REVIEW OF LITERATURE
2. SLEEP: AN OVERVIEW

Sleep-wakefulness is a behavioural phenomena: Sleep has been defined as a reversible behavioural state of perceptual disengagement from and unresponsiveness to the environment (Carskadon and Dement, 1989). It can also be called the higher form of BRAC (Basic Rest Activity Cycle). Sleep-wakefulness has been objectively classified and quantified primarily based on electrophysiological signals recorded from the brain, the electroencephalogram (EEG), the neck muscles, the electromyogram (EMG) and the eye muscles, the electroocculogram (EOG).

Sleep is characterized by certain electrophysiological patterns which are observed in all mammals. It is usually accompanied by postural recumbency, quiescence and closed eyes. Mammalian sleep is a biphasic phenomenon consisting of two distinct phases known as NREM (Non Rapid Eye Movement) and REM (Rapid Eye Movement), which alternate with each other during the sleep cycle. Normally, sleep is initiated with the NREM state.

**NREM sleep state:**

It is also known as SWS (Slow Wave Sleep) which occupies about 65% of the total sleep time. NREM sleep is characterized by EEG synchronization, high voltage, low frequency waves and spindles and is divided into four stages in man with a progressive increase in arousal thresholds as sleep progresses from stage I to stage IV (Rechtschaffen and Kales, 1968). The characteristics of the four stages are as follows:

Stage I is associated with sleep onset and a low arousal. The sleep in stage I persists for a short duration (1-7 min) and is easily discontinued by a low intensity stimulus. In addition to its role in the initial wake-sleep transition, stage I occurs as a transitional stage throughout the night.

Stage II NREM sleep is signalled by sleep spindles or K-complexes in the
EEG. This stage follows stage I and continues for 10 to 25 min. The same stimulus that produced an arousal in Stage I produces an evoked K-complex in Stage II, but no awakening is observed i.e. a more intense stimulus is required for arousal.

Stage III NREM sleep is characterized by gradual appearance of high voltage (≥ 75μV) and slow-wave (≤ 2 Hz) activity in the EEG, which accounts for more than 20 per cent but less than 50 per cent of the EEG activity. It lasts only a few minutes.

Stage IV NREM sleep is identified when the high voltage slow wave activity is more than 50 percent of the EEG activity and generally lasts for 20-40 min in the first cycle. An incrementally larger stimulus strength is generally required to produce an arousal from stages III and IV sleep than from stages I and II sleep.

**REM sleep state**

REM sleep is also known as *paradoxical sleep* (PS) as it reveals an EEG pattern similar to that of waking during behavioural sleep. Although mention of a similar behavioural state can be found in ancient Hindu literature the Upanishads, however, REM sleep state, with its present characteristic features, was experimentally identified a little over half a century by Aserinsky and Klietman in 1953. This state is objectively identified by the characteristic electrophysiological signs viz. desynchronization of the EEG, rapid eye movement, atonia in the antigravity muscles and pontogeniculo-occipital (PGO)waves. This state has also been termed as *rapid eye movement (REM) sleep* or *active sleep* or *desynchronized sleep*. REM sleep occupies about 15-20% of the total sleep time. This is the deepest sleep state when the threshold for arousal is maximum and it mostly follows deep sleep state. The mental activity of human REM sleep is associated with dreaming, based on vivid dream recall from approximately 80 per cent of arousals from this state of sleep. In short, REM
state represents a highly activated brain in a paralysed body.

2.2 PHYLOGENY AND ONTOGENY OF REM SLEEP

REM sleep has been identified in mammals and birds. It has been identified across species, however, all the identifying characteristic signs may not be uniformly expressed in different species in the evolutionary ladder. Until recently, it was considered to be relatively of later origin because it could not be identified in monotreme (Allison, 1972). However, with the recent findings that REM sleep like state is also present in the platypus, the egg laying mammal, it is proposed that this state might have evolved more than 300 million years ago (Siegel, 1997).

REM sleep is proposed to be ontogenetically primitive as it is found maximally in early life. In mammals, REM sleep has been consistently found to occur in its greatest amounts in the foetus or immature newborn animal, where the major portion of sleep is occupied by active sleep (Roffwarg et al., 1966; Jouvet-Monnier et al., 1968). In human infants and in many young animals, "active sleep", the ontogenic precursor of the REM sleep state, is not accompanied by tonic cortical EEG desynchrony or muscle atonia and has been identified by the phasic muscle twitches and eye movements that recur during quiescent state. As NREM sleep and wakefulness emerge with maturation, the time spent in REM sleep is reduced. REM sleep has also been found to decrease with advancing age.

2.3 CHARACTERISTICS OF REM SLEEP

REM sleep is characterized by both tonic and phasic events. Tonic events are those which occur throughout the REM sleep episode. These tonic events are EEG desynchronization, hippocampal theta rhythm and atonia of postural muscles. Phasic events are those which occur sporadically in REM sleep, but these events largely
coincide with each other. These are rapid eye movements, field potentials in the pontine tegmentum, lateral geniculate and occipital cortex- together known as PGO waves, myoclonic twitches and cardiorespiratory fluctuations. Although the cortical EEG is desynchronized in the REM sleep state, the hippocampal EEG is highly synchronized at 4-10 Hz (theta frequency).

REM sleep episodes repeat a number of times during sleep, however, its frequency of occurrence and duration per bout increase with the progress of sleep. In humans, REM sleep appears in a cyclic manner following a period of SWS of about 45-85 minutes. As the sleep progresses, the length of SWS bouts decreases, while the length of REM bouts increases. The alteration of non-REM -REM cycle constitute the basic organizational unit of mammalian sleep (Zeplin, 1989). Klietman (1939) established that average length of sleep cycle beginning with NREM and ending with REM is approximately 90 minutes in man. This reliable alteration of NREM and REM sleep represented an ultradian rhythm and corresponds to the BRAC.

Although the actual and precise role of REM sleep has not been established, its importance cannot be overlooked. Since interruption of sleep in rats by non-traumatic means for periods of 5-30 days has been shown to produce severe pathological changes and is often fatal, it may be said that sleep serves a vital physiological function (Rechtschaffen et al., 1989). REM sleep deprivation were followed by longer periods of recovery sleep, where the animal was found to spend longer periods in the REM sleep state. This is known as "rebound REM". This observation also emphasizes the importance of REM sleep in our daily life.

Some important functions attributed to REM sleep include: cell maturation, energy or drive dissipation, development and refinement of oculo-motor control, maintenance of catecholamine levels, consolidation of memory and learning and maintenance of neuronal excitability. These are explained in detail later in the chapter.
2.4 BRAIN AREAS INVOLVED IN GENERATION OF REM SLEEP

Transection studies, lesion studies and single neuronal recording studies have contributed tremendously to identify the structural substrates involved in the generation and maintenance of REM sleep. Atonia, rapid eye movements (REMs) and PGO spike bursts and REM sleep like activation of reticular formation could be recorded caudal to the cut, when the transections are placed at the midbrain level. When these transections were made at the midpontine level, an alteration of the synchronized and the desynchronized states could be recorded rostral to the cut. When the cut is made more caudal i.e. at the junction of spinal cord and medulla, all the REM sleep signs except muscle atonia could be observed rostral to the cut. Thus, structures caudal to the midbrain and rostral to the spinal cord were found to be necessary for the generation of REM sleep. Further, transection studies at various other levels showed that when pons was connected to midbrain and forebrain, most of the REM sleep characteristics could be recorded rostral to the cut and when pons was connected to the medulla and spinal cord, most of the REM sleep signs could be recorded caudal to the cut. Moreover, isolated acute pons preparation containing pontine tegmentum also could elicit REM sleep like signs of periodic episodes of REMs and PGO spikes. These studies confirmed the role of pontine structures in generation and maintenance of REM sleep (Siegel et al., 1984, 1986).

Lesion studies were done to study if REM sleep or its signs could be recorded after destruction of certain cell groups. A study by Jouvet and Delorme (1965) found that electrolytic lesions of locus coeruleus (LC) prevented REM sleep. However, Carli and Zanchetti (1965) reported no loss of REM sleep following lesion of the LC. Large electrolytic lesion of the pontine tegmentum resulted in a total loss of the state of REM sleep, thus confirming its role in the generation of REM sleep state (Jones, 1979).
Recording studies from single neurons revealed two populations of neurons viz. REM-ON and REM-OFF cells. REM-ON neurons are those which discharge only during REM sleep phase. These were detected in the laterodorsal pontine tegmentum and pedunculopontine tegmentum (LDT-PPT nuclei). Some of the neurons in the peri-LCα also show a dramatic state dependent increase in discharge rate, just prior to and throughout REM sleep (Sakai, 1988). REM-OFF neurons are those which discharge during waking, decrease their firing rate during SWS or NREM and are completely silent during REM sleep. These were detected in dorsal raphe (serotonergic) and LC (noradrenergic) nuclei and posterior hypothalamic (histaminergic) nuclei.

2.5 ROLE OF NEUROTRANSMITTERS IN REM SLEEP

Different neurotransmitters play important roles in the generation and maintainence of REM sleep.

Role of Acetylcholine

There are various evidences to prove the importance of the role of acetylcholine in REM sleep.

Jouvet (1972) showed that REM sleep could be suppressed by atropine and enhanced by acetylcholinesterase inhibitor- eserine in the pontine cat. This led him to propose that the generation of REM sleep depended, at least partly, upon cholinergic mechanism. Earlier in 1963, Hernandez-Peon et al. showed that a REM-sleep like state could be induced by applying acetylcholine (ACh) crystals directly into the limbic- forebrain limbic midbrain circuit. Administration of ACh synthesis inhibitor, hemicholinium, which blocks reuptake of choline led to a reduction in REM sleep (Hazra, 1970), while carbachol, an acetylcholine agonist or inhibitor of
acetylcholinesterase-physostigmine caused an increase in REM sleep (Jacobs and Jones, 1978).

While the noradrenergic LC neurons stopped firing during REM sleep, cells in gigantocellular tegmental field (FTG) increased firing during REM sleep state (McCarley and Hobson, 1971). Cholinergic neurons were identified in the dorsolateral mesencephalic tegmentum using choline acetyltransferase (ChAT) immunohistochemistry (Jones, 1990). Later, Siegel and McGinty (1977) showed that FTG neurons also showed increased firing rates during active waking in association with movement. Certain neurons in the LDT-PPT area were found to fire only during the REM sleep state, i.e. they were REM-ON in nature. In the cat, there is a considerable overlap between the cholinergic (LDT-PPT) cell group and noradrenergic (LC) cell group (Jones, 1991). When the lateral and dorsolateral pontine tegmentum were lesioned, REM sleep was eliminated (Friedman and Jones, 1984; Sastre et al., 1981).

The concentration of ACh was found to increase in a cortical cup in the cat during REM sleep, when compared to NREM sleep (Jasper and Tessier, 1971) and in the effluent of the push-pull cannulae in the striatum (Gadea-Circia et al., 1973). Electrical stimulation of LDT and PPT increased ACh release in the medial pontine reticular formation (mPRF) (Lydic and Baghdoyan, 1993). Recently, extracellular in vivo microdialysis was used to monitor ACh release across behavioural state in the rat thalamus, a major projection site of mesopontine cholinergic neurons. ACh concentration in the thalamus was found to be high during both wake and REM sleep and significantly lower during slow-wave sleep (Williams et al., 1994). In vitro intracellular recordings have shown that most neurons in the mPRF are depolarized by carbachol (Greene et al., 1989).

The involvement of M2 receptor in the carbachol induced immediate state
independent PGO and subsequent long term enhancement of REM sleep has been reported (Datta et al., 1993). In other studies, ACh has been found to participate in REM sleep generation through the muscarinic cholinergic receptors (mAChRs) (Roth et al., 1996) of a non-M1 subtype. The signal transduction pathway activated by M2 and M4 mAChRs involves a pertussis toxin-sensitive G protein, adenylate cyclase (AC), adenosine 3',5'-cyclic monophosphate (cAMP), and protein kinase A (PKA). In order to test the role of cAMP and protein kinase A, mPRF was microinjected with compounds known to either facilitate the effects of cAMP (dibutyryl cAMP and 8-bromo-cAMP), stimulate PKA (Sp-cAMP[S]) or inhibit PKA (Rp-cAMP[S]). The results showed that compounds that fostered the intracellular effects of cAMP significantly decreased cholinergic REM sleep, while having no effect on spontaneously occurring REM sleep (Capace and Lydic, 1997).

ABV [(+/-)-4-aminobenzovesamicol], a vesamicol-like compound, is known to non-competitively inhibit vesicular packaging of ACh in the presynaptic terminals. This compound was microinjected alone or before administration of the acetylcholinesterase inhibitor neostigmine to evaluate the effects on natural REM sleep and on the neostigmine-induced REM sleep-like state. ABV decreased (24.8%) REM sleep and significantly reduced (33.6%) the neostigmine-induced REM sleep, thus indicating that REM sleep deprivation possibly leads to an impairment in the ACh vesicular packaging (Capace et al., 1997).

Nitric Oxide (NO) has been shown to modulate the release of ACh in a number of brain regions (Leonard and Lydic, 1995) and studies indicate that NO may participate in the modulation of sleep/wake states (Kapas et al., 1994). Inhibition of local nitric oxide synthase (NOS) by microdialysis delivery of N(G)-nitro-L-arginine (NLA), a nitric oxide synthase inhibitor, significantly reduced ACh release in the cholinergic cell body region of the pedunculopontine tegmental nucleus and in the
cholinoceptive mPRF (Kapas et al., 1994). Microinjection of NLA into the mPRF also significantly reduced the amount of REM sleep (duration and not the frequency) and the REM sleep-like state caused by mPRF injection of the acetylcholinesterase inhibitor neostigmine (Kapas et al., 1994).

The induction of immediate early gene, c-fos, has been associated with increased neuronal activity. Shiromani et al. (1992; 1996) performed a series of experiments to ascertain if the neurons which showed increased c-fos expression after REM sleep were cholinergic in nature. When REM sleep was induced using carbachol, the animals showed a significantly higher number of Fos-Like Immunoreactivity (FIL) cells in the pontine regions which have been implicated in REM sleep generation. More importantly, 11.2% of cholinergic neurons in the LDT and PPT nuclei were determined to be also FIL positive. In the vehicle treated animals, very few FIL cells were found and none of these were found to be cholinergic. These findings indicate that during carbachol induced REM sleep, a transcriptional cascade involving c-fos occurs in a subpopulation of pontine cholinergic neurons (Shiromani et al., 1992; 1996).

**Role of Norepinephrine**

Jouvet (1972) proposed that the noradrenergic group of neurons present within the nucleus LC may be playing a major role in the generation of REM sleep. Jouvet and Delorme (1965) observed that electrolytic lesion in the region of the LC could disrupt the state of REM sleep, interrupting particularly the neck muscle atonia. They also reported that very large lesions destroying entire noradrenergic neurons resulted in elimination of the REM sleep state altogether. However, in a subsequent study, it was observed that more selective lesion restricted to area within the region of LC did not eliminate REM sleep (Jones et al., 1977). Nevertheless, mild electrical stimulation
of LC neurons led to a decrease in REM sleep, which was followed by a rebound increase after the cessation of the stimulation (Singh and Mallick, 1996).

Pharmacological studies showed that depletion of catecholamines by inhibition of their synthesis by α-methyl para tyrosine (AMPT) caused an increase in both SWS and REM sleep (Jacobs and Jones, 1978). An enhancement in REM sleep was observed in rats (Hartmann et al., 1971) and in the cats (Stern and Morgane, 1974), when the synthesis of NE is blocked using AMPT, an analog of tyrosine that inhibits tyrosine hydroxylase (TH) activity. Conversely, treatments which enhanced release or increased synaptic concentrations of catecholamines, such as tricyclic antidepressants (Hartmann, 1969), monamine oxidase (MAO) inhibitors (Jouvet, 1972) and electroconvulsive shock (Cohen and Dement, 1965), were found to suppress REM sleep and enhance arousal. NE has been found to hyperpolarize identified mesopontine cholinergic neurons in vitro (Williams and Reiner, 1993). The role of different adrenoceptors have also been proposed in the generation and maintenance of REM sleep. Receptor agonist and antagonist studies showed that the α-1 antagonist, prazosin, increased (Hilakivi and Leppavuori, 1984), while its agonist, methoxamine, decreased (Pellejero et al., 1984) REM sleep. On the other hand, the β-adrenoceptor antagonist, propranolol, decreased (Lanfumney et al., 1985) while its agonist, isoproterenol, increased (Monti, 1983) REM sleep.

TH is the rate-limiting step in the synthetic pathway of NE. Its activity increased after 96 hrs of REM sleep deprivation in lower brain stem and cerebral cortex, but remained unaltered in the upper brain stem (Sinha et al., 1973). Later on, Porkka-Heiskanen et al. (1995) showed an increase in the TH gene expression in the LC in REM sleep deprived rats. In another related study, a decrease in MAO-A activity, the enzyme specifically involved in degradation of NE, after REM sleep
deprivation has been reported (Thakkar and Mallick, 1993). Recently, the effect of REM sleep deprivation on the levels of TH and norepinephrine transporter (NET) mRNA in the LC of rats were studied (Basheer et al., 1998). The animals were deprived of REM sleep for 1, 3 or 5 days and changes in the mRNA levels were determined using in situ hybridization. The levels of both TH and NET mRNA increased in LC of animals deprived of REM sleep for 3 days or longer whereas no change was observed in control animals.

Single neuronal recording studies showed that the noradrenergic LC neurons showed a decrease in firing rate during SWS and ceased firing altogether during REM sleep (Aston-Jones and Bloom, 1981). This indicates that the concentration of NE in the projection areas of LC neurons would increase during waking and decrease during REM sleep.

**Role of Serotonin**

The serotonergic dorsal raphe nucleus is considered an important modulator of state-dependent neural activity and thus the state of REM sleep. These neurons are postulated to modulate REM sleep via projections to the cholinergic neurons of the PPT. The role of serotonin [5-hydroxytryptamine (5-HT)] in sleep wakefulness mechanism was first proposed by Jouvet in 1972, where a long-lasting insomnia was observed on lesioning the dorsal raphe containing serotonergic neurons. The serotonergic neurons show a REM-OFF pattern of firing i.e. they show higher firing rate during waking which decrease during sleep and cease during the REM sleep state (McGinty and Harper, 1976). Serotonin is believed to play an important inhibitory role in the regulation of REM sleep. Inhibition of serotonin synthesis by para chlorophenyl-alanine (PCPA) treatment in cats resulted in insomnia (Koe and
Intraperitoneal injection of 5-hydroxytryptophan was found to restore SWS and suppress REM sleep in cats (Jouvet, 1972; Koella et al., 1968). In rat brainstem in vitro, 5-HT was found to hyperpolarize and inhibit the bursting properties of LDT neurons assumed to be involved in generating REM sleep and PGO waves (Leubke et al., 1992). In vivo 5-HT microinjection into the LDT was found to suppress REM sleep (Horner et al., 1997). The 5-HT may exert this effect on neurons of the LDT, which are proposed to be important for the generation of REM sleep, particularly the phasic REM sleep events such as PGO waves and respiratory variability (Horner et al., 1997). In another study, sleep/waking stages were studied following administration of selective 5-HT1A agonist, 8-hydroxy-2-(di-n-propyamino) tetralin (8-OH-DPAT), either systemically or as continuous perfusion for 6 hours into the dorsal raphe nucleus (DRN) using microdialysis. Given systemically, 8-OH-DPAT increased waking to 149% and reduced SWS to 86%, transition to 76% and REM sleep to 73%. The effect on deep SWS (SWS-2) was biphasic, with an increase after 2 hrs. Continuous perfusion of 8-OH-DPAT in DRN produced a fourfold increase in REM sleep compared to perfusion of artificial cerebrospinal fluid (Bjorvatn et al., 1997). The other sleep/waking stages were not significantly affected by 8-OH-DPAT perfusion in DRN. This is consistent with the hypothesis that reduced 5-HT neurotransmission following 5-HT1A autoreceptor stimulation would disinhibit cholinergic REM sleep-promoting mesopontine neurons and thereby lead to an increase in REM sleep. REM sleep suppression by the 5HT agonist, eltoprazine was followed by a rebound which was abolished by muscarinic antagonist, scopolamine, thus suggesting a reciprocal interaction between cholinergic and serotoninergic neurons (Stickgold et al., 1993).
Frank and Heller (1997) studied the effect of serotonin reuptake inhibitors administered in neonates and adult rats. Neonatal treatments with the serotonin uptake inhibitors clomipramine (CMI) and zimelidine (ZMI) were found to disrupt sleep patterns in adult rats, but had no effect on the "active sleep" as found in the neonates. ZMI and CMI increased the frequency and decreased the duration of REM sleep bouts, increased the number of non REM-REM sleep transitions, and increased sigma power in REM and non REM sleep EEGs in adulthood (Frank and Heller, 1997). Although the firing patterns of the DRN neurons suggest a decrease in the serotonin levels in the brain during REM sleep, the actual measurement of serotonin metabolite 5-hydroxyindole acetic acid (5-HIAA) in the posterior hypothalamus was done recently using in vivo voltametry with carbon fiber microelectrodes and polygraphic recordings in free-moving rats. The level of 5-HIAA was found to increase with wakefulness and decreased with sleep and it was significantly lower during REM sleep than SWS (Imeri et al., 1994).

**Role of Dopamine**

Dopamine (DA) plays a modulatory role in the regulation of REM sleep. Centrally acting agonist apomorphine interacts with the pre or post synaptic receptors and cause a biphasic effect on sleep-wakefulness. While a high dose of apomorphine has been found to reduce REM sleep, a low dose was found to increase REM sleep (Monti, 1983). The increase at low doses may be due to activation of presynaptic receptors of dopamine. Changes in dopaminergic transmission have been associated with REM sleep deprivation and elevated DA turnover has been reported during REM sleep rebound (Wojcik and Radulovacki, 1981). Chlorpromazine, a D1 and D2 receptor antagonist increased the amount of REM sleep over a short dose range, in
addition to enhancing the total sleep time (Kafi and Gaillard, 1978; Monti, 1983). In rats, D1 receptor antagonist SCH 23390 increased (Trampus and Ongini, 1990) and its receptor agonist SKF38393 reduced REM sleep (Trampus et al., 1993). Thus suggests the involvement of D1 receptors in the regulation of REM sleep. The behavioural response of rats to apomorphine has been found to be intensified by REM sleep deprivation. It was hypothesized that the observed hyper-responsiveness of REM sleep deprived rats to apomorphine could be due to a state of supersensitivity of post synaptic dopaminergic receptors in the brain (Tufik, 1981).

Role of GABA

The role of gamma-amino-butyric acid (GABA) in the generation and maintenance of REM sleep has been implicated by various studies. GABA is degraded by the enzyme GABA transaminase. Intraperitoneal injection of L-cycloserine, an inhibitor of GABA transaminase, was found to significantly increase REM sleep (Scherschlicht, 1985). An increase in REM sleep in cats was observed when GABA was perfused locally into the ventroposterolateral thalamic nuclei (VPL) by in vivo microdialysis technique (Juhasz et al., 1990). Intraperitoneal injection of muscimol, a GABA agonist led to an enhancement in both NREM and REM sleep (Lancel et al., 1997). Nitz and Siegel (1997) measured GABA release as a function of sleep-wake stages and reported an increase in GABA in the LC during REM sleep. Similarly, GABA level has been found to change in the posterior hypothalamus (Nitz and Siegel, 1996) and in the septum (Mallick et al., 1997) during sleep-wake cycle. Microinjection of GABA-antagonist picrotoxin into LC (Kaur et al., 1997a) and preoptic area (Ali et al., 1999) induced a significant reduction in REM sleep. GABAergic neurons and terminals have been localized in the LC cells (Iijima and
Ohtomo, 1988; Ford et al., 1995; Shipley et al., 1996; Jones, 1990). Based on all these observations, it has been proposed that the inhibition of LC REM-OFF neurons is mediated by GABA (Mallick et al., 1999b). The results of various combination studies of microinjections into the LC led the authors to propose that the duration of REM sleep episodes was regulated by GABAergic mechanism, while the frequency of occurrence of REM sleep episodes was regulated by cholinergic mechanism (Kaur et al., 1997b).

**Role of Other Peptides**

A number of peptides like delta sleep inducing factor (DSIP), enkephalin, substance P, cholecystokinin (CCK-8) somatostatin, neuropeptides, vasoactive intestinal peptide etc have been reported to modulate the activity of LC neurons (Aston-Jones et al., 1990), which is involved in the regulation of REM sleep. Intracerebroventricular injection of vasoactive intestinal peptide (VIP) also increases REM sleep. Since VIP has been found to co-exist with acetylcholine in the central nervous system, it was administered at the oral pontine reticular nucleus to study if that area was the target site for VIP action. Injections of both 1ng and 10ng produced significant increases in REM sleep at the expense of wakefulness (Bourgin et al., 1997). Intraventricular administration of cerebrospinal fluid (CSF) obtained from sleep deprived (SD) animals and VIP have been shown to increase REM sleep. It has thus been suggested that VIP may accumulate in the CSF as a consequence of waking and might thus be partly responsible for the subsequent rebound of REM sleep which follows prolonged wakefulness (Jimenez-Anguiano et al., 1993). Similarly, when cerebrospinal fluid from normal cat was intraventricularly injected, a restoration of REM sleep was observed in the PCPA pretreated insomniac cats, indicating that
"paradoxical sleep inducing factor" may be stored in the central nervous system during sleep deprivation (Sallanon et al., 1982). Several of the putative sleep factors like muramyl peptide, endotoxin and tumor necrosis factor have been found to enhance interleukin (IL1) production. Thus, the somnogenic action of these substances could be mediated in part, via their ability to alter IL1 production (Krueger et al., 1993). IL1 in turn alters prostaglandin (PG) production (Dinarello, 1984).

Different types of PG have been found to have different effects on REM sleep. PGD2 is somnogenic while PGE2 promotes wakefulness (Hayaishi, 1988).

The effect of CLIP [corticotropin-like intermediate lobe peptide; ACTH(18-39)] and its shorter fragments ACTH(25-39), ACTH(18-24) and ACTH(20-24) on sleep were investigated by intracerebroventricular injections. While SWS was not influenced, REM sleep was increased by CLIP as well as by the N-terminal CLIP fragment ACTH(18-24) and by the pentapeptide ACTH(20-24), whereas the C-terminal fragment ACTH(25-39) was ineffective. Thus, CLIP and its N-terminal fragments have been found to have selective REM sleep-enhancing effects (Wetzel et al., 1994).

The role of prolactin (PRL) has been implicated in the regulation of sleep. Intracerebroventricular injection of PRL has been found to enhance REM sleep in rats and stimulation of endogenous PRL secretion by VIP also has been found to promote REM sleep (Roky et al., 1995). In control and REM sleep deprived rats, galanin coding mRNA was visualized using in situ hybridization, and cells expressing galanin mRNA were counted. In REM sleep-deprived animals, the number of cells expressing galanin was higher in the preoptic area and periventricular nucleus which led the authors to conclude that REM sleep deprivation can induce galanin gene expression in some brain areas, but galanin alone does not modify spontaneous sleep.
The effects of REM sleep deprivation on somatostatin (SRIF) and growth hormone releasing hormone (GHRH) gene expression were studied using oligonucleotide probes to the mRNA. Fewer cells were found to express GHRH in the paraventricular nucleus and arcuate nucleus of animals subjected to 24 and 72 h of REM sleep deprivation than in control animals. The number of cells expressing SRIF was elevated in the arcuate nucleus after 24 h of REM sleep deprivation but not after 72 h. In the periventricular nucleus, the number of cells expressing SRIF was higher after 72 h of deprivation when compared to expression in animals maintained on large platforms. Serum GH level was also found to decrease in animals maintained on either small or large platforms (Toppila et al., 1997).

2.6 MECHANISM OF GENERATION OF REM SLEEP

Administration of acetylcholinesterase inhibitor -eserine led to increase in ACh concentrations which produced a state of wakefulness. However, the same drug, eserine produced a state of REM sleep, when the monamines had been previously depleted by the treatment of reserpine (Karczmar et al., 1970). This study proposed an interaction between the cholinergic and aminergic neuronal groups in REM sleep generation.

Reciprocal Interaction Model

Based on single neuronal activity and pharmacological studies, Hobson et al. (1975) proposed a reciprocal interaction model between pontine FTG REM-ON and LC and dorsal raphe REM-OFF neurons for the generation of REM sleep. They reported an opposite pattern of discharge rates in the REM-ON and REM-OFF cells.
during the sleep-wakefulness cycle.

The modified version of this model proposes that, REM-OFF aminergic neurons of DR and LC exert an inhibitory influence on the cholinergic neurons of LDT-PPT area and to themselves. The LDT-PPT neurons are excitatory to the REM-OFF neurons of LC as well as to themselves. As the animal or subject enters into NREM, REM-OFF cells decrease their discharge rate and the REM-ON cells are progressively disinhibited to the point of autoactivation. Thus, during REM sleep phase, the REM-ON cells, which are cholinergic in nature, through their anatomical projections to the ascending reticular activating system, may provide the necessary conditions for eliciting REM sleep (Steriade and McCarley, 1990).

**Mutual Inhibitory Interaction Model**

According to the **mutual inhibitory theory**, the putative REM-OFF neurons of LC are inhibitory to the putative REM-ON cholinergic/cholinoceptive neurons and the REM-ON cells are also inhibitory to REM-OFF cells. The REM sleep can therefore occur either by excitation of REM-ON cells or by inhibition of the REM-OFF cells (Sakai, 1988). Immunohistochemical studies have shown that the cholinergic neurons in the LDT-PPT areas and aminergic neurons in the LC are in close proximity, which would allow an interaction between these two cell types (Jones, 1990). Small GABA containing neurons are also found intermingled with these neurons and may thus contribute in facilitating the direct inhibition of aminergic neurons by cholinergic neurons (Jones, 1990). Recently, it has been proposed that cholinergic neurons inhibit the LC noradrenergic neurons through GABAergic interneurons (Kaur et al., 1997a).
Genetic Regulation of REM sleep generation

Recently, quantitative trait loci (QTL) approach was used to identify candidate regions on the mouse genome that influence sleep. REM sleep during the light period was associated with markers on chromosome 7 between 7 and 20 centimorgan from the centromere. The REM sleep during the dark period was associated with QTL identified on chromosome 5, present near the Clock gene. The 24 hour amount of REM sleep was influenced by markers on chromosome 2, 17 and 19 (Tafti et al., 1997).

2.7 REM SLEEP DISORDERS

REM sleep has been found to be disturbed in various pathological conditions. One of the most common example of REM sleep disorder is narcolepsy. Narcolepsy is characterized by i) excessive daytime sleepiness ii) episodes of cataplexy brought on by emotional excitation iii) hypnogogic hallucinations and iv) sleep paralysis. These four symptoms are often referred to as "Narcolepsy Tetrad". Brain stem cholinergic mechanisms, which normally act to induce atonia in REM sleep, are proposed to be triggered during waking resulting in Cataplexy and sleep paralysis. Cataplexy is also described as the inappropriate isolated intrusion of REM sleep atonia into wakefulness, usually induced by an emotionally laden event (Guilleminault et al., 1974). A comparison was made of cholinergic cell numbers in the brain stems of normal and narcoleptic canines. An increased number of cholinergic neurons at the R6-R7 level of the LDT and PPN was observed in narcoleptic canines (Nitz et al., 1995). This abnormality can explain alterations in cholinergic receptor number, acetylcholine release and the occurrence of cataplexy and sleep paralysis that characterize narcolepsy. A link between a class II antigen of
the major histocompatibility complex (MHC) known as DR2 and narcolepsy was discovered (Matsuki et al., 1987).

In patients suffering from Parkinson's disease, sleep fragmentation is the most consistent abnormality observed. Polysomnographic recordings have shown an increase in sleep latency and frequent awakenings, and these symptoms tend to increase in proportion to the severity of waking parkinsonian symptoms (Friedman, 1980). Patients with schizoaffective illness have been found to typically demonstrate extremely fragmented sleep, diminished SWS and extremely abbreviated REM sleep latencies.

REM sleep parameters have been found to be disturbed in patients suffering from endogenous depression. The most predictable sleep abnormalities of endogenous depression include sleep continuity disturbances, decreased REM sleep latency, increased REM sleep time and REM activity earlier in the night and increased density of REMs during REM sleep (Foster et al., 1976). The mechanisms of these abnormalities has been attributed to supersensitivity of cholinergic muscarinic receptors. REM sleep deprivation via arousals at the onset of REM periods was found to improve endogenous depression (Vogel, 1983). The antidepressant action of REM sleep deprivation by arousals supported the hypothesis that the REM sleep deprivation is a mechanism of action of antidepressant drugs (Vogel, 1975).

REM sleep is proposed to protect infants from developing SIDS (Sudden Infant Death Syndrome). The frequent occurrence of respiratory pauses in REM burst-free periods of REM sleep suggests that tonic REM mechanisms inhibit respiratory neurons, while phasic REM mechanisms are facilitatory and protect an infant from prolonged sleep apnea.
REM sleep behaviour disorder is characterized by excessive augmentation of chin and/or limb phasic muscle tone, limb and body jerking, complex violent behaviour. Thus, disturbances in REM sleep is a common feature in a number of disorders and psychiatric illnesses. This indicates the importance of REM sleep in maintenance of good health in our daily life.

2.8 REM SLEEP DEPRIVATION

The different phasic and tonic components of REM sleep are generated and regulated by different areas in the brain. It is difficult to elicit REM sleep by stimulation as different areas have to be stimulated simultaneously and probably with different intensities to obtain the normal REM sleep patterns. Although some studies exist, which elicit REM sleep by stimulation, it often is termed as the REM sleep like state. The other way to study the possible function of REM sleep is by studying various physiological parameters after depriving the animal of this particular phase of sleep. In fact, most of the studies which have contributed to elucidating possible function(s) of REM sleep are interpretations and conclusions based on experiments related to REM sleep deprivation.

2.9 METHODS USED FOR REM SLEEP DEPRIVATION

**Hand arousal technique**

Dement (1960) first applied the hand arousal technique in humans for REM sleep deprivation. Electrophysiological parameters signifying sleep-wakefulness were constantly monitored from human subjects and as soon as rapid eye movements as well as an "activated" EEG pattern appeared (characteristic of REM sleep), the subjects were awakened. This technique was subsequently applied on animals also.
Experimental subjects/animals were aroused by external stimulation at the onset of each REM sleep episode and immediately after awakening, the experimental subjects were allowed to resume sleep. Nonspecific concomitants of this technique were reasonably controlled by "yoked" control animals which were aroused concurrently with the experimental animals. The limitations and disadvantages of this technique are, first, it needed a constant human intervention and second, that although initially this technique could effectively prevent the animals from entering into or continue REM sleep, the frequency of awakening (vis-a-vis going into REM sleep by the animals) increases rapidly with increase in length of deprivation making it extremely difficult to continue deprivation for more than a few hours.

*Treadmill arousal technique*

In this method, the rats are put on a treadmill where due to the continuous movement of the latter, the rats cannot sleep. In this method, theoretically, the animals should not get any form of sleep (i.e. total sleep deprivation) since they are always in motion. However, in practice, in a short period of time, the rats learn to run in the opposite direction to that of the movement of the treadmill and have a nap till it reached the other end of the treadmill. This time the rats have to wake up to prevent from falling. The time of sleeping depends on the speed of the treadmill. It has been found that the cats could have almost 40% of the time spent in the treadmill in non-REM sleep. Hence, a combination of treadmill and hand arousal was used (Ferguson and Dement, 1967) for REM sleep deprivation. The rats were kept for 16-22 hs per day on the treadmill. The remaining 2-8 hrs were spent in cages from which polygraphic recordings were possible. Thus, in treadmill-arousal studies, the experimental and control animals do differ in REM sleep, but as this technique
deprives the animal of a large amount of total sleep, this technique has not frequently been used for REM sleep deprivation studies.

**Pendulum technique**

The pendulum technique was designed to avoid the restriction and other stress experienced by other methods (Van Hulzen and Coenen, 1980). In this method, the animals were kept in their home cages (thus they were not transferred to a new environment as in water tank procedure), which were made to swing like a pendulum. At the extremes of motion due to postural imbalance the animals are forced to walk downward to the other end of cages. A minimal amount of REM sleep (0-2%) and a moderate amount of non-REM sleep (19-30%) were detected by the authors during 72 hrs deprivation.

**Flower-pot technique**

A popular and rather effective technique for long term REM sleep deprivation is the Flower-pot technique. This method is also known as water tank, platform, or pedestal technique. It was first used in cats (Jouvet et al., 1964) and has since been extensively used on cats, rats and mice for REM sleep deprivation (Hicks et al, 1977; Oniani et al., 1988). In this method, the animals are maintained on small raised platforms, typically inverted flower pots, surrounded by water. On this artificial island, the animals can sit, crouch and move around freely. However, the animals do not get enough room for complete relaxation. As soon as the animals enter into REM sleep, due to atonia in the postural muscles, they cannot stay on the small platform and fall into the surrounding water. Consequent to coming in physical contact with the surrounding water the animals wake up. Thus, although the animals
can enjoy sleep, occurrence of REM sleep is prevented. In this method, the animals quickly learn to wake up at the onset of REM sleep and hence do not frequently fall into the water. Thus, in this method the animals can have NREM sleep but not REM sleep. This technique is very effective, inexpensive, procedurally simple and at the same time allows a large number of animals to be deprived of REM sleep simultaneously.

A large platform enabling the animals to relax and have both NREM as well as REM sleep without falling into the water is most effectively used as control. Movement restriction controls are carried out to rule out the effects of restricting the movement of the animals on small platforms. In this method, the animals are maintained in dry smaller cages, ideally of similar size to that of the small platform, where the animals can have both slow wave sleep and REM sleep. Another control which has frequently been used to eliminate the effect of increased muscular activity on the small platforms is the swimming control. However, it is difficult to equate the increased muscle activity on the small platforms and that of by swimming. This platform technique has been most widely used for REM sleep deprivation studies. Mendelson et al. (1974) recorded the EEG for 96 hrs from four groups of rats a) normal or baseline, b) on small platform (diameter 6.5 cm), c) on large platform (diameter 12.5 cm), and d) swimming (1 hr). He reported that rats on small platform had 57% as much REM sleep as baseline during the first 24 hrs, without any change in non-REM sleep, while rats on large platform, on the first day had 55% REM sleep (compared to baseline). The REM sleep increased to baseline value by the fourth day in the large platform control animals. On the fourth day the rats on the small platform continued to have significantly reduced REM sleep. The swimming control rats had no reduction in REM sleep or non-REM sleep at any stage. It was concluded that it
is the ratio of platform size to the body weight of animal which decides the amount of reduction in REM sleep (Mendelson et al., 1974; Hicks et al., 1977).

**Multiple platform technique**

Immobilization stress and inadequate feeding (due to constant swinging movement) were the problems associated with platform and pendulum technique respectively. In order to overcome these problems, the classical platform technique was modified to multiple platform technique (Van Hulzen and Coenen, 1981). In this, seven small (6.5 cm) platforms were placed in one water tank, permitting movement of the animals from one platform to another thus reducing forced immobility. The effects on sleep in this condition were identical to that of the classical platform technique, since only one platform can be used for sleeping purpose. In the multiple platform control, one platform was made a little bigger so that the animal could have REM sleep as well.

**Rotating Disc technique**

More recently, Bergmann et al. (1989) designed a computerised procedure for REM sleep deprivation, the rotating disc technique. In this technique, a pair of clear, smooth plastic cages house the experimental rat and its yoked control (in different cages). A smooth plastic disc, with its center in an alley between the cages, protrudes under each cage to provide a partial floor. Beneath each side of the disc and beyond it upto the walls of each cage is a tray of water. Both the rats are connected to polygraph for continuous recording of EEG and other electrophysiological signals signifying sleep-wakefulness. This polygraph is linked to a computer programmed to rotate the disk at randomly chosen direction at a rate of 3.33 rev/min whenever an
experimental animal entered into REM sleep. The rotation of the disk awakens the rat which starts walking opposite to disc rotation to avoid being carried into the water. When the polygraphic signals indicate that the experimental rat is out of REM sleep, the disc rotation stops. Thus, the animal is selectively deprived of REM sleep.

10. EFFECTS OF REM SLEEP DEPRIVATION

Behavioural changes

Dement (1960) performed the first deprivation studies in humans to investigate its physiological effects. He constantly monitored the classical parameters of sleep from human subjects and when the person displayed activated EEG patterns and rapid eye movement, the person was awakened. The subjects displayed more number of attempts to go into REM sleep phase as the period of deprivation increased. On recovery nights, the subjects spent a larger percentage of sleep in REM sleep compared to the baseline. This is known as "Rebound" REM sleep. REM sleep deprivation has been found to cause various behavioural changes including fatigue, irritability, inability to concentrate and anxiety (Dement and Fisher, 1963). Rats, deprived of REM sleep for 16-54 days by the disk method, showed a debilitated appearance, lesions on their tails and paws, and weight loss in spite of increased food intake, indicating an increase in energy expenditure (Kushida et al., 1989).

REM sleep deprivation has been shown to alter behavioural effect of some drugs. Heise and Boff (1962) studied the effect of D- Amphetamine on the rate of shock avoidance in control and REM sleep deprived rats. They reported that amphetamines, which release catecholamines enhanced the rate of shock avoidance in control rats but not in REM sleep deprived rats indicating an impairment of catecholaminergic systems.
REM sleep deprivation has been found to affect memory. The effect of REM sleep deprivation on story retention was studied in human subjects. It was found that recall accuracy following REM sleep deprivation was significantly poorer when compared to the recall accuracy in the control subjects (Tilley and Empson, 1978). Posttraining REM sleep amount was found to increase after spatial learning in a Morris maze (Smith and Rose, 1997). In another study, the rats were trained for 10 days in an eight-arm radial maze, where food rewards were available in four of the arms and other four arms were never baited. After each training session, the rats were deprived of REM sleep either immediately, after 4 hrs or 8 hrs. It was observed that the group that received REM sleep deprivation for 4 hrs immediately following the training sessions showed significant impairment of spatial memory when compared to other groups (Smith et al., 1998).

**REM sleep deprivation and excitability**

REM sleep deprivation also causes many physiological changes like decrease in threshold to electroconvulsive shock in rats (Cohen and Dement 1965). The ratio between amplitude of potentials evoked by a pair of clicks was greater in nondeprived than in deprived rats. This change was reversed when the rats were allowed to recover the lost sleep (Dewson et al., 1967). An increase in shock induced fighting behaviour was observed in rats after REM sleep deprivation (Morden et al., 1968). Loss of sleep, mainly the paradoxical phase resulted in an increase in brain excitability as seen by a decrease in threshold to seizures (Owen and Bliss, 1970). Evoked potential as an indicator of neural excitability was studied during progressive REM sleep deprivation and an increase in amplitude of entorhinal potentials was observed as the duration of deprivation increased. Since there was a decrease in excitability of primary sensory
afferent pathways, it was concluded that REM sleep deprivation may lead to paleocortical excitability and to increase in inhibition responsible for sensory filtering (Satinoff et al., 1971). It also has been reported to increase in aggressiveness in male rats (Hicks et al., 1979). Mallick et al. (1989) showed that the neurons of LC, which are REM-OFF in nature, donot stop firing during REM sleep deprivation. Similarly, REM sleep deprivation was also found to decrease auditory evoked inhibition in dorsolateral pontine neurons (Mallick et al., 1991). Thus, REM sleep deprivation is likely to have a generalized effect on neuronal responsiveness and excitability.

**Biochemical changes**

REM sleep deprivation leads to a variety of changes, including changes in the activities of various enzymes and changes in gene expression.

An increase in NE turnover (Pujol et al., 1968; Mark et al., 1969) and TH (Sinha et al., 1973; Basheer et al., 1998) has been reported after REM sleep deprivation. The effect of REM sleep deprivation on the total content and proportion of different mucopolysaccharides (AMPS) containing uronic acid in rat brain was studied. A significant increase of AMPS was found in the cerebral hemispheres, cerebellum and brainstem of REM sleep deprived rats. A further increase of AMPS was found in the cerebral hemispheres and brainstem after the rebound of REM sleep following its deprivation (Levental et al., 1975).

REM sleep deprivation of 24 hrs was found to produce a fall in glucose, glucose 6-phosphate and pyruvate in cerebral frontal lobes. After three hours of recovery sleep, all values were found to return toward their predeprivational levels (Djuricic et al., 1977). Peder et al. (1986) studied the effect of REM sleep deprivation on regional brain metabolic activity of rats using 2-[^14C]deoxyglucose
REM sleep deprivation for 5 days caused an increase in metabolic activity in the lateral habenula, the caudal and middle parts of the limbic system and in the corpus callosum. In a related study, a decrease in glucose-6-phosphatase activity and an increase in hexokinase activity was reported after REM sleep deprivation, which returned to baseline level on recovery (Thakkar and Mallick, 1993).

REM sleep deprivation caused a decrease in β adrenergic sites in rat brain. Also, a marked decrease in $[^3H]$-cyclic AMP synthesis after REM sleep deprivation was observed indicating adrenergic receptor down-regulation (Troncone et al., 1986). REM sleep deprivation led to a significant increase in the adenosine A1 receptor binding in the cortex and corpus striatum (Yanik and Radulavacki, 1987). Recently, quantitative receptor autoradiography was used to map alterations in binding to α1-, α2-, β1- and β2-adrenergic receptors throughout the brain of rats deprived of REM sleep for 96 h. While α adrenergic receptor binding was not affected, β-adrenergic binding was significantly reduced throughout the brain (Hipolide et al., 1998).

REM sleep deprivation caused an increase in acetylcholinesterase (Thakkar and Mallick, 1993) and a decrease in MAO-A (Thakkar and Mallick, 1993) activity. Also, REM sleep deprivation led to an increase in Na-K ATPase (Gulyani and Mallick, 1993) and chloride sensitive ATPase (Mallick and Gulyani, 1993) activity.

The effect of chronic REM sleep deprivation on brain PGE2 and PGD2 biosynthesis in mouse was evaluated, since they are known to have opposite actions as respectively wake- and sleep-inducing substances. REM sleep deprivation for 5 to 10 days caused an enhancement in the ratio of PGE2/PGD2 in the hypothalamus and hippocampus, indicating a shift from PGD2 toward PGE2 (Moussard et al., 1994). Changes in the level of glutamine synthetase (GS), an enzyme mainly located
in astrocytes, were investigated in rat brain after REM sleep deprivation and during recovery. A significant increase in GS protein was observed both in the frontoparietal cortex and in the LC. Both GS mRNA and GS protein tended to return to control values 4 hrs later during recovery (Sallanon-Moulin et al., 1994).

REM sleep deprivation did not cause any change in 5'-nucleotidase activity (Thakkar and Mallick, 1996). A decrease in microsomal and synaptosomal membrane fluidity (Mallick et al., 1995) and free Ca++ levels (Mallick and Gulyani, 1996) have also been observed on REM sleep deprivation.

Changes in gene expression

Differential screening of a subtractive rat forebrain cDNA library with cDNA probes derived from either sleep-deprived or control rats resulted in the isolation of several transcripts which showed altered hybridization profiles (Rhyner et al., 1990). One of the transcripts was characterized to be 70-90 kD protein which was named dendrin and was found to contain potential sites for phosphorylation by various kinases (Neuner-Jehle et al., 1996).

In both cats and rats, immediate early genes such as c-fos and NGF1-A are activated after short periods of spontaneous waking or sleep deprivation (Pompeiano et al., 1992, 1995). Total sleep deprivation studies showed an increase in the mRNA levels of transcription factors like c-fos and NGF1-A in cerebral cortex of rats after waking when compared to after an equal duration of sleep (Cirelli et al., 1993). Injection of antisense c-fos mRNA into the medial preoptic area led to a decrease in REM sleep and an increase in wakefulness, while injection of sense mRNA or saline did not alter the sleep patterns (Cirelli et al., 1995). An increase in Fos Like Immunoreactivity (FIL) was found in a number of structures in the brainstem and diencephalon during recovery from REM sleep deprivation (Merchant-Nancy et al., 1995).
1995). Also, phosphorylation of cyclic adenosine monophosphate response element-binding protein was also reduced (Cirelli et al., 1996). The increased transcription of c-fos was found to be modulated by noradrenergic LC neurons as no increase in mRNA levels were found after waking if the brain was deprived of noradrenergic innervation (Cirelli et al., 1996). In order to systematically investigate differences in gene expression during sleep and waking, mRNA differential display was used to examine mRNAs from the cerebral cortex of rats who had been spontaneously asleep, spontaneously awake, or sleep deprived for a period of 3 h. It was found that, while the vast majority of transcripts were expressed at the same level among these three conditions, the expression of a subset of mRNAs was modulated by sleep and waking. Half of these transcripts had known sequences in databases. RNAs expressed at higher levels during waking included those for the transcription factors c-fos, NGFI-A, and rlf, as well as three transcripts encoded by the mitochondrial genome, those for subunit I of cytochrome c oxidase, subunit 2 of NADH dehydrogenase, and 12S rRNA. This finding suggests the hypothesis that an increase in the level of mitochondrial RNAs may represent a rapid regulatory response of neural tissue to adapt to the increased metabolic demand of waking with respect to sleep (Cirelli and Tononi, 1998a).

Immunocytochemical mapping of cells labelled with anti-phosphoserine and anti-phosphothreonine antibodies showed that the number of cells labelled with either antibody in the cerebral cortex was markedly higher in rats sacrificed after 3 hours of waking than after 3 hours of sleep. In animals with unilateral LC lesion, the number of labelled neurons after 3 hours of waking was decreased on the ipsilateral side of the lesion only. This indicated a variation in the phosphorylation status across the sleep-wakefulness cycle and LC plays a role in its maintenance (Cirelli and Tononi, 1998b).
2.11 HYPOTHESESIZED FUNCTIONS OF REM SLEEP

Since, REM sleep is a process which is absolutely essential to survival, it is likely to serve one or more very important functions. Although many theories of REM sleep function have been proposed, an exclusive role/function of REM sleep has not yet been demonstrated.

According to some initial theories, REM sleep was proposed as being periods of heightened vigilance oriented to urgencies of waking life. According to the **restorative theory**, sleep is a period of recovery or restoration of physiological, neurological and/or psychological states. NREM sleep was viewed as restoration of physical or general body tissue and REM sleep was ascribed cortical or brain restorative functions (Hernandez-Peon and Sterman, 1966). Snyder (1966) suggested a **sentinel hypothesis** which proposes that REM sleep, with its heightened neural arousal and decreased sensory threshold, serves the animal in the wild by allowing for brief periods of environmental scanning embedded in the less engaged and deeper states of non active sleep. Another theory called **homeostasis hypothesis** was put forward by Ephorn and Carrington (1966), who suggested that REM sleep provides a periodic restoration of cortical tonus or vigilance to compensate for reduction of sensory stimulation from the environment during sleep. The **functional theory** was proposed by Roffwarg and coworkers (1966) based on the predominance of REM sleep in new born mammals. As neuronal activity is known to play an important role in the establishment of connections and functional synapses, they proposed that the intense neural activity indicative of REM sleep may assist in neuronal differentiation, maturation and myelination in higher centres.

In 1969, Berger proposed his **occulomotor innervation hypothesis**. He proposed that REM sleep provides a mechanism for the establishment of the neuromuscular pathways involved in voluntary conjugate eye movements in both
phylogenesis and ontogenesis. Also, REM sleep was proposed to furnish periodic innervation of the oculomotor system during extended periods of sleep throughout mammalian life. Stern and Morgane (1974) proposed that REM sleep plays a role in maintaining the availability of catecholamine neurotransmitter levels for synaptic action on CNS. Jouvet (1975) has suggested that the dreams that often accompany active sleep in adults act as a practice forum for species-typical behaviours. The *motivational theory* was proposed by Vogel in 1975. He suggested that dampening of "basic behaviours" by REM sleep releases the animal from its instincts, give more flexibility to other behaviours. Since REM sleep is also known to be associated with increased protein synthesis (Bobillier, 1971), McGinty and Drucker-Colin (1982) proposed a protein synthesis function for REM sleep. Vertes (1986) has suggested that REM sleep serves as a state of *neural activation* interspersed within the other quieter states of sleep. This neural activation, it is hypothesized, is essential for preventing the brain from shutting down during sleep and thus leading to death. In 1986, Mirmiran put forward a hypothesis that REM sleep in early life serves as (1) an indicator for the degree of brain maturation and (2) the promotor of further brain development. Siegel and Rogawski (1988) presented a theory of REM sleep as a regulator of noradrenergic receptor sensitivity. According to this theory, the cessation of activity of noradrenergic LC neurons in REM sleep is a key functional aspect of REM sleep.

*Cognitive theory* for sleep have also been proposed. Crick and Mitchison (1983) proposed that REM sleep may function to remove undesirable modes of interaction in networks of cells in the cerebral cortex. Winson (1993) proposed a *memory processing hypothesis* suggesting that REM sleep aids in the integration of individual experience into a strategy for further behaviour. This theory centres on theta rhythm of the hippocampus, an electrophysiological correlate of REM sleep. The
theta rhythm is observed during REM sleep and it is postulated that the animal sorts and integrates the sensory information obtained and stores them as a part of species-specific behaviour. A number of studies have argued that REM sleep is necessary for the consolidation of memory (Smith and Rose, 1996; 1997). Rats were made to learn a specific task like the Y-maze. These were tested for short and long term memory when the rats were subjected to REM sleep deprivation after task acquisition. It was observed that while short-term memory was not affected, long term memory seemed to be impaired after REM sleep deprivation immediately after task acquisition. Interestingly, the rats showed increased amounts of REM sleep after the learning task while non-learners did not show the increase in REM sleep.

*Maintenance of neuronal excitability: A REM sleep function*

REM sleep has also been attributed a function of *maintenance of neuronal excitability* (Mallick et al., 1999). REM sleep deprivation is accompanied by increase in neuronal excitability as shown by decrease in threshold to seizures, electroconvulsive shocks and a generalized increase in aggressiveness and irritability.

Unit activity studies of pontine neurons were studied after REM sleep deprivation. The pontine REM-OFF neurons, which normally stop firing during REM sleep, were found to continue firing during REM sleep deprivation (Mallick et al., 1989). Also, REM sleep deprivation was found to reduce the auditory evoked inhibition of unit discharge in dorsolateral pontine neurons (Mallick et al., 1991). Inhibition levels were found to return to baseline after recovery. Thus, these studies indicate that REM sleep deprivation affects brain and neuronal activities. One of the possible reasons for the increase in neuronal excitability is the observed increase in the membrane bound Na-K ATPase activity (Gulyani and Mallick, 1993) observed after REM sleep deprivation. Na-K ATPase is known to maintain the transmembrane
potential (Trachtenberg et al., 1982). This increase in Na-K ATPase activity was found to be mediated by $\alpha$-1 adrenoceptor (Gulyani and Mallick, 1995) and Ca\textsuperscript{+}+ (Mallick and Gulyani, 1996). This increase in enzyme activity was not observed in large platform controls and was found to return to baseline levels after recovery. Based on these studies, Mallick et al. (1999a) proposed that possibly the basic function of REM sleep is to maintain neuronal excitability, which in turn, affects most of the other functions.

### 2.12 NEURONAL EXCITABILITY

**Neuron at rest:** Resting transmembrane potential of a neuron is around -60mV with the cytoplasm being more negative than the outside. The membrane potential arise due to relative unequal distribution of ions across the neuronal membrane. Of the four major ions in cells, Na\textsuperscript{+} and Cl\textsuperscript{-} are more concentrated outside the cells, and K\textsuperscript{+} and organic anions (A-) are more concentrated inside the cell. As a result of different permeabilities of K\textsuperscript{+} and Na\textsuperscript{+}, an equilibrium is attained when the outward movement of K\textsuperscript{+} balances the inward movement of Na\textsuperscript{+}. Although these steady leaks tend to cancel each other, it does not happen continuously as it would lead to depletion of intracellular K\textsuperscript{+} and ionic gradients would run down gradually, decreasing the membrane potential. Dissipation of these ionic gradients is prevented by the ATPase driven Na\textsuperscript{+}-K\textsuperscript{+} pump. This pump derives energy from hydrolysis of ATP and drives the Na\textsuperscript{+} and K\textsuperscript{+} ions against their net electrochemical gradient. Thus, when the cell is at rest, the active fluxes (driven by the pump) and the passive fluxes (due to diffusion) are balanced for Na\textsuperscript{+} and K\textsuperscript{+}, so that the net flux of each of these two ions is zero. Also, different ionic channels open and close even at rest and several types of channels open simultaneously. Under these circumstances, the ionic gradients are dissipated constantly, albeit slowly and ionic pumps, like Na-K ATPase
are always needed to maintain a steady state.

After an action potential: After the generation of an action potential, the resting membrane potential and the ionic concentrations are brought back to the resting state mainly by the action of Na-K ATPase.

2.13 TYPES OF ATPases

Ion-pumps can be grouped into three principal classes of ATP-powered ion pumps, called P, V and F. All the three classes of ATPases have one or more binding site(s) for ATP on the cytosolic face of the membrane. Table 1 summerizes their different properties.

2.14 Na-K ATPase

Skou (1957) initially discovered that the Na⁺ and K⁺ fluxes across the crab neuronal membrane were dependent on ATP and that the pump could be inhibited by cardiac glycosides. Later studies showed that Na-K ATPase is a membrane associated enzyme responsible for maintaining the high internal [K⁺] and low internal [Na⁺] which is the characteristic of most animal cells. This enzyme couples hydrolysis of ATP to transport of Na⁺ and K⁺ across the plasma membrane, against their respective electrochemical gradients. The cation gradients created by the Na-K ATPase are fundamental to such diverse cellular functions as the regulation of cell volume, nutrient uptake and membrane excitability. The Na-K ATPase is important as upto one-third of the animal cell's energy requirement is consumed in fuelling the Na-K ATPase. Upto 70% of the total energy requirement of the brain is consumed by Na-K ATPase.

2.15 STRUCTURE OF Na-K ATPase

Na-K ATPase is formed by at least two noncovalently linked polypeptides, a
<table>
<thead>
<tr>
<th>PROPERTY</th>
<th>CLASS P</th>
<th>CLASS F</th>
<th>CLASS V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of types of subunits</td>
<td>2</td>
<td>8 (minimum)</td>
<td>7 (minimum)</td>
</tr>
<tr>
<td>Ions transported</td>
<td>H⁺, Na⁺, K⁺, Ca²⁺</td>
<td>H⁺ only</td>
<td>H⁺ only</td>
</tr>
<tr>
<td>Characteristic features</td>
<td>Large α subunit phosphorylated</td>
<td>Generally functions to synthesize ATP, powered by movement of H⁺ down the electrochemical gradient.</td>
<td>Generally functions to hydrolyse ATP and to generate a transmembrane H⁺ electrochemical gradient.</td>
</tr>
<tr>
<td>Location of specific pumps</td>
<td>a) Plasma membrane of plants, fungi (H⁺ pump). b) Plasma membrane of higher eukaryotes (Na⁺-K⁺ pump). c) Plasma membrane of mammalian stomach cells (H⁺-K⁺ pump). d) Plasma membrane of all eukaryotic cells (Ca²⁺ pump). e) Sarcoplasmic reticulum membrane in muscle cells (Ca²⁺ pump).</td>
<td>a) Bacterial plasma membranes.</td>
<td>a) Vacuolar membranes in plants and fungi. b) Endosomal and lysosomal membrane in animal cells. c) Plasma membrane of certain acid-secreting animal cells (e.g., osteoclasts and some kidney tubule cells.</td>
</tr>
</tbody>
</table>
catalytic α subunit (90-135kDa) and a small glycosylated β subunit (35-57kDa) respectively (Jorgensen, 1982; Mercer, 1993). A small peptide with a molecular weight of approximately 10kDa termed the γ, has also been identified in the purified preparations of the enzyme (Hokin et al., 1973).

Na-K ATPase belongs to a widely distributed class of E1/E2 or P-type cation transporting proteins. The E1/E2 designation refers to the two general enzymatic conformations E1 and E2, which are formed during the catalytic cycle. The unique characteristic of such proteins is that a transient, phosphorylated aspartyl residue is formed during the reaction cycle. The regions around the phosphorylated aspartyl residue and several regions involved in ATP binding exhibit a high degree of amino acid homology. The α and β chains are non-covalently linked on the membrane.

The α peptide gets phosphorylated by ATP, undergoes ligand-dependent conformational changes accompanying the binding, occlusion and translocation of ions across the membrane. The β subunit is a glycosylated polypeptide whose exact function is yet unknown. In ouabain-resistant HeLa cells, both α and β subunits are amplified suggesting that both are required for ouabain resistance (Schneider et al., 1985; Mercer et al., 1986). Both the α and β subunits are found to be required for the functional expression of the Na-K ATPase in Xenopus oocyte (Noguchi et al., 1987). The β subunit may also facilitate the correct assembly of α subunit into the membrane (Noguchi et al., 1990)

Apart from regulation of Na⁺ and K⁺ ion concentrations across the membrane, Na-K ATPase may also play a role in the release of neurotransmitters, regulation of cell volume changes, energy dependent activities such as uptake of metabolites and neurotransmitters, regulation of intracellular Ca²⁺, indirectly via the Na⁺/Ca²⁺ exchange in muscles and neurons (Hernandez, 1992).
A. MODEL OF THE $\alpha$ SUBUNIT OF THE HUMAN Na-K ATPase

TRANSMEMBRANE ORIENTATION

B. MODEL OF THE $\beta$-SUBUNIT OF THE HUMAN Na-K ATPase

Fig 1 [Taken from “Structure of Na-K ATPase” (Mercer, 1993)]
α- SUBUNIT

Structure

The deduced primary structure of the α-subunit reveals a polypeptide consisting of approximately 1000 amino acids with a molecular weight of about 113 kDa. Studies with ATP analogs, identification of the catalytic phosphorylation site has given an indication of the cytoplasmic domains and residues that contribute to the ATP binding site. The amino acid which gets phosphorylated during the catalytic cycle is aspartate at 369 position. This was confirmed by the study that substitutions of Asp369 to Asn, Glu, Thr or Ala abolished ATP hydrolysis (Pedersen, 1996). The hydropathy profiles indicate that the α subunit is a multipass transmembrane protein. The α subunit may contain up to 10 transmembrane regions. Its cytoplasmic amino terminus is followed by four membrane-spanning regions, which is followed by a large cytoplasmic domain consisting of roughly one-third of the polypeptide (Fig 1A). This large domain contains the aspartyl residue (369) which gets phosphorylated during translocation of ions (Lingrel and Kuntzweiler, 1994; Lutsenko and Kaplan, 1994). Analysis of the C-terminal portion of the enzyme indicates the presence of two to six membrane spanning regions.

ATP binding and hydrolysis occur in one region of the protein, while transport and cation occlusion occur at spatially distinct sites on the protein. The presumptive cation binding sites are present in the transmembrane segments IV, V and VI, and the amino acid residues important are Glu327, Glu779, Asp804 and Asp808 (Pederson et al., 1997; 1998). Studies have shown that removal of K⁺ ions from the preparation led to the loss of M5M6 hairpin from the membrane to the aqueous phase (Lutsenko et al., 1995). This redistribution from the membrane phase to the aqueous phase was prevented by K⁺ ions.
Isoforms

Three isoforms of α subunit have been identified which are named as α1, α2 and α3. The degree of similarity between identical isoforms across species is over 92%. Each rat or chicken isoform differs from other two isoforms by approximately 14%. Greatest similarities occur around the site of phosphorylation and the major hydrophobic regions (Mercer, 1993). Determination of the nucleotide sequence and hybridization analysis of rat genomic DNA shows that each α subunit isoform is the product of a different gene. Chromosome mapping localizes the gene coding for α1 isoform to chromosome 1p in human, α2 isoform to chromosome 1q and α3 isoform to chromosome 19 (Kawakami et al., 1986).

Developmental and Tissue-Specific Regulation

The α1 and α2 isoforms exhibit a diverse expression pattern in various tissues, while α3 is found exclusively in the adult neural tissue. α-1 subunits have been detected in virtually all rat tissues (Shyjan and Levenson, 1989). Substantial differences in the cell-specific pattern of expression were found for the genes encoding three isoforms of the α subunit. Monoclonal antibodies (Mab) to isoforms of the Na-K ATPase α subunit were used to study the distribution patterns of the enzyme. It was observed that α-3 specific MAb - McBX3 recognises an unusual epitope which is not present in the α-3 of the heart. DNA sequencing of the reverse-transcribed PCR products from α-3 mRNA and hydroxylamine sensitivity studies indicated that the difference in epitope was not due to alternate splicing of RNA, but because of the Ab recognizing a post-translational modification (Arystarkhova and Sweadner, 1996). Thus, the differences in post-translational modification may also contribute to the variability of the enzyme in different tissues. Transcripts of α1-subunit gene were detected in virtually all cell types and structures of the brain.
Expression of α 2-subunit mRNA was characteristic of glia, whereas α 3-subunit transcripts were predominant in neurons (Watts et al., 1991).

In addition to the tissue-specific regulation, the isoforms of Na-K ATPase are regulated developmentally. In adult rat brain, all the three α isoforms are present in roughly equivalent amounts, however in fetal brain, α3 is the most abundant isoform. In the adult rat, the multiple α isoform and β-subunit mRNA appear to be coordinately regulated with maximum expression occurring between 15 and 25 days of age (Orlowski and Lingrel, 1988). Levels of α3 mRNA increase over ten fold during first seven days of development. In contrast, α1 and α2 increase more gradually reaching levels similar to α3 at approximately 25 days of age (Orlowski and Lingrel, 1988).

The developmental pattern also differs in a tissue specific manner. For example, in the skeletal muscle, α1 is the major form in the fetus and its amount remains constant throughout development. α2 mRNA remains relatively low in early stages of development, however following birth, α2 increases ten fold to become the predominant form (Orlowski and Lingrel, 1988).

It is speculated that the α-subunit isoforms may have functional properties that are important in regulation of Na-K ATPase activity in different tissues. But, the exact function(s) of these different α-subunits and their physiological significance are unknown. As predicted from comparisons of the cDNA and protein sequences, α-1 appears to undergo posttranslational modification of cleavage at its amino terminus, whereas α2 and α3 do not (Pressley and Petrosian, 1997).

**β SUBUNIT**

*Structure*

It is a glycosylated polypeptide consisting of approximately 302-305 amino
acids and has a molecular weight of approximately 35kDa. The mammalian β subunit has three potential sites for N-linked glycosylation (Fig 1B). Hydropathy plots indicate the presence of a single transmembrane region (Girardet et al., 1983). The polypeptide contains seven cysteines—all of which are predicted to be involved in disulphide bond formation except one which is predicted to be in the membrane spanning domain (Miller and Farley, 1990). This formation of disulphide bonds seem to be important for the activity of the enzyme, since β-mercaptoethanol treatment can render the enzyme inactive (Kawamura and Nagano, 1984).

**Isoforms**

Rat and human β-subunit gene encodes four distinct mRNA species that are expressed in a tissue-specific manner. Transcripts encoding the β 1 subunit were detected in neurons, whereas β 2-subunit mRNA expression was characteristic of glia (Watts et al., 1991). A third isoform of β subunit was identified and characterized recently in humans. The cDNA of β-3 subunit was found to encode a polypeptide of 279 amino acids, which exhibited primary sequence and secondary structure similarities to other β subunits.

**Developmental and Tissue specific regulation**

Different mRNAs are found to produce a single protein, so it is assumed that this is probably derived from the same gene through alternate splicing in the noncoding region (Young et al, 1987). The physiological significance of such a process is unknown.

The mRNA levels of β2 subunit may vary in a tissue specific manner too. β2 shows approximately 58% amino acid similarity with β1. β2 has eight potential N-linked glycosylation sites in the rat. The tissue specific expression of β2 is different from that of β1. Expression of β1 subunits has been detected in brain, heart and
kidney (Shyjan and Levenon, 1989), whereas $\beta_2$ subunits are expressed primarily in brain, pineal gland and photoreceptor cells (Schneider et al., 1991). Northern blot analysis revealed that the $\beta_3$ subunit gene encodes two transcripts that are expressed in a variety of tissues like testis, brain, kidney, liver etc.

These isoforms are also regulated developmentally. Immunohistochemical studies reveal that both $\beta_1$ and $\beta_2$ Na-K ATPase subunits show the same pattern of cellular expression in both neurons and glia in the neonatal stage. After birth, $\beta_1$ protein expression increases reaching the adult level at day 6, after which the subcellular patterns remains constant in both cell types (Martin-Vasello et al., 1997).

**$\gamma$ SUBUNIT**

It is a hydrophobic peptide of 58 amino acids having a molecular weight of 6497 daltons (in the kidney) and has no sites for glycosylation. The evidence that this subunit is associated with Na-K ATPase is that it can be photoaffinity labelled with ouabain derivatives. Reeves et al. (1980) has suggested that the $\gamma$ subunit is present in the enzyme in stoichiometric amounts. Earlier, it was assumed that the $\gamma$ subunit was a breakdown product of the $\alpha$ and $\beta$ subunits. However, partial sequencing of the HPLC-purified $\gamma$ subunit has clearly established that this polypeptide is different from the $\alpha$ and $\beta$ subunits (Collins and Lesznk, 1987). The amino terminal of the $\gamma$ peptide is cytoplasmic, which is followed by one membrane spanning domain and a highly charged extracellular domain. The exact role played by this peptide is unknown, although it is postulated to induce cation channel activity (Minor et al., 1998). The peptide is coded for by a gene which encodes two mRNA species of approximately 1.5 and 0.8 kb (Mercer, 1993). There is little expression of the $\gamma$ subunit in the brain.
2.16 OCCURRENCE AND LOCALIZATION

Na-K ATPase is ubiquitous amongst animal species and present in all animal cells but its activity ranges widely. In the rabbit CNS, the mesencephalon has the highest levels of ATPase activity, cerebellar cortex followed by cerebral cortex, medulla spinal cord and medulla and lowest in optic chiasma. The nervous structures which are richer in myelinated fibers exhibit a lower Na-K ATPase activity. In myelinated axons, the enzyme is restricted in portions of the axolemma not covered by the myelin sheath, including the presynaptic terminal membrane. Synaptosomal membranes prepared from rat brain homogenate stained intensely for Na-K ATPase whereas synaptic vesicles and mitochondria did not (Phillis and Wu, 1981).

Inhibition of the Na-K ATPase by ouabain increases the cytosolic Na+ concentration and thereby decreases the plasma membrane Na+ gradient. This leads to enhanced Ca++ entry and reduces Ca++ exit mediated by the plasma membrane Na+/Ca++ exchanger. The net Ca++ gained by the cells is then effectively sequestered in the sarcoplasmic/endoplasmic reticulum (S/ER) via the S/ER Ca++ pump, so that the cytosolic free Ca++ concentration rises only minimally. Using monoclonal antibodies, it was observed that Na+ pump α2 and α3 subunit isoforms and Na+-Ca++ exchanger in the plasma membrane are present in close proximity. α2 and α3 isoforms indirectly helps to regulate S/ER Ca++ stores and thus influence the numerous processes that depend on mobilization of Ca++ from these stores (Juhanszora and Blaustein, 1998).

Using baculovirus expression system in Sf-9 insect cells, the structure and function of Na-K ATPase α and β isoforms were studied by infecting and expressing α1, α2, α3, β1 and β2 cDNA. All α isoforms were found to be stably assembled with either β1 or β2 polypeptides and all αβ combinations were found to be
catalytically active with different kinetic properties. The assembly of αβ subunits can occur at the endoplasmic reticulum level or at the plasma membrane level and these subunits can be delivered to the plasma membrane independently (Blanco et al., 1997).

2.17 MECHANISM

One of the properties of the plasmalemmal Na-K ATPase which allows the translocation of specific ions across the plasma membrane is that the enzyme exhibits sidedness i.e. has separable ligand foci facing the cytosol and facing the extracellular fluids (Trachtenberg, 1982).

Three classes of sites are evident on the enzyme which is present on the membrane:

-Ionophoric sites: where specific substrates undergo translocation (ions).
-Catalytic sites: where substance to be hydrolysed (nucleotides) bind to the enzyme.
-Permissive sites: which binds ions/molecules that are unaltered by the action of the enzyme but whose occupation appears to be essential for enzyme activity. There are sites for K⁺ and ouabain in the external portion of the α subunit and sites for Na⁺ and ATP in the cytoplasmic portion of the α subunit (Robinson and Flashner, 1979). The enzyme translocates 3 Na⁺ ions from inside to the outside and 2 K⁺ from outside to inside the cell. Thus the pump is electrogenic in nature. The protein can exist in two major conformational states designated as E1 and E2. E1 form prefers to bind Na⁺ and ATP and is stabilized by these ligands, whereas E2 form binds K⁺ and /or a covalent phosphate and is stabilized by these ligands. The monovalent cation binding sites are accessible from the cytoplasmic side of the membrane only when the protein is in E1 state and are accessible from the extracellular side only when the protein is in E2 state (Fig 2).
Fig 2: The sequence of events in the reaction cycle of Na-K ATPase is shown in this figure. [Taken from Basic Neurochemistry, 1989 Siegel, G.J. (Ed)]
SCHEMATIC DIAGRAM OF PROPOSED MECHANISM OF TRANSITION
OF Na-K ATPase BETWEEN E1 AND E2 STATES

Fig 3 (Taken from Biochemistry by Stryer, 1975)
When 3 Na⁺ ions bind to the cytoplasmic side of the protein which is in the E1 state, the enzyme gets phosphorylated in the presence of ATP. The phosphorylated enzyme undergoes a conformational change and assumes the E2 state. The Na⁺ ions bound to the cation binding sites are extruded out and K⁺ ions occupy the sites from the extracellular side. In the presence of K⁺, the ATPase activity is stimulated and the enzyme undergoes dephosphorylation. This causes another change in the conformation forming back E1 state where Na⁺ ions replace the K⁺ ions (Fig 2 & 3). Thus the pump acts in a cyclic manner transporting Na⁺ ions to the outside and K⁺ to the inside with ATP hydrolysis (Fig 2 & 3) (Albers et al., 1963).

2.18 REGULATION OF Na-K ATPase

*Regulation by cations*

Under steady-state conditions, the rate of ATP hydrolysis by the Na-K ATPase is equal to the sum of rates of Na⁺ entry processes. Pump rate can be regulated both by internal [Na⁺] and external [K⁺]. However, under physiological conditions, [Na⁺] inside the cell is usually less than that required for 50% occupation of the pump (internal) sites for [Na⁺], whereas [K⁺] is sufficient to occupy the external K⁺ sites maximally. Thus, the pump rate is more responsive to internal [Na⁺] than to external [K⁺]. The cation flux during action potentials is 2-3 orders of magnitude greater than the resting state. Na⁺ entry and K⁺ efflux during a single action potential, which lasts for about 1 m sec is 3x10⁻¹² mol/cm² membrane. Resting membrane flux in this tissue is 12x10⁻¹² mol/cm²/sec. Thus it would take the pump about 0.25 sec to discharge the flux of one spike at the rate of the pump in the resting membrane state. The Na-K ATPase would have to respond through a range of 2.5 to 25 times higher rate in order to maintain a steady state when the neuron is conducting at frequencies of 10-100 impulses per second.
Regulation by K⁺

Incubation of cultured cells in low concentrations of K⁺ (<1mM) results in a net loss of K⁺ and a net gain of Na⁺ (Kim et al., 1984). But, these changes are subsequently reversed within 12 to 24 hrs by significant increases in both abundance of Na-K ATPase and active transmembrane transport of Na⁺ and K⁺ in cells in culture (Pressley et al., 1988; Ismail-Beigi et al., 1988). Low K⁺ also leads to a transient induction of c-fos, which may act as a cofactor necessary for induction of Na-K ATPase (Cayanis et al., 1992).

Regulation by Na⁺

In cultured rat vascular smooth muscle cells, Na-K ATPase α and β isoform mRNA expressions were evaluated by Northern blot analysis. Transfection studies were conducted using chimeric plasmids containing the 5'-flanking sequences of α-isoform and luciferase reporter gene. Stimulation with 10μM of veratridine, a Na⁺ channel activator, caused a three fold increase in luciferase activity. This indicated that Na⁺ responsive elements are located within the 5'-flanking sequences of the gene (Yamamoto et al., 1994).

Regulation by Ca²⁺

Apart from Na⁺ and K⁺ ions, the Na-K ATPase is also regulated by calcium. Using Sf-6 cells infected with recombinant baculoviruses, the effect of different Ca²⁺ concentration on inhibition constants of α1β1, α2β1 and α3β1 forms of the enzyme were found to be 1.0±0.2X10⁻⁴ M, 7.3±4.6x10⁻⁶ M and 1.9±1.0x10⁻⁵ M respectively. In excitable cells, when the intracellular Ca²⁺ concentration reaches 5-10μM, the α-1 isozyme remains active, while the α2 and α3 are functioning at half their capacity (Blanco et al., 1997). Thus, intracellular Ca²⁺ may regulate cellular excitability by
selectively inhibiting specific Na-K ATPase isozymes.

The 5'-regulatory region of the α-1 subunit isoform manifests two sequences which would provide distinct mechanisms by which changes in free cytosolic Ca$^{++}$ levels might elicit changes in the transcription rate of α-subunit. A consensus sequence to which the transcription factor CREB binds and which mediates both cAMP and Ca$^{++}$-induced transcription activation and second, the AP-1 consensus sequence to which the AP-1 transcription factor binds (Montminy and Bilezikjian, 1987). This AP-1 consensus sequence, would provide a mechanism, which is less direct than the CREB mediated process. The stimulation of synthesis of the proto-oncogene products, Fos and possibly, Jun, elicited by elevations in free cytosolic Ca$^{++}$ levels would stimulate DNA binding activity of the AP-1 transcription factor and cause transcription of α-1 subunit.

**Regulation by ouabain and ouabain-like substances**

The classical inhibitors of Na-K ATPase are the cardiac glycosides derived from the extracts of the plants of the genera Digitalis, Strophanthus and Urigenia i.e digoxin from the leaves of Digitalis lanata and ouabain from the seeds of Strophanthus gratus.

Several endogenous digitalis-like factors from various mammalian organs have been partially isolated and characterized. Many groups have proposed the existence of endogenous ouabain like substances. Rodriguez de Lores Arnaiz and Pena (1995) reported the isolation and subsequent purification of two peaks from the cerebral cortex, which stimulated and inhibited Na-K ATPase respectively. The peak II isolate was found to compete with ouabain binding in different brain areas (Antonelli *et al.*, 1991). Upon characterization, the endouabain or ouabain like substance was found to be susceptible to carboxypeptidase digestion and charring by acids. A cationic
component was found to be necessary for the maintenance of inhibitory activity, since EGTA and EDTA treatments abolished the inhibitory activity. Using HPLC columns to purify, it was found to be a non-peptide and non-lipidic in nature (Rodriguez de Lores Arnaiz and Pena, 1995). Recently, an endogenous digitalis like factor (EDLF) was extracted from human umbilical cord plasma which inhibited Na-K ATPase in the rat kidney, heart and brain and also competed with digoxin and ouabain for placental digitalis binding sites (Crambert et al., 1997). EDLF was found to inhibit α1 (in kidney) and α-2 (in brain and heart) rat isoforms with similar potency. It appeared to have no effect on rat brain α-3 isoform which is known to have the highest affinity for ouabain (Crambert et al., 1997).

Incubation of rat glial cultures with 1mM ouabain for two hours led to a stimulation of α1 and β1 and inhibition of α2 and β2 mRNA expression (Hosoi et al., 1997). This increase in α1 expression was mimicked by Na+ channel activator-monesin and inhibited by cycloheximide, intracellular Ca++ chelator-BAPTA-AM and calcineurin inhibitor- FK506 (Hosoi et al., 1997). This indicated the role of newly synthesized proteins, intracellular Ca++ and calcium calmodulin dependent phosphatase-calcineurin in stimulating α1 mRNA expression in glial cells.

**Regulation by lipid and membrane parameters**

The Na-K ATPase needs 100-200 molecules of phospholipid per molecule of enzyme and the activity can be modulated by the fluidity of the acyl chains of the lipids and is promoted by negatively charged head groups (Kimelberg and Papahadjopoulos, 1972). Swann (1982) showed that a step in the regulation of K+ phosphatase activity corresponding to dissociation of K+ and binding of ATP is sensitive to temperature and agents that alter membrane fluidity. The effect of age on the integrity and functioning of brain synaptosome was studied by comparing the lipid
composition, membrane fluidity, Na-K ATPase activity and susceptibility to in vitro lipid peroxidation in young, adult and old rats. An age related increase in synaptosomal free fatty acids, with no modification in acyl chain composition, a corresponding age-dependent decrease in membrane fluidity and a reduction of Na-K ATPase activity and overall greater susceptibility to in vitro lipid peroxidation was observed (Viani et al., 1991). Further, lipid peroxidation promoted strong modifications in the membrane fluidity, lipid composition and Na-K ATPase (Viani et al., 1991). In peroxidized membranes, the affinity for ATP and strophanthidin was increased (two and seven fold, respectively), whereas affinity for K+ and Na+ was decreased (to one tenth and one seventh of control values, respectively). Changes in the affinity of active sites would affect the phosphorylation and dephosphorylation mechanisms of Na- K+-ATPase reaction. An increased affinity for ATP favors the phosphorylation of the enzyme at low ATP concentrations whereas, decreased affinity for K+ will not favour the dephosphorylation of the enzyme-P complex resulting in unavailability of energy for transmembrane transport processes. Thus, lipid peroxidation alters Na- K+-ATPase function by modification at specific active sites in a selective manner, rather than through a non-specific destructive process. Several lysophospholipids (LPLs) and metabolites of the PLA2 type inhibited the synaptic membrane Na-K ATPase activity in a dose-dependent manner (Nishikawa, 1989).

**Regulation by Phosphorylation**

The short term regulation of the Na-K ATPase pump is still poorly understood although a phosphorylation/dephosphorylation regulatory mechanism via protein kinases and phosphatases has been suggested. Lingham and Sen (1982) showed that cAMP could inhibit the enzyme in crude rat brain membrane preparation. They
proposed that the mechanism involved membrane bound cAMP dependent protein kinase which phosphorylated a substrate protein which in turn regulates Na-K ATPase activity. The α-subunit of Na-K ATPase is found to serve both in intact cells and in subcellular fractions as a substrate for protein kinases. Phosphorylation of the enzyme by PKC and cAMP-dependent PKA in subcellular fractions has been described. With either kinase, phosphorylation is associated with an inhibition in the enzyme activity. Activation of PKC with phorbol ester- phorbol 12-myristate 3-acetate (PMA) resulted in a decrease in the activity of α1β1, α2β1 and α3β1 isozymes which were expressed in a recombinant baculovirus system in Sf-9 insect cells. Effect of PMA could be reversed by treating the cells with specific inhibitors of PKC, staurosporin and H7. Moreover, Na-K ATPase activity could be restored by treatment with protein phosphatase A (Blanco and Mercer, 1997). In another study, the cerebellar neurons showed an increase in Na-K ATPase activity in the presence of glutamate. This increase was blocked in the presence of PKC activator- phorbol esters and in the presence of calcineurin inhibitors- W-7 and cyclosporin. Thus the activation resulted from decreased phosphorylation of the enzyme (Marcaida et al., 1996). When phosphorylation studies were done in sciatic nerves with protein kinase modulators, it was observed that PKC activator phorbol 12-myristate 13-acetate (PMA) had little effect on the α-subunit 32-P labelling. In contrast, staurosporine, a PKC inhibitor and extracellular calcium omission decreased it. However, in calcium-free conditions, PMA restored the labelling to basal levels. The cAMP increasing agent forskolin reduced the 32-P labelling of the α-subunit. This led the authors to conclude that nerve Na-K ATPase is tonically phosphorylated by PKC in a calcium-dependent manner and PKA modulates the phosphorylation process (Borghini et al., 1994).
Phosphorylation by PKA has been mapped to Ser943 for the rat α-1 isoform, while PKC phosphorylates the polypeptide on Ser16 and Ser23. Ser943 is conserved in the α2 and α3 polypeptides, however, Ser16 is not and Ser23 is present in α1 and α3. Thus, the effect of PKA and PKC activators may depend on phosphorylation of additional groups on the α polypeptides, on differential modifications of the structure and hence function of the proteins, or on the involvement of another second messenger(s). As the Na-K ATPase isoforms have unique kinetic characteristics (Blanco et al., 1995), the differential modulation of their activity may be important in adapting Na-pump function to specific cellular requirements.

**Regulation of Na-K ATPase by monamines**

Several factors, including hormones, regulate the activity of Na-K ATPase by two distinct mechanisms: i) induction of Na-K ATPase gene expression and ii) post translational modifications of the enzyme from pre-existing pool. It has been observed that the steroid hormones (aldosterone, corticosterone, cortisol etc.) whose action is mediated by DNA-binding, mainly control the transcriptional activity of the Na-K ATPase gene, and to a lesser extent the processing and stability of corresponding mRNAs and their translation (Rossier et al., 1987). However, other hormones including polypeptides (insulin, glucagon, catecholamines) act on pre-existing pumps by modulating their activity (Rossier et al., 1987).

NE can hyperpolarize a variety of excitable tissues including neurons in various regions of the central nervous system. As early as 1967, Nishi and Koketsu observed that slow inhibitory postsynaptic potentials (SIPSP) of sympathetic ganglia were associated with an electrogenic Na⁺ pump because of a highly specific action of ouabain on the SIPSP. A possibility of an interaction between NE and the Na⁺ K⁺ pump was also suspected. Herd et al (1970) reported that NE stimulated the activity
of Na-K ATPase in rat brown adipose tissue membranes. In relation to the CNS, Clausen and Formby (1967) reported that the presence of NE enhanced the activity of Na-K ATPase. Schaefer et al. (1972, 1974) were the first to demonstrate that catecholamines could stimulate particle-bound Na-K ATPase from rat brain synaptosomal membranes in the presence of soluble fraction only. Later, it was shown by many investigators that the activity of the ouabain sensitive Na-K ATPase was enhanced in brain homogenates and microsomal fractions (Hernandez, 1992). These isolated studies were done in either homogenate, microsome, nerve ending particles or synaptosome, but a comparison of microsomal and synaptosomal Na-K ATPase activities from the same animal was not available.

Phillis and Wu (1981) discovered that the Na-K ATPase inhibitors like ouabain and azide antagonized the depressant action of NE on the cerebral cortical neurons. Hence, it was hypothesized that NE induced hyperpolarizing action by stimulating the Na-K ATPase.

2.19 MECHANISM OF AMINERGIC STIMULATION OF Na-K ATPase

Apart from the in vitro studies, noradrenergic stimulation in vivo has also been found to increase Na-K ATPase activity. An increase in the enzyme activity in the rat brain was observed after electrical stimulation of the LC and after administration of piperoxane. The increase due to administration of piperoxane was prevented when the noradrenergic fibres from the LC were destroyed with 6-hydroxy dopamine (6-OHDA) (Swann et al., 1981; Swann, 1983).

Desaiah and Ho (1977) described a dose-dependent increase of the Na-K ATPase by NE in mice brain synaptosomal preparations. These authors reported a change in the enzyme kinetics, producing an increase in $V_{\text{max}}$. Many mechanisms have been proposed to explain the aminergic activation of Na-K ATPase (Phillis and Wu,
Hexum (1977) proposed that the activation of the enzyme by NE could be due to the removal of divalent metal ions through the formation of a catecholamine-metal complex, since Na-K ATPase activity is inhibited in vitro by such biologically significant divalent metal ions as Ca++, Cu++, Zn++ and Fe++. Godfraind et al (1974) observed that the effect of NE upon Na-K ATPase activity was the same as that produced by EGTA. Thus, it was proposed that Ca++ inhibition of the enzyme was neutralized by NE, leading to an increase in Na-K ATPase. NE could be neutralizing the inhibition by chelating Ca++. Sulakhe et al (1977) showed that catecholamines stimulate the Na-K ATPase activities in hypothalamic, cortical, cerebellar, caudate nucleus and superior cervical ganglia homogenates of rat and rabbit. However, Wu and Phillis (1979) demonstrated that although 10⁻⁴ M EDTA has a maximal stimulatory effect on Na-K ATPase, the addition of 10⁻⁵ M NE to an enzyme preparation containing 10⁻⁴ M or 10⁻³ M EDTA can further enhance enzyme activity. Thus, the removal of divalent metal ion inhibition can only partially account for catecholamine stimulation of Na-K ATPase. Swann and Steketee (1989) suggested that NE regulates the number of ouabain binding sites and cAMP also has a significant role in its regulation. Forskolin (stimulator of adenylate cyclase) was also able to increase the ouabain binding sites.

The other proposed mechanism for catecholamine induced stimulation of Na-K ATPase is the involvement of α-adrenergic receptor (Phillis and Wu, 1981). The first indication of the involvement of a receptor was given by studies of Herd et al. (1970) who reported that in brown adipose tissue, the stimulation of Na-K ATPase by NE could be blocked by propranolol, a β adrenoreceptor antagonist. Iwangoff et al. (1974) were able to demonstrate antagonism of NE stimulation of Na-K ATPase with phentolamine, an α adrenoreceptor antagonist. Yohimbine is known to increase NE
by inhibiting \( \alpha-2 \) presynaptic adrenoceptors. Swann (1983) observed stimulation of Na-K ATPase and increase in ouabain binding, by repeated yohimbine treatment. The effects of selective \( \alpha-1 \) and \( \beta \) noradrenergic antagonists were studied on yohimbine stimulated increase in Na-K ATPase. Prazosin was found to prevent the increase in enzyme activity. So, it was concluded that NE binds to \( \alpha-1 \) receptors for increasing the enzyme activity. Na-K ATPase did not seem to be directly coupled to the receptor, since changes in enzyme activity or its sensitivity to acute stimulation does not parallel changes in receptor number (Swann, 1983). Stimulation could therefore, be indirect, with secondary to \( \alpha-1 \) receptor mediated changes in phospholipid turnover or fluidity. Later, a number of studies reported antagonism with both \( \alpha \) and \( \beta \) adrenoreceptor antagonists. L-norepinephrine was found to be more potent than the D-form in eliciting pharmacological responses. Studies with receptor antagonists showed that whereas the activation of Na-K ATPase, induced by L-noradrenaline could be antagonized by phentolamine or propranolol, that evoked by D-noradrenaline could not be blocked by these antagonists (Phillis and Wu, 1981). NE is proposed to act through \( \alpha-1 \) adrenoceptor, which on stimulation causes phosphoinositide hydrolysis. Calcium seems to play an important role as removal of extracellular calcium ions with EGTA blocked the receptor mediated phosphatidylinositol turnover (Gusovsky et al., 1986).

Thus, although a receptor mediated mechanism has been proposed, the possibility of a non-receptor action cannot be ruled out. These suggest a possibility that two disparate mechanisms are operating in the catecholamine stimulation of Na-K ATPase. The two mechanisms- receptor and non-receptor may ultimately act synergistically or involve a common pathway.

A non-stereospecific activation appears to be involved in action of agents like D-noradrenaline, since the effect is not blocked by \( \alpha \) or \( \beta \) antagonists. The
mechanism involved in this action is likely due to non-receptor mediated effects such as chelation of inhibitory metal ions and antilipoperoxidation. High concentrations of noradrenaline and isoprenaline elicit this kind of response as it was not blocked by phentolamine or propranolol. However, at low concentrations, the stimulation by NE could be blocked by phentolamine or propranolol (Phillis and Wu, 1981). The NE induced increase in Na-K ATPase activity in rat brain microsome was blocked by \(\alpha\)-1 adrenoceptor blocker- prazosin and not with \(\beta\)-adrenoceptor blocker- propranolol (Gulyani and Mallick, 1995).

Thus, more than one mechanism may be functional in eliciting this response. It is thus hypothesized that NE causes hyperpolarization of the cortical neurons by stimulating the activity of Na-K ATPase. Thus, the stimulation of the pump causes hyperpolarization which is associated with an increase in membrane resistance. This could account for the depression of neuronal spontaneous firing and signal to noise ratio enhancing action of these amines. Inhibition of this pump would lead to depolarization and thus enhanced excitability (Phillis, 1992).

Since the presence of ouabain-like compounds have been proposed to modulate Na-K ATPase activity, the effect of 6-OHDA on brain and plasma ouabain-like compound (OLC) levels and brain catecholamine levels were studied. In centrally sympathectomized rats, hypothalamic OLC content and plasma OLC level were found to be significantly decreased, in accordance with reduced brain NE content. On the other hand, peripheral sympathectomy did not affect plasma OLC level. These findings suggest that central adrenergic neurons may be involved in the synthesis and/or release of circulating OLC (Yamada et al., 1995) and may thus regulate Na-K ATPase activity.

Schaefer et al (1972) showed that NE induced increase in Na-K ATPase activity could be elicited only in the presence of soluble fraction. They proposed that
NE increased the Na-K ATPase activity by inhibiting the membrane lipid peroxidation caused by cations; the effect being compounded by the presence of ascorbic acid in the soluble fraction (Schaefer et al., 1974).

Aperia et al (1992) showed an increase in Na-K ATPase activity in the rat renal tubules in the presence of α-adrenergic agonist, oxymetazoline. This activation was prevented by prazosin and was mimicked by calcium ionophore A23187. The agonist induced increase was blocked in the presence of specific peptide inhibitor of a calcium calmodulin dependent phosphatase -calcineurin. Dephosphorylation of the enzyme was proposed as a mechanism of activation of Na-K ATPase by NE or oxymetazoline.
SUMMARY OF REVIEW OF LITERATURE

♦ Sleep-wakefulness can be divided into two distinct stages-NREM and REM sleep.

♦ REM sleep is characterized by EEG desynchronization, muscle atonia, hippocampal theta rhythm, PGO waves, rapid eye movements and muscle twitches.

♦ Distinct neuronal groups in the pons, LDT-PPT, LC are responsible for generation and maintenance of REM sleep phase.

♦ ACh and NE are important neurotransmitters, whose interaction plays an important role in the generation and maintenance of REM sleep. Other neurotransmitters like dopamine, serotonin, GABA etc are known to modulate REM sleep.

♦ REM sleep deprivation is one of the important ways to investigate the function(s) of REM sleep.

♦ The flower-pot technique has been most widely used to investigate the effects of REM sleep deprivation.

♦ One of the basic function of REM sleep is to maintain brain and neuronal excitability.

♦ Membrane bound Na-K ATPase is one of the factors which is known to regulate the transmembrane potential and thus, the neuronal excitability.

♦ REM sleep deprivation is likely to increase NE, which is known to increase Na-K ATPase. Hence REM sleep deprivation induced increase in Na-K ATPase activity may be mediated by NE.

♦ Na-K ATPase, a heterodimer consisting of α and β subunits, belongs to E1/E2 class of transporting proteins.
The α and β subunits are found to exist in multiple isoforms, which show distinct developmental and tissue specific regulation.

Na-K ATPase has been shown to be regulated by cations, ouabain-like substances, lipid components and phosphorylation. Hormones like NE are also known to regulate Na-K ATPase activity.

At the cellular level, the NE induced increase in the enzyme activity is proposed to be mediated by various mechanisms:
- disinhibition by divalent cations
- involvement of α, β or both the adrenoceptors
- inhibition of lipid peroxidation
- regulation of synthesis and / or release of circulating ouabain-like compounds.

LACUNAE

i) The molecular mechanism of REM sleep deprivation induced NE mediated increase in Na-K ATPase activity was not known.

ii) The role of Ca++ in NE mediated increase in Na-K ATPase activity was not known.

iii) The change in kinetics of Na-K ATPase after REM sleep deprivation was also not known.
OBJECTIVES

The objectives of this study were to investigate:

- the effect of REM sleep deprivation on synaptosomal Na-K ATPase activity in whole brain and in brain areas; and its comparison with microsomal Na-K ATPase activity.
- the effect of NE on synaptosomal Na-K ATPase activity.
- the mechanism of REM sleep deprivation induced NE mediated increase in synaptosomal Na-K ATPase activity, which included
  - kinetics of synaptosomal Na-K ATPase after REM sleep deprivation, NE and Ca²⁺ treatment.
  - involvement of second messenger, if any, in NE induced increase in Na-K ATPase activity
  - comparison of lipid peroxidation after REM sleep deprivation and NE treatment.