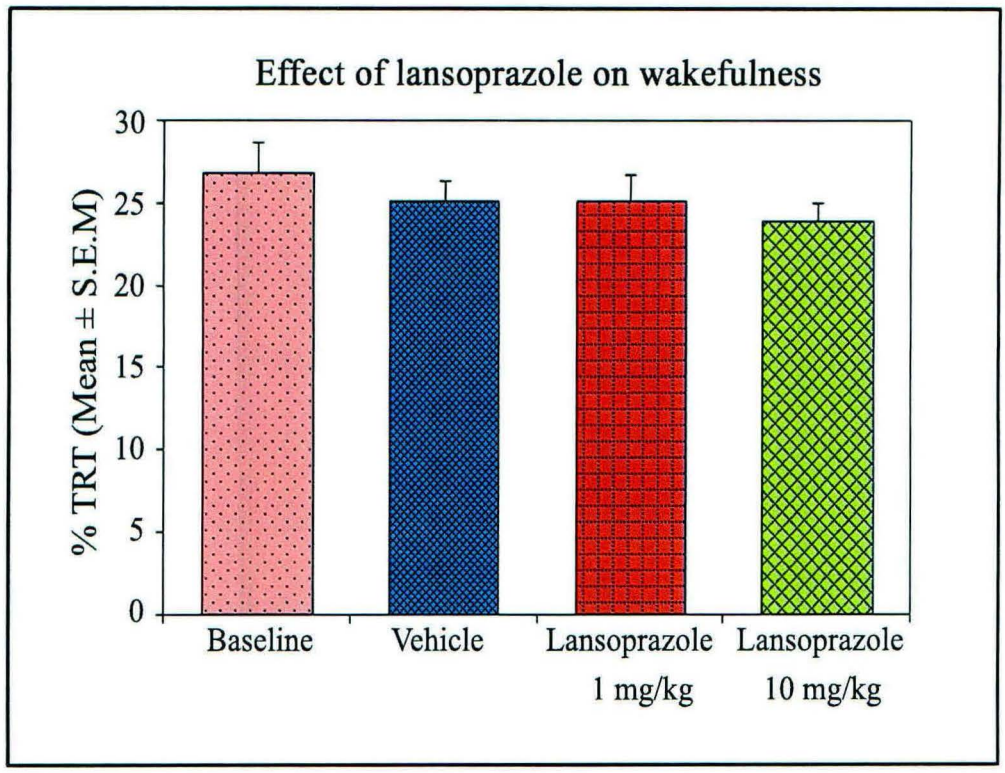


***Figure 3.1.1. Percent wakefulness amount out of total recording time (TRT).*** *Lansoprazole did not induce significant changes in wakefulness.The wake amount in all four conditions, baseline, vehicle, lansoprazole (1 mg/kg) and lansoprazole (10 mg/kg) was comparable.*

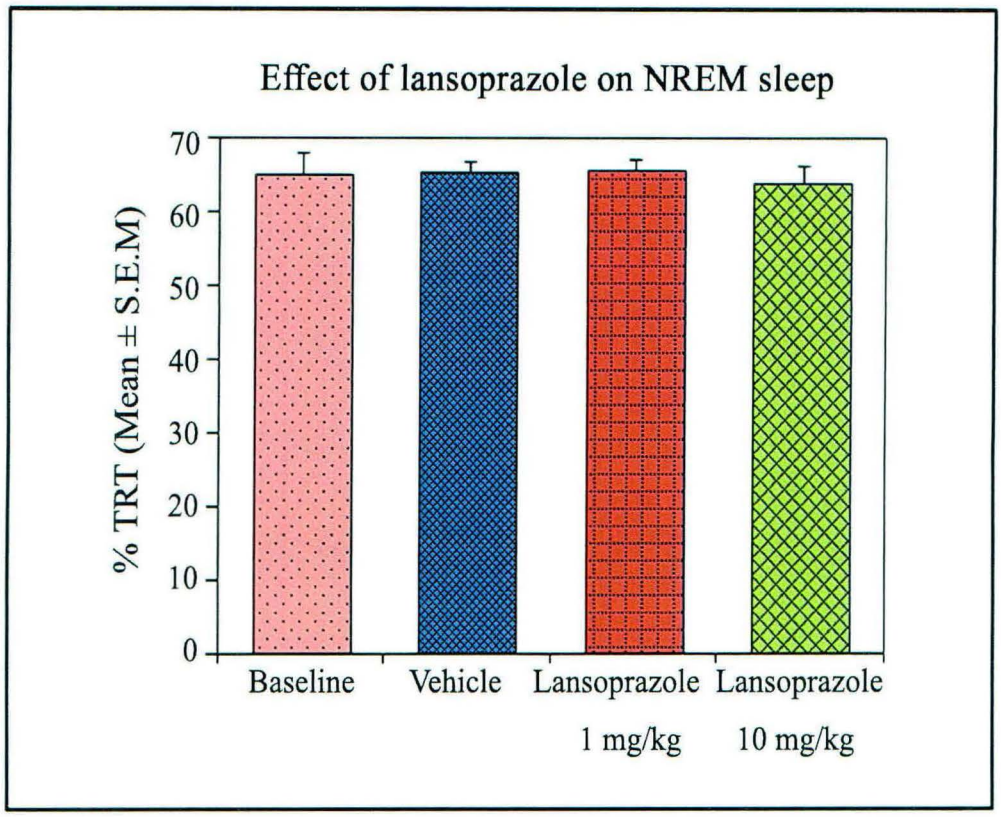


***Figure 3.1.2. Percent NREM sleep amount out of total recording time (TRT).*** *Lansoprazole did not alter the amount of NREM sleep. The amount of NREM sleep in different conditions, baseline, vehicle, lansoprazole (1 mg/kg), lansoprazole (10 mg/kg) was comparable.*

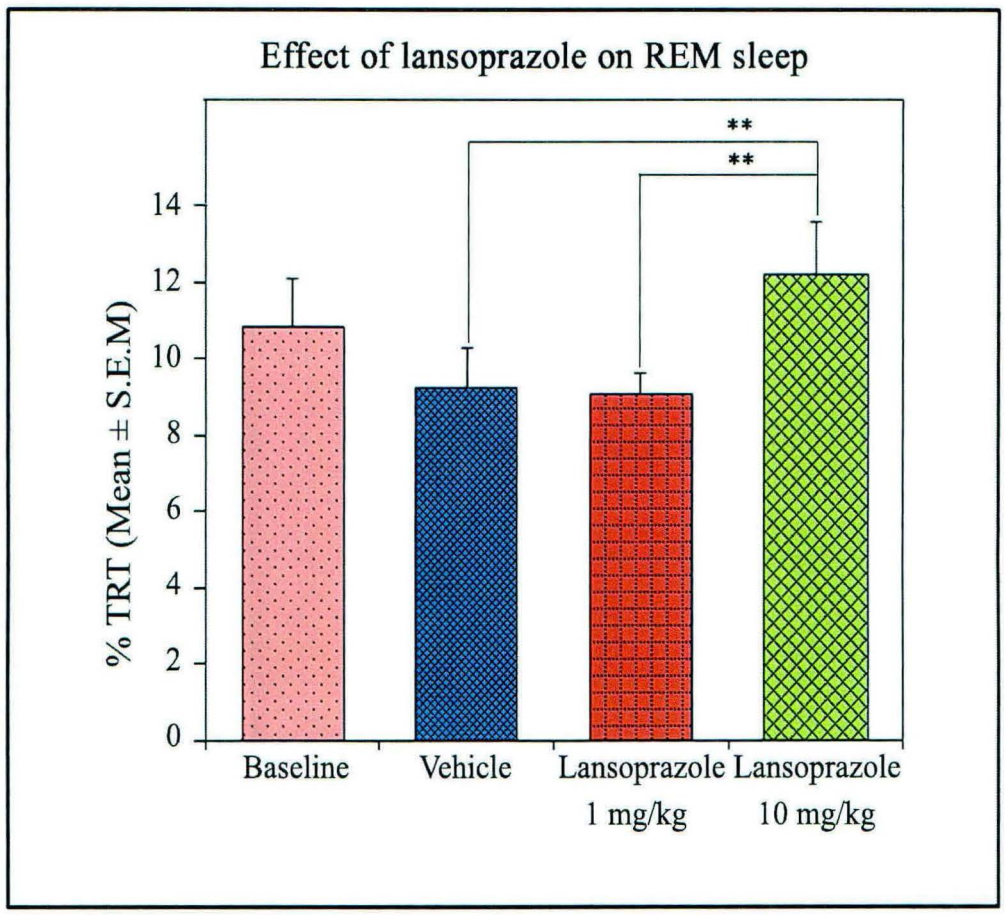


***Figure 3.1.3. Percent REM sleep amount out of total recording time (TRT).*** *Lansoprazole (10 mg/kg) significantly increased REM sleep amount. Percent REM sleep amount in lansoprazole (10 mg/kg) treated animals was significantly more compared to the vehicle control ($F_{(3,27)}$= 6.53, $p < 0.01$) as well as lansoprazole (1 mg/kg) treated animals ($p < 0.01$).*

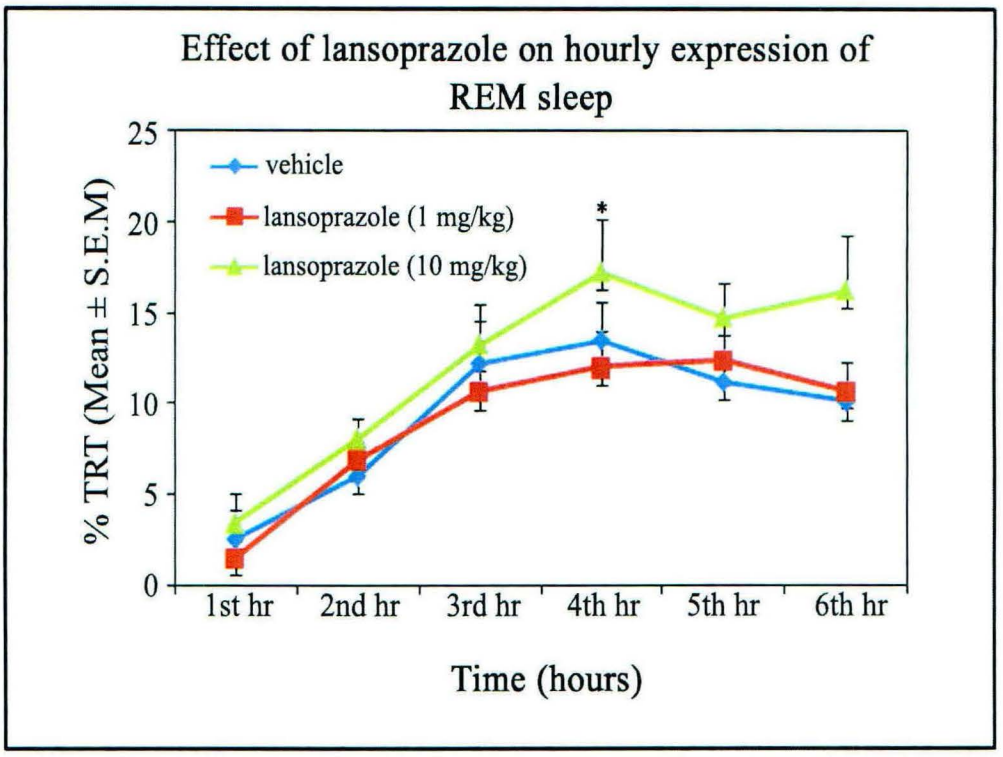


***Figure 3.1.4. Hourly percent REM sleep amount out of total recording time (TRT).*** *Lansoprazole (10 mg/kg) persistantly increased REM sleep every hour of the recording. However, increased percent REM sleep amount at 4th hour was statistically significant* ($F_{(2,20)} = 5.046$, $p < 0.05$).

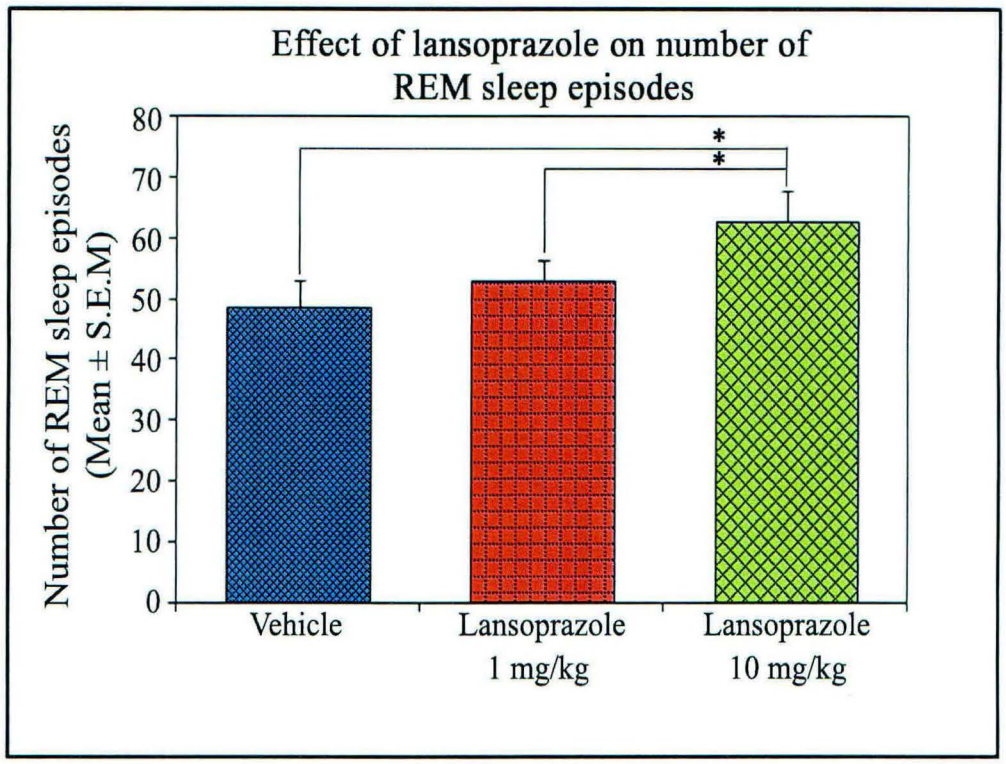


***Figure 3.1.5. REM sleep episode number.*** *Lansoprazole (10 mg/kg) significantly increased REM sleep episode number compared to vehicle and lansoprazole (1 mg/kg) treated rats ($F_{(2,20)}$= 4.82, $p < 0.05$).*

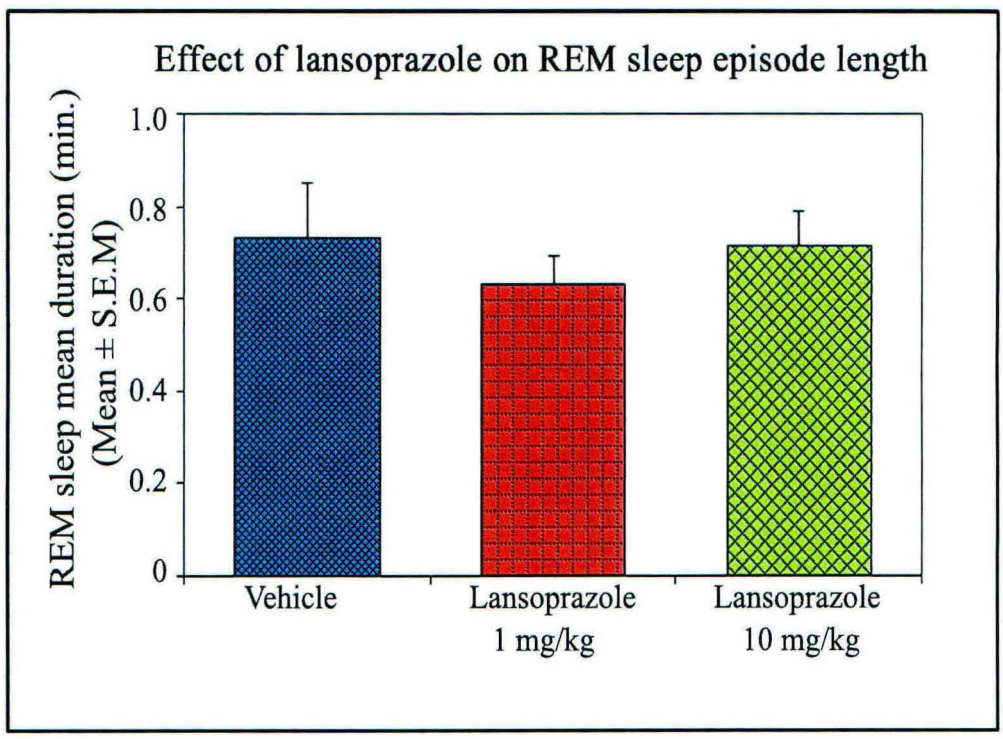


***Figure 3.1.6. REM sleep episode duration.*** *Lansoprazole did not alter the average REM sleep episode duration. It was almost comparable in vehicle, lansoprazole (1 mg/kg) and lansoprazole (10 mg/kg) treated rats.*

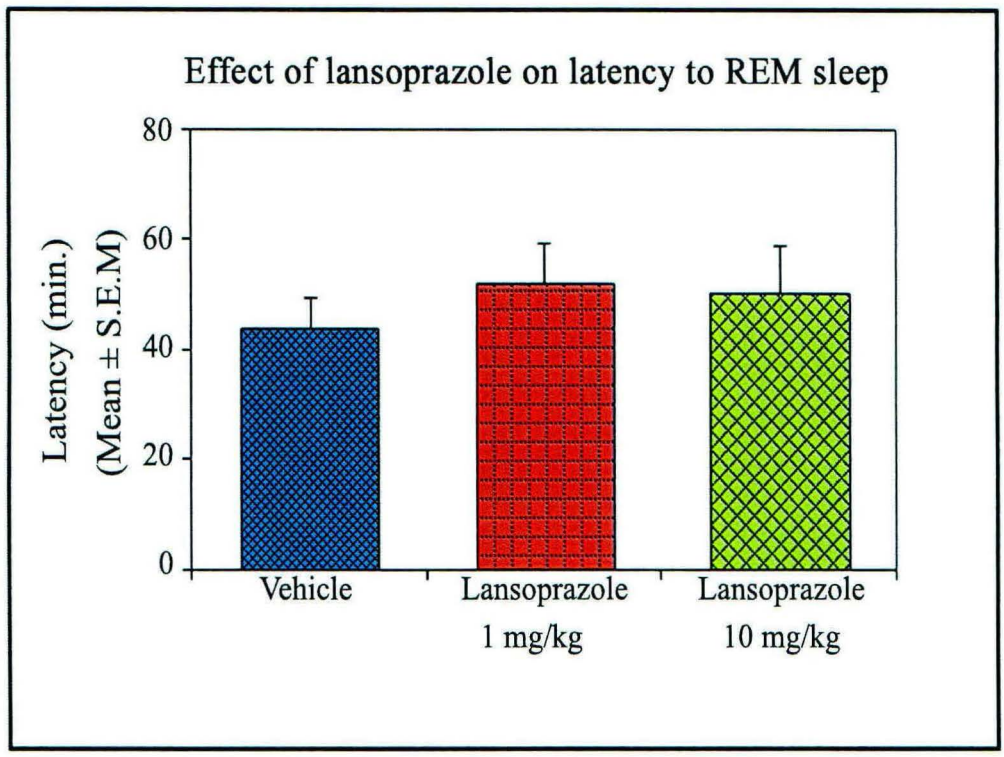


***Figure 3.1.7. Latency to REM sleep.*** *Lansoprazole ( 1 mg/kg and 10 mg/kg ) did not alter REM sleep latency. It was comparable in vehicle, lansoprazole (1 mg/kg) and lansoprazole (10 mg/kg) treated rats.*

## 3.2. Effect of inhibitor of sodium/proton exchanger '*Amiloride*' on vigilant states:

Two different doses of *Amiloride*: 50 mg/kg (n = 4) and 10 mg/kg (n = 5) were injected in rats. All animals treated with *Amiloride* at 50 mg/kg died. One animal died after 24 hrs and three died 36 hrs later. These animals died possibly due to ionic stress and dehydration. Although 100 mg/kg dose has been used previously for an anticonvulsant effect in rodents with no reports of animal casualty (Luszczki et al. 2009); however, this dose may cause intestinal perforation and peritonitis (Archer and Roth 1999). Therefore, we decided a dose of 50 mg/kg but even at this dose animal suffered with dehydration and ultimately died. Signs of dehydration appeared 10-15 hrs after *Amiloride* injection (50 mg/kg) and even supplementing saline or dextrose saline did not improve their condition. Further, we noticed that animals exhibited increased breathing rate and were sluggish, but, 6 hrs S-W recordings exhibited that these animals (n = 3; data from one animal was not taken for analysis because the animal was more stressed and died 24 hrs later) were significantly more awake ($p < 0.05$; $F_{(1,5)} = 12.46$) **(Fig. 3.2.1.)**. They spent significantly less time in NREM sleep ($p < 0.05$; $F_{(1,5)} = 9.46$) compared to vehicle control group ( n = 3) **(Fig. 3.2.2.)**, REM sleep amount, however, did not change **(Fig. 3.2.3.)**. Hourly analysis of % S-W (n = 3) demonstrated that % wakefulness increased ($p < 0.05$; $F_{(2,11)} = 12.33$), while % NREM sleep decreased ($p < 0.05$; $F_{(2,11)} = 6.64$) significantly during initial two hrs after *Amiloride* (50 mg/kg) injection. Nevertheless, in further hourly time points, these were comparable to the vehicle control group **(Fig. 3.2.4, Fig. 3.2.5.)**.

Low dose of *Amiloride* (10 mg/kg) neither caused animal death (n = 5) nor induced any physiological changes. We did not notice stress or dehydration signs in these animals and they were active, alert and exhibited normal S-W patterns. Amounts of wakefulness, NREM sleep and REM sleep in these animals [treated with *Amiloride* (low dose)] did not change and were comparable to the vehicle control group **(Fig. 3.2.6. – Fig. 3.2.8.)**.

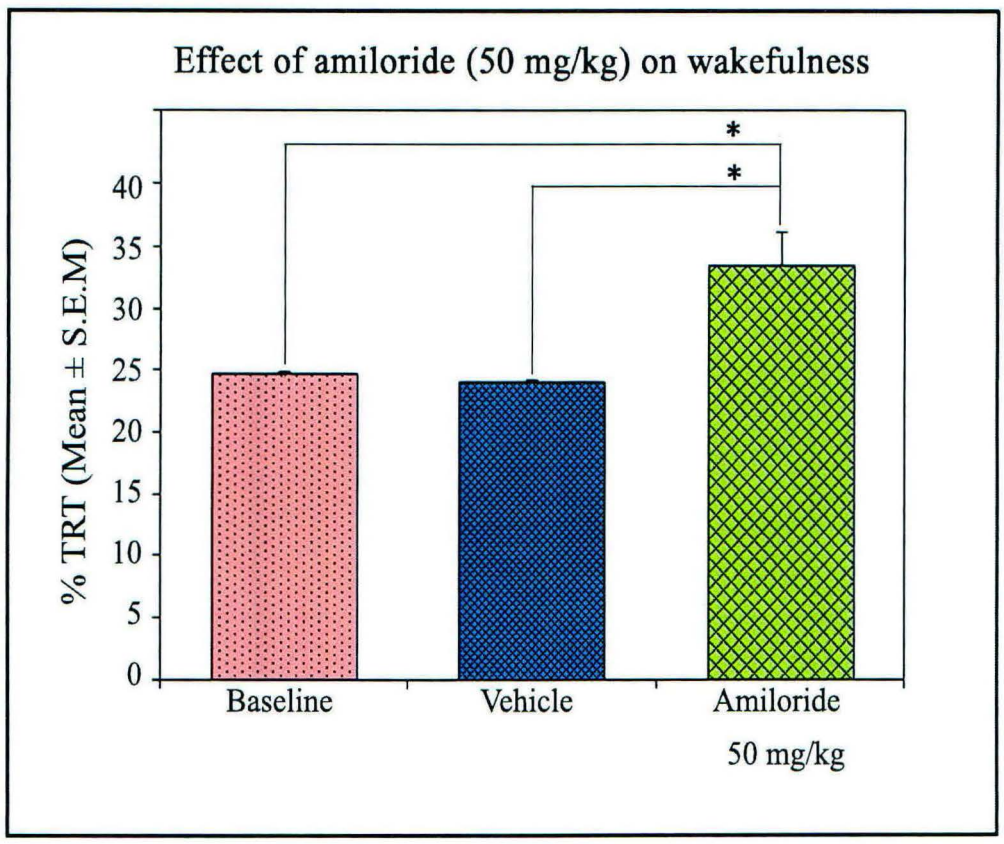


***Figure 3.2.1. Percent wakefulness out of total recording time (TRT).*** *Amiloride (50 mg/kg) significantly increased wakefulness compared to baseline ($F_{(1.5}$= 10.903, $p < 0.05$) and vehicle groups ($F_{(1.5)}$= 12.458, $p < 0.05$).*

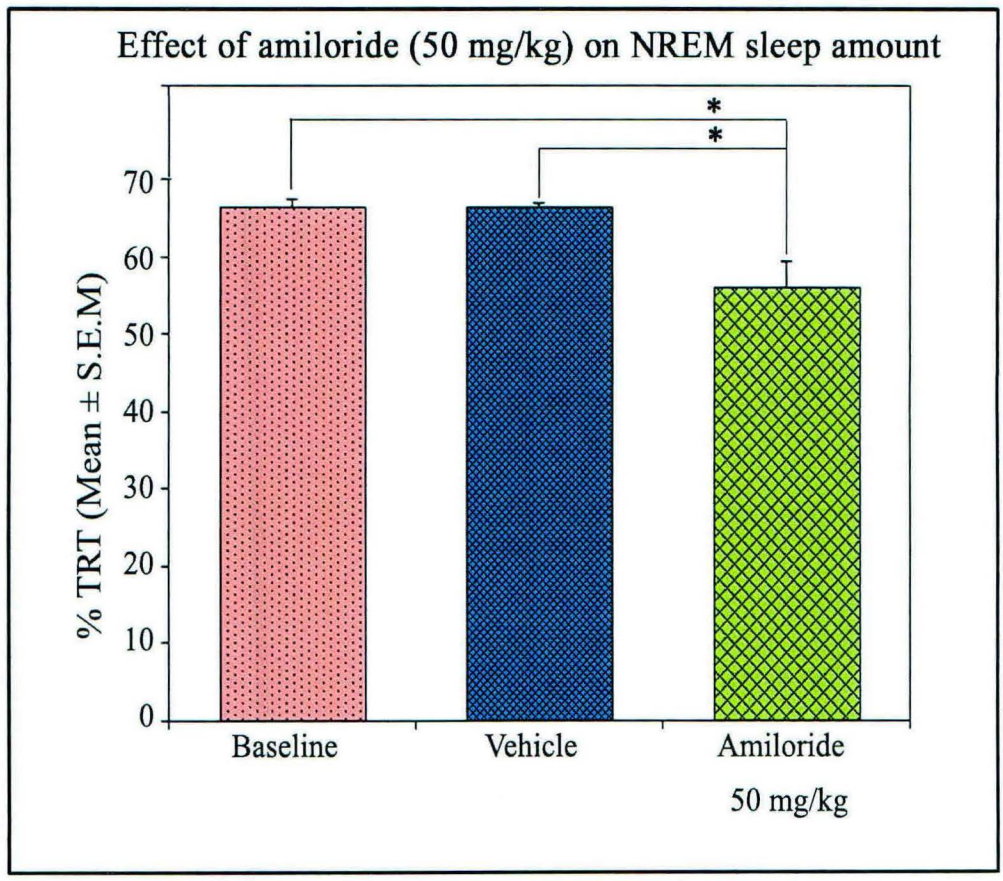


***Figure 3.2.2. Percent NREM sleep out of total recording time (TRT).*** *Amiloride (50 mg/kg) significantly decreased NREM sleep amount. Percent NREM sleep in high dose amiloride (50 mg/kg) treated group (n = 3) was significantly less than baseline ($F_{(1.5)}$= 9.536, $p < 0.05$) and vehicle ($F_{(1.5)}$= 9.461, $p < 0.05$) groups.*

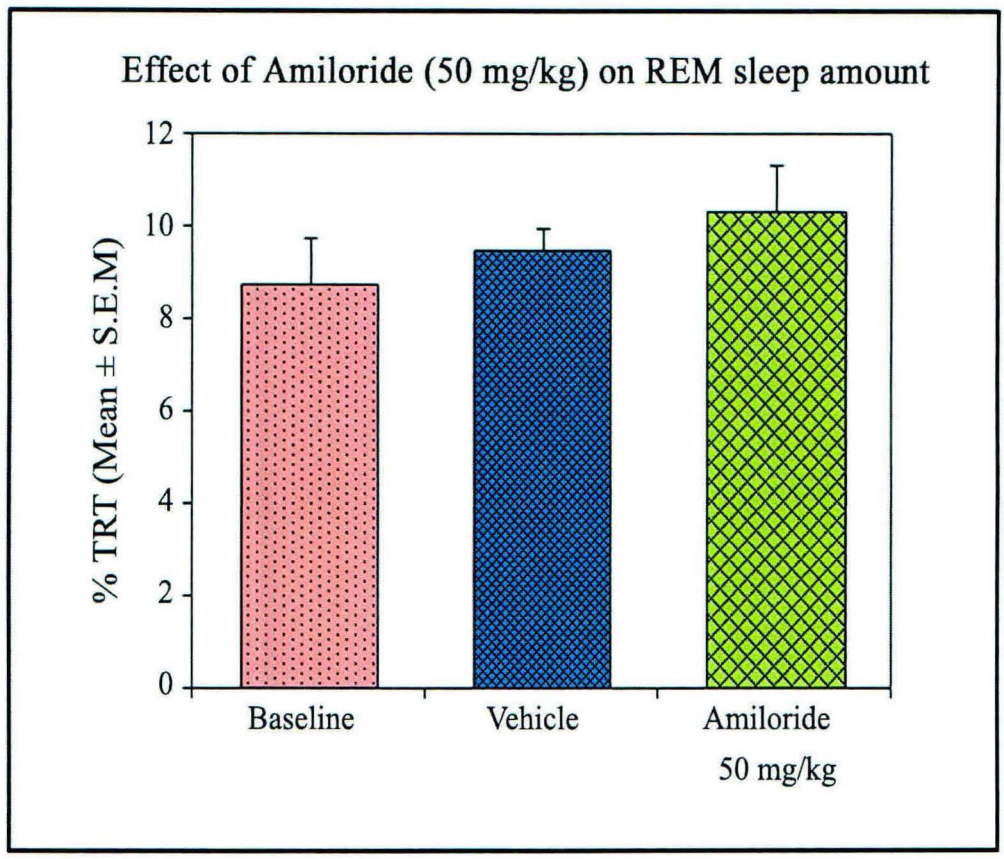


***Figure 3.2.3. Percent REM sleep amount out ot total recording time (TRT).*** *Amiloride (50 mg/kg) did not alter REM sleep. Percent REM sleep amount in baseline, vehicle and amiloride (50 mg/kg) treated animals (n = 3) was comparable.*

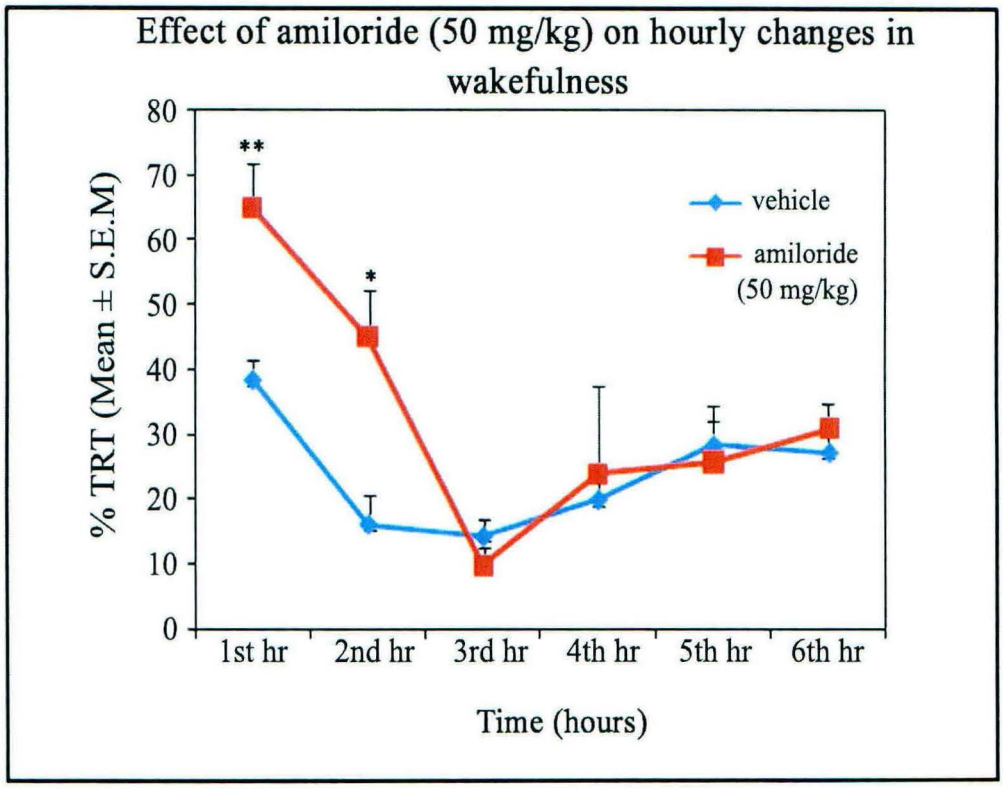


***Figure 3.2.4. Hourly percent wakefulness out of total recording time (TRT).*** *Lansoprazole (50 mg/kg) significantly induced wakefulness during the first ($p < 0.01$) and second hour ($p < 0.05$) ($F_{(2,11)} = 12.33$) compared to vehicle treated animals. It was however comparable during other hourly time points.*

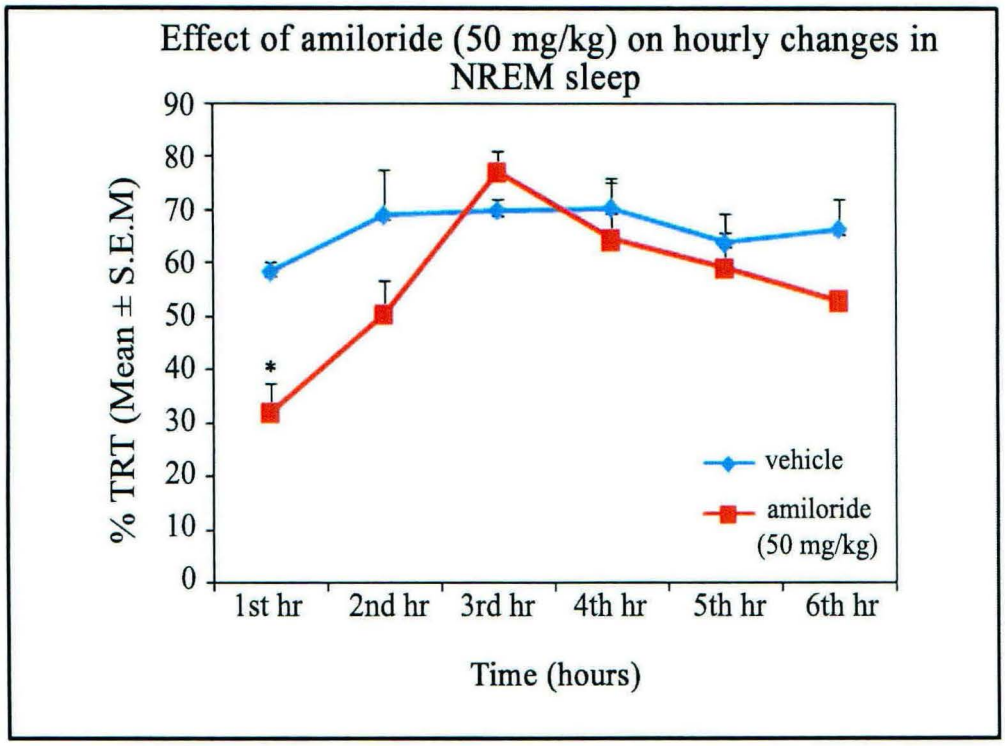


***Figure 3.2.5. Hourly percent NREM sleep amount out of total recording time (TRT).*** *NREM sleep amount in animals treated with amiloride high dose (50 mg/kg) significantly decreased during the initial first hour compared to vehicle treated animals.It was also less during the second hour (statistically not significant) but was comparable during other hourly time periods* ($F_{(2,11)} = 6.644$, $p < 0.05$).

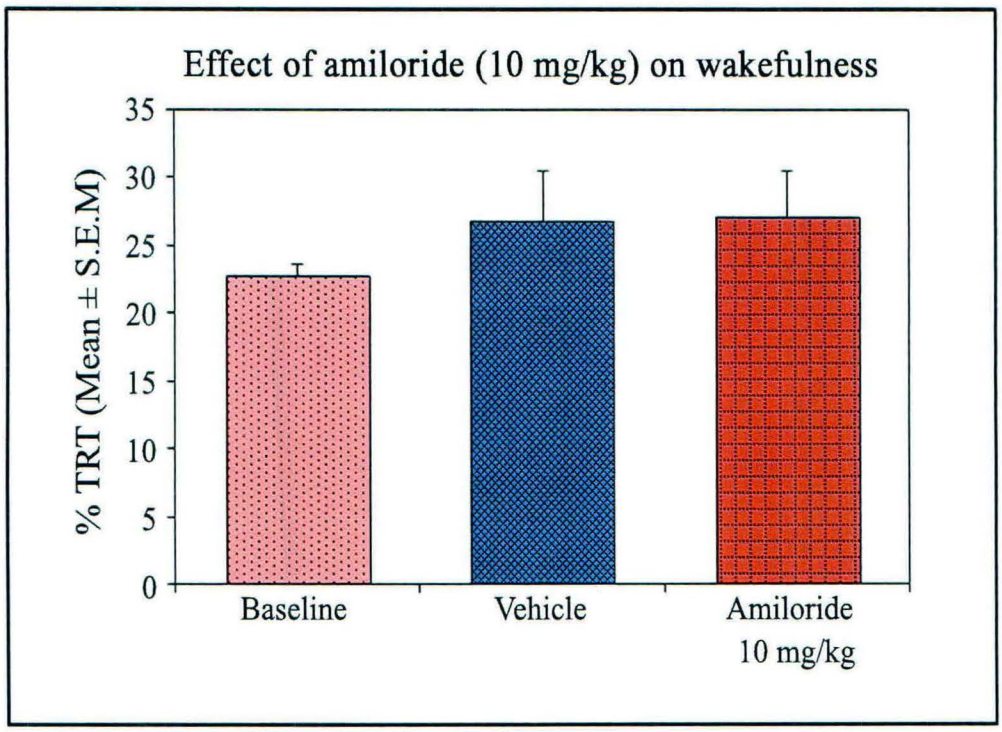


***Figure 3.2.6. Percent wakefulness out of total recording time (TRT).*** *Amiloride (10 mg/kg) did not induce any changes in the wakefulness amount. Percent wake in baseline, vehicle and amiloride (10 mg/kg) treated animlas (n = 5) was comparable.*

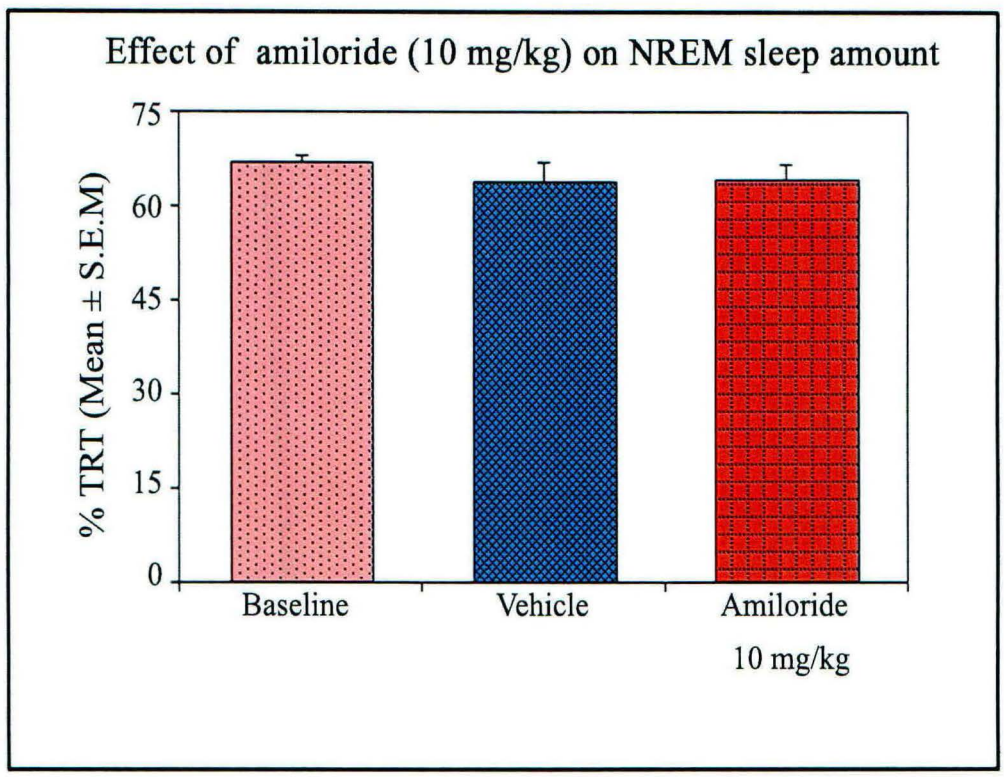


***Figure 3.2.7. Percent NREM sleep amount out of total recording time (TRT).*** *Amiloride (10 mg/kg) did not induce any changes in the NREM sleep amount. Percent NREM sleep in baseline, vehicle and amiloride (10 mg/kg) treated animals (n = 5) was comparable.*

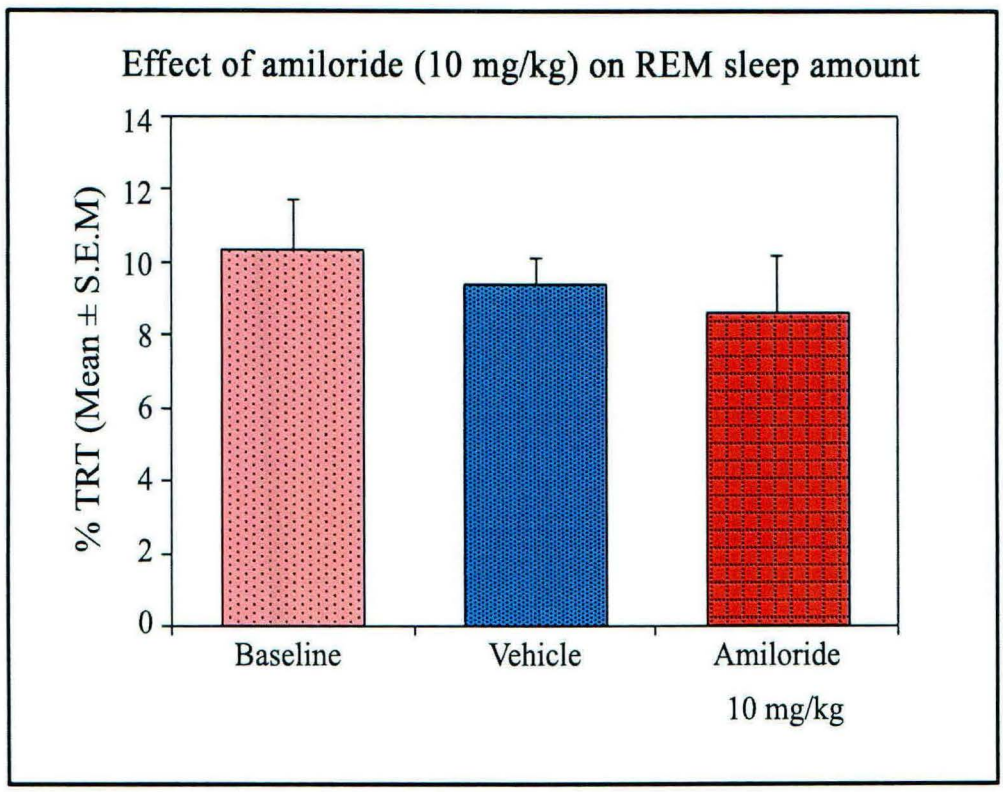


***Figure 3.2.8. Percent REM sleep amount out of total recording time (TRT).*** *Amiloride (10 mg/kg) did not alter the REM sleep amount. Percent REM sleep in baseline, vehicle and amiloride (10 mg/kg) treated animals (n = 5) was comparable.*

# *Discussion and Conclusion*

## Discussion and Conclusion.

We examined the effect of proton pump inhibitor (PPI) '*Lansoprazole*' and sodium-hydrogen exchanger (NHE) blocker '*Amiloride*' on S-W. *Lansoprazole* (10 mg/kg) did not alter NREM sleep but REM sleep was significantly increased. A trend of consistent increase in REM sleep continued throughout the recording period compared to vehicle and low dose of *Lansoprazole*, however, REM sleep was significantly high during the fourth hour of recording. Additionally, *Lansoprazole* significantly increased REM sleep episode number, but REM sleep average duration length and its latency did not change. On the other hand, NHE blocker '*Amiloride*' (10 mg/kg) was ineffective in inducing any change in S-W architecture. These results suggest that PPI, which is widely used for the treatment of gastric and duodenal ulcers (Hawkey et al. 1993) as well as reflux esophagitis (Castell et al. 1996), also has a potency to induce REM sleep.

The NHE blocker '*Amiloride*' (10 mg/kg) did not alter sleep architecture. Although, high dose (50 mg/kg) did significantly increase wakefulness and inhibited NREM sleep in three animals, but all died 24 – 36 hrs after injection. It has been reported earlier that 100 mg/kg *Amiloride* enhances anticonvulsant action of several antiepileptic drugs (Luszczki et al. 2009); but may also cause intestinal perforation and peritonitis (Archer and Roth 1999). Therefore, we preferred 50 mg/kg as high dose, but unfortunately all animals died even at this dose. We, however, could not determine the actual cause of death of these animals (n = 4), but to observation, it could be attributed to acute dehydration. *Amiloride* also acts as the potassium-sparing diuretic (Benos 1982) and all animals treated with 50 mg/kg *Amiloride* were urinating copiously. The apparent signs of dehydration were noticed 24 hrs after injection in these animals. The animals could not even recover with a periodic supplement of saline/dextrose-saline and ultimately died. The data obtained from three animals exhibited a significant increase in wakefulness. We could not ascertain if the change in sleep architecture in *Amiloride* treated (50 mg/kg) animals was indeed due to the direct action of drug or because of an associated acute stress induced in response to drug. The low dose of *Amiloride* (10 mg/kg) neither induced any physiological changes nor altered

sleep-wake architecture, suggesting that it may not have any direct modulating effect on sleep-wakefulness.

The proton pump and NHE are associated in the modulation of several physiological functions including chemoreception. The brainstem chemosensory neurons discern change in brain pH and activate cardio-respiratory response to regulate pH (Mulkey et al. 2010). The proton pump is located within neurons, whereas NHE is found on the membrane in several brainstem nuclei including the locus coeruleus (LC) (Dean 2010; Dean et al. 2001; Kersh et al. 2009; Mulkey et al. 2010; Nattie 1995). LC neurons are highly sensitive to changes in $CO_2$/ $H^+$ ion concentration (Dean et al. 2001; Nattie 1995). It has been reported that raising the concentration of $CO_2$ in the inspired gas mixture, in anesthetized rats, results in rapid increase in the firing rate of LC neurons (Elam et al. 1981). On the other hand, it has also been observed that if the increased $CO_2$ concentration persists, it hyperpolarizes LC neurons (Dean et al. 2001). $CO_2$-induced alteration in the firing of LC neurons in part depends on the presence of $CO_2$ level outside the neurons, nevertheless, it is also modulated in part, by the chemoreceptors located within LC neurons (Pineda and Aghajanian 1997). Some studies suggest that proton pump plays an important role in neuronal firing as well as in neurotransmitter release through a biphasic pH change; a brief acidification followed by a prolonged alkalization of cytoplasm. If this biphasic pH change is perturbed, it inhibits neuronal firing (Tabares and Betz 2010; Zhang et al. 2010). It is known that the PPI '*Lansoprazole*' crosses blood brain barrier and affects intracellular machinery (Cronican et al. 2010; Rojo et al. 2010) and therefore, may alter neuronal activity. Besides the chemosensory neurons, LC also contains a unique group of neurons called 'REM-OFF" neurons. These neurons remain silent during REM sleep (Aston-Jones and Bloom 1981; Jha and Mallick 2011; Mallick et al. 2004) and if LC is electrically or pharmacologically activated, it does not allow REM sleep to occur (Jha and Mallick 2011; Kaur et al. 2004; Kaur et al. 1997; Singh and Mallick 1996). It has further been proposed that cessation of LC's REM-OFF neurons disinhibits brainstem REM-ON neurons, which further help initiate REM sleep over NREM sleep (Jha and Mallick 2011). During NREM sleep, breathing rate slows down and as a result $CO_2$ level increases (Robin et al. 1958). Recently, we have proposed that increased $CO_2$ level may activate LC's neurons including

'REM-OFF' [which fire mostly during wakefulness], which in turn would activate the brainstem reticular ascending pathways to induce wakefulness over NREM sleep (Madan and Jha 2012). However, moderately increased $CO_2$, if persist it may hyperpolarize LC neurons including 'REM-OFF' which would facilitate REM sleep, during which the breathing rate again goes up (Madan and Jha 2012). Now an intriguing question is how does PPI modulate REM sleep? There could be both or either of the two mechanisms: 1] *Lansoprazole* might have impaired the neuronal biphasic pH alteration by blocking vacuolar proton pump and persistent intracellular acidic condition may cause hyperpolarization of LC neurons (Dean et al. 2001) including 'REM-OFF' cells, a condition that facilitates REM sleep (Jha and Mallick 2011). 2] *Lansoprazole* has a potency to induce anti-inflammatory action by inhibiting the production of pro-inflammatory cytokines (Hashioka et al. 2009), which modulates LC neurons (Borsody and Weiss 2002) and sleep-wakefulness (Kapsimalis et al. 2005; Opp 2005). We have injected the drug intraperitoneally, hence the role of some other pathways or factors can't be ruled out. In addition to the use of *Lansoprazole* for the treatment of gastric/duodenal ulcer, our results suggest that the drug has a potency to induce REM sleep.

In summary, the brainstem LC's neurons play an important role in REM sleep regulation as well as in chemoreception. It is however not clearly known if some of the chemosensory neurons are also part of REM sleep executive machinery and this needs further experimental verification. Nevertheless, our data suggest that chemosensory neurons either directly or indirectly through cytokine may modulate REM-Off neurons. This supports our view that REM sleep might act as a sentinel to help maintain normal $CO_2$ level for unperturbed sleep.

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