DISCUSSION...
In this part, the significance and relevance of the results obtained in this study have been discussed in relation to existing knowledge in the field. First, the focus of the study, the rationale for choosing the brain areas and the REM sleep deprivation method has been discussed followed by discussion of the results obtained along with their significance.

6.1 FOCUS OF THIS STUDY:

It was known that REM sleep deprivation caused aggressiveness, irritability, confusion, lack of concentration etc. It was proposed that all these could possibly be due to alteration in brain excitability. Further, as a mechanism of action at the cellular level it has been reported that REM sleep deprivation increased Na-K ATPase activity (Gulyani and Mallick, 1993) but reduced membrane fluidity (Mallick et al., 1995). It was known that Na-K ATPase is required to maintain the osmotic balance within the cells and to stabilize cell volume (Alberts et al., 1994). Since, the activity of Na-K ATPase increased and the membrane fluidity decreased after REM sleep deprivation, it was proposed that deprivation might affect the size and morphology of neurons. Hence, the effects of REM sleep deprivation was investigated on the perimeter and area of neurons in four brain areas viz locus coeruleus (LC), laterodorsal tegmentum/ pedunculopontine tegmentum (LDT/PPT), medial preoptic area (mPOA) and lateral septum. It had been reported that cell death is preceded by alterations in cell size and shape. Sizes of cells have been reported to increase in necrosis and decrease in apoptosis (Wyllie et al., 1980). Therefore, neurons from deprived and control brain areas were studied under the electron microscope. Since the electron micrographs of neurons after deprivation showed signs of neuronal damage, further studies were conducted through deprived and control brain areas by staining degenerated cells with amino-cupric-silver, bcl2/bax and TUNEL techniques. The structural proteins are responsible for maintaining the neuronal structure, size and shape.
Any change in the size of neurons is likely to affect them and vice versa. Moreover, there are reports that during apoptotic process, the cytoskeletal proteins are cleaved by caspases (Yin and Stull, 1999). Hence, the structural protein actin was immunohistochemically stained and quantified densitometrically in control and REM sleep deprived rat brain neurons.

6.2 RATIONALE FOR CHOOSING THE BRAINAREAS FOR THIS STUDY:

Four brain areas viz. locus coeruleus (LC), laterodorsal tegmentum and pedunculopontine tegmentum (LDT/PPT), medial preoptic area (mPOA) and lateral septum were selected for this study. Earlier studies reported that REM sleep is generated within the brainstem particularly in the pons. An interaction between the cholinergic REM-ON neurons in the LDT/PPT and the noradrenergic REM-OFF neurons in LC for the generation of REM sleep had been proposed (Hobson and McCarley, 1975; Sakai, 1981). Several reports suggested that GABA in LC and LDT/PPT plays an important role in the regulation of REM sleep (Mallick et al., 1999). Hence, noradrenergic, cholinergic and GABAergic neurons in LC and LDT/PPT were investigated in this study. The third area chosen was mPOA, that is known as a sleep inducing area (Mallick et al., 1992). However, it is not directly related to REM sleep though it also receives projections from LC neurons (Osaka and Matsumura, 1994). The fourth area was lateral septum that has no apparent relation with sleep and wakefulness. This area was taken as a control to study if the effects observed were specific to neurons located only in REM sleep related areas or generalized effects across the brain. All these areas were first nissl stained and the cell sizes estimated. Then the neurons in LC, LDT/PPT and mPOA were stained with antibody specific to enzymes responsible for synthesis of NE, ACh and GABA, respectively and cell size as well as the synthesizing enzyme concentrations were estimated. Finally,
neuronal degeneration and actin concentration were studied in all these areas after staining with the respective antibodies.

6.3 REM SLEEP DEPRIVATION:

In order to study the effects of REM sleep on any physiological parameter, two approaches may be used i.e. either by increasing or by decreasing the amount of REM sleep. The former is difficult to attempt as different parts of the brain are involved for REM sleep generation and stimulation of all these areas simultaneously for increasing REM sleep is nearly impossible. Further, when REM sleep is induced using chemicals or by electrical stimulation, it often does not exhibit all the classical signs and hence is termed as “REM sleep like state”. The techniques would also be invasive as they would involve surgical implantations of cannula and electrodes and would result in mechanical damage to the brain tissue. Added to this, in nature, the amount of a physiological event has evolved to an optimum level after a great deal of trial and error. An increase in the phenomenon (REM sleep in this case) by artificially stimulating the brain may cause concomitant changes in other physiological processes by the activation of diverse and multiple neuronal circuits. It will therefore become difficult to segregate the changes due to an increase in REM sleep from those due to stimulation of neuronal circuits of other physiological phenomena. Hence, experimental study by increasing REM sleep may not be an effective approach.

On the other hand, the deprivation or the so-called negative approach has the advantage of overcoming the drawbacks of the positive approach mentioned above. The major advantages are that it is a noninvasive method, the parameters under study, if related to REM sleep, are likely to be amplified proportional to the period of deprivation, and most important that REM sleep loss (to different degree) is experienced by almost
everybody. Hence, it can be considered a near natural phenomena. However, the experimental approach to REM sleep deprivation suffers from the drawback that a certain amount of stress and loss of slow wave sleep is unavoidable. This can be overcome by the use of appropriate and effective controls.

REM sleep deprivation was carried out in this study by the classical flowerpot method (Jouvet et al., 1964). This technique induces almost total REM sleep deprivation without significantly affecting other stages, it does not involve any special gadget, suitable controls can be designed to rule out the effects of stress and other non-specific effects (large platform control) and many experimental as well as control rats can be used simultaneously. Nevertheless, this method is not suitable for REM sleep deprivation for 24 hours or less since 24 hours large platform control animals are also deprived of REM sleep to a significant extent as the experimental animals. However, beyond 24 hours, this method is very effective for deprivation and has been widely used in rats (Mendelson et al., 1974; Hicks et al., 1977; Oniani et al., 1988) and cats (Jouvet et al., 1964; Mallick et al., 1989). This method has been standardized and successfully used in this lab in rats for quite sometime (Gulyani et al., 2000; Mallick et al., 2002).

In the present study, for estimating the size of neurons the rats were subjected to 6 days of REM sleep deprivation. For neuronal degeneration, no baseline data was available where the effect of different days of REM sleep deprivation on neuronal damage was reported. Lack of suitable literature for REM sleep deprivation associated neuronal damage necessitated standardization of the number of days for which the animal was to be deprived of REM sleep. Previously it had been reported from this lab that after 4 days of REM sleep deprivation the activities of various enzymes in the brain were altered (Gulyani et al., 2000). Therefore, for this study rats were deprived for 4, 6 and 10 days and neuronal damage in LC, LDT/PPT, mPOA and lateral septum was assessed after amino-cupric-
silver staining. It was observed that compared to free moving control, after 4 days of REM sleep deprivation, the neuronal degeneration did not consistently increase in any of the areas to make it statistically significant. However, after 6 and 10 days the neuronal degeneration was significantly higher in all areas except lateral septum compared to free moving control. Since, it was not desirable to subject the rat to unwanted deprivation but at the same time to subject them to minimum deprivation to get significant effect on the parameter to study, it was decided to continue further experiments after 6 days of REM sleep deprivation. Suitable large platform control (for 6 days) and recovery for 3 days after 6 days of deprivation were also carried out.

6.4 EFFECTS OF REM SLEEP DEPRIVATION ON CELL SIZE AND NEUROTRANSMITTER SYNTHESIZING ENZYME CONCENTRATION:

The effects of REM sleep deprivation were investigated on nissl stained and immunostained neurons in LC, LDT/PPT, mPOA and lateral septum. Nissl staining was done in all the four areas in free moving control (FMC), REM sleep deprived (REMSD), large platform control (LPC) and recovery (REC) group of rats. Additionally, cell size was estimated after the rats were injected (i.p.) with α-1 adrenoceptor blocker prazosin, during REM sleep deprivation. The enzymes stained immunohistochemically were tyrosine hydroxylase (TH, enzyme required for biosynthesis of NE) in LC, choline acetyl transferase (ChAT, enzyme for acetylcholine biosynthesis) in LDT/PPT and glutamic acid decarboxylase (GAD, enzyme for synthesis of GABA) in LC, LDT/PPT and mPOA. The sizes of the neurotransmitter specific neurons were measured and the synthesizing enzyme content estimated densitometrically. The results from these experiments have been discussed below.
6.4.1(a)  **Effects on cell size in LC:**

The neurons in the LC increased in size in nissl stained sections. TH antibody, that specifically stained noradrenergic neurons, revealed that TH positive neurons also showed a similar increase in size after REM sleep deprivation. However, a few larger sized neurons could be identified in TH stained sections that were not observed in nissl stained sections in all four groups. No explanation could be found for this observation. By following a similar approach using GAD it was found that the GABAergic neurons also increased in size in LC. However, they were smaller in size as compared to the TH stained neurons. The frequency distribution histograms also revealed that most of the GABAergic neurons in all four groups were found in bins of smaller size, which indicated that they were smaller in size. This is supported by other reports in which GABAergic neurons have been proposed to act as interneurons in LC that lie within the majority of noradrenergic neurons (Ijima and Ohtomo, 1988; Jones, 1991).

The size of the neurons in LPC did not increase as compared to FMC. This indicated that the effects were specific to REM sleep deprivation and not due to any non-specific factors. The increase in cell size also returned to FMC levels after recovery. Thus, the effects were not permanent and could be reversed. An interesting observation was that the increase in LC neuronal size after deprivation was not observed if the effects of norepinephrine (NE) were blocked by injection (i.p.) of prazosin during the deprivation period. The size of these neurons was comparable to FMC neurons. These results suggest that NE plays a significant role in increasing the cell size after REM sleep deprivation. During REM sleep deprivation, the noradrenergic neurons in LC have been reported to fire continuously (Mallick et al., 1989) and NE levels have been reported to be elevated after 72 hours of REM sleep deprivation (Porkka-Heiskanen et al., 1995). Thus, there would be production of excess NE during deprivation. Hence, the increase in cell size observed after
deprivation in LC may be due to the increased levels of NE. This may further be supported by the fact that, NE has been reported to induce hypertrophy of cultured rat myocardial cells through alpha-1 adrenoceptors (Simpson et al., 1983) and in neonatal cardiac myocytes (Zheng et al., 1995). Additionally, it is known that, increased activity of the noradrenergic neurons removes the feedback inhibition on TH production by NE which may lead to increased transcription of TH enzyme’s gene (Kandel, 2000). This would in turn lead to increased production of TH enzyme molecules and consequently increased norepinephrine synthesis and release. The increase in TH enzyme concentration was also observed in our study as discussed below.

6.4.1(b) Effect on neurotransmitter synthesizing enzyme concentration in LC:

This study showed that TH and GAD concentrations increased in LC neurons after REMSD as compared to FMC. No such increase was observed in the LPC rats. The increase in the concentration of TH and GAD returned to the basal levels after recovery from REMSD. Increase in the TH concentration in the LC neurons suggests that there was an increase in the synthesis of NE in the LC neurons after REM sleep deprivation. The findings provide explanation at the molecular level for possible mechanism of sustained increase in NE concentration in the brain after REM sleep deprivation (Porkka-Heiskanen et al., 1995). It also confirms earlier findings that REM sleep deprivation increased turnover of NE (Pujol et al., 1968) and TH-mRNA (Porkka-Heiskanen et al., 1995; Basheer et al., 1998) in the brain. The results of this study also support previous observations that low frequency long term stimulation of LC neurons, that presumably increased release of NE in the brain, induced REM sleep deprivation like state (Singh and Mallick, 1996).
The increased GAD concentration in the LC neurons after REMSD suggests that there is likely to be increased GABA synthesis in LC neurons. The findings confirm earlier reports that GABA levels increased in LC during REM sleep (Nitz and Siegel, 1997) and GABA in LC increased while its blocker reduced REM sleep (Kaur et al., 2001; Mallick et al., 2001). Since, GABA is reported to inhibit the noradrenergic LC neurons (Gervasoni et al., 1998), it is likely that increased GABA inhibits REM-OFF neurons for REM sleep regulation. This may explain the observation that after REM sleep deprivation although there was continuous firing of the REM-OFF neurons (Mallick et al., 1989) unlike cessation of firing of those neurons during spontaneous REM sleep (Aston-Jones and Bloom, 1981) in the LC, the firing rate was lower than that during awake period. Thus, it is possible that during REM sleep deprivation an increased concentration of GABA tries to reduce the firing of the noradrenergic neurons possibly to withstand the adverse effects of deprivation.

6.4.2(a) Effect on cell size in LDT/PPT:

Nissl and ChAT immunostaining of sections through LDT/PPT revealed that the neuronal size decreased after REM sleep deprivation. However, GAD immunostaining showed that there was an increase in the size of GABAergic neurons in LDT/PPT after REM sleep deprivation. The decrease in size in ChAT positive neurons and increase in size in GAD positive neurons were reversed after recovery and after prazosin injection. Grouping of the cells into bins according to cell size revealed that some large sized neurons observed among the ChAT stained ones in all four groups were not observed in the nissl stained preparations. A valid explanation for this observation could not be found. The GABAergic neurons in the LDT/PPT also belonged to the smaller size groups. This is
supported by a recent report that GABAergic neurons act as interneurons along with the predominant cholinergic neurons in the LDT/PPT (Torterolo et al., 2001).

During REM sleep deprivation, the firing rate of the cholinergic REM-ON neurons decrease while the noradrenergic REM-OFF neurons in LC fire continuously (Mallick et al., 1989). The noradrenergic LC neurons have been reported to activate the GABAergic neurons in the LDT/PPT and those in turn are likely to keep the REM-ON neurons inhibited (Mallick et al., 2001). During REM sleep deprivation the REM-OFF neurons are continuously activated and they in turn continuously activate the GABAergic neurons in LDT/PPT. The GABAergic neurons are likely to continuously inhibit the REM-ON neurons resulting in reduced firing rate of the REM-ON neurons during REM sleep deprivation (Mallick et al., 1989). It has been reported that when neurons are in a relative inactive state, the $\text{Na}^+$ concentration inside the neurons remains low (Kandel, 2000). A low firing rate would also mean that the $\text{Na}^+$ concentration inside the cells would be lower than in case when the cells are continuously firing. If the activity of the cell has been suppressed for long, the $\text{Na}^+$ concentration may become very low. Low intracellular $\text{Na}^+$ levels have been reported to cause shrinkage of cells (Bortner et al., 1997). Since GABAergic neurons inhibit the activity of REM-ON neurons during deprivation, the decreased $\text{Na}^+$ concentration inside these cells would explain the cell shrinkage observed in these cells.

6.4.2(b). *Effect on neurotransmitter synthesizing enzyme concentration in LDT/PPT*:

Since REM-ON neurons show opposite behavior than those of REM-OFF neurons, it was expected that the enzyme concentration would also show opposite behavior than that in the LC-neurons. The present findings do not indicate the same as in LDT/PPT.
neither ChAT nor GAD concentration was affected in the neurons after REM sleep deprivation. A slight decrease in the ChAT concentration was observed in LDT/PPT (the site of REM-ON neurons) however it was not significant. It is possibly because during REM sleep deprivation continuous activity of the LC neurons would increase NE release that would activate the GABA-ergic neurons in the LDT/PPT and they in turn would keep the REM-ON neurons inhibited as proposed recently (Mallick et al., 2001). This proposed model may be supported by the fact that there was a slight increase in GAD levels in LDT/PPT neurons after deprivation but it was not statistically significant. Therefore, it is likely that increased NE in LC would increase GABA synthesis in the neurons that would keep the activity of REM-ON neurons low leading to reduced acetylcholine levels in the projected areas.

6.4.3(a)  *Effect on cell size in mPOA:*

In the mPOA, nissl staining showed a decrease in the neuronal size after REM sleep deprivation. GAD immunostaining showed a similar decrease in size after deprivation. It is evident from the frequency distribution histograms that both the nissl stained and GAD stained neurons represented the same population, hence they are likely to be the same neurons. However, neither TH nor ChAT was studied in mPOA as previous reports suggested that this area lacks ChAT stained neurons (Gritti et al., 1994) and only a few fiber terminals have been found to be TH positive (Simerly et al., 1986). GAD was studied in mPOA because GAD stained neurons have been reported in this area (Gritti et al., 1994) and GABA antagonist picrotoxin in this area has been found to affect REM sleep (Ali et al., 1999).

The decrease in the cell size in mPOA after REMSD was reversed after the recovery from REMSD. Also there was no decrease in cell size when prazosin injections
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(i.p.) were given to the rats during deprivation indicating a possible role of NE in regulating their cell size. The noradrenergic neurons in LC have been shown to have projections to the mPOA (Osaka and Matsumura, 1994). The continuous firing of the REM-OFF neurons may inhibit the sleep active neurons in the mPOA resulting in increased wakefulness. The decreased firing of the neurons in mPOA might decrease the Na\(^+\) concentration of these cells resulting in cell shrinkage as mentioned in case of the neurons in LDT/PPT.

6.4.3(b) Effect on neurotransmitter synthesizing enzyme concentration in mPOA:

The concentration of GAD in mPOA showed a non-significant decrease after deprivation. This may be because this area is not directly related to REM sleep.

6.4.4(a) Effect on cell size in lateral septum:

There was no significant difference between the neuronal sizes in all the five conditions (FMC, REMSD, LPC, REC and PRZ) in the lateral septum. Only nissl staining was done in the lateral septum. Immunostaining was not done as no ChAT or GAD immunoreactive cells have been reported to be present in the lateral septum (Gritti et al., 1993). The results obtained from the lateral septum show that the effects of REM sleep deprivation observed in LC, LDT/PPT and mPOA are not generalized effects observed throughout the brain, rather they are localized to areas related to REM sleep regulation.

6.4.5 Other studies on effect of sleep deprivation on cell size:

Very few reports have investigated the effects of sleep/REM sleep deprivation on neuronal structure. The results of the small number of reports that are available are contradictory. Bubenik et al. (1972), studied the nuclear size variations in cells of locus
coeruleus during sleep, arousal and sleep deprivation and found them to be larger during arousal and sleep deprivation as compared to sleep. Pompeiano et al. (1995) coupled sleep deprivation with monocular deprivation in kittens to study the effects on plasticity of neurons in the lateral geniculate nucleus (LGN) in kittens. Similar experiments were also performed by Oksenberg et al. (1996) and Shaffery et al. (1998, 1999) in which it was reported that when REM sleep deprivation was combined along with monocular deprivation, the decrease in cell size in the segment of LGN receiving input from the closed eye was much more than only after monocular deprivation. These studies provide support to our results and suggest that REM sleep deprivation affects the size and morphology of the neurons.

6.4.6 Role of Na-K ATPase in regulating cell size:

It was known from our previous studies that REM sleep deprivation alters membrane fluidity (Mallick et al., 1995) and neuronal excitability (Mallick et al., 1999). One of the possible reasons for increase in neuronal excitability after REMSD, could be an increase in activity of the enzyme Na-K-ATPase (Gulyani et al., 1993). Na-K ATPase is crucial for maintaining the transmembrane potential (Trachtenberg et al., 1982). It is also known that Na-K ATPase is required to maintain the osmotic balance within the cells and stabilize cell volume (Alberts et al., 1994). The importance of Na-K ATPase in controlling cell volume is indicated by the fact that when treated with ouabain, animal cells swell and sometimes burst (Glynn, 1985). Inhibition of Na-K ATPase by ouabain has also been reported to induce swelling in cells in neuroblastoma cell line CHP-100 (Basavappa et al., 1998). Other studies have also shown that there is a link between neuronal excitability and changes in size of cells. Kuppermann et al. (1983) reported that in the monocular deprivation model in cats, injection of tetrodotoxin into the eye blocks the tonic firing of
the retinal ganglionic cells and causes changes in the size of the lateral geniculate cells. Thus, the effect of altering the neuronal excitability is seen on the size of cells. Since both Na-K ATPase and membrane fluidity are altered with REM sleep deprivation, it is possible that the continuously firing REM-OFF neurons and increased NE during REM sleep deprivation affect the excitability status of the neurons that receive projections from LC which in turn alters the size of these neurons. Thus, both the ionic balance inside the cells and the membrane fluidity are altered by REM sleep deprivation and affect the size of the neurons in the areas that are involved in REM sleep generation.

6.5 EFFECT OF REM SLEEP DEPRIVATION ON NEURONAL DAMAGE:

It was reported that alterations in the size and shape of cells precede cellular degeneration (Wyllie et al., 1980). Therefore, studies were undertaken in which neuronal structure was studied using electron microscopy. Since it was observed that after deprivation, neurons exhibit signs of degeneration, the control and deprived brain sections were stained using amino-cupric-silver technique. Though amino-cupric-silver technique stains neurons undergoing degeneration, it does not distinguish between apoptosis and necrosis. Further, to distinguish apoptosis from necrosis, brain areas were immunostained for bcl-2 (an anti-apoptotic protein) and bax (a pro-apoptotic protein). Finally, cells were stained with TUNEL technique that stains nuclei having nicks in the DNA as found in the apoptotic cells. The amino-cupric-silver technique was performed in four areas viz. LC, LDT/PPT, mPOA and lateral septum. The rest of the studies were conducted in LC, LDT/PPT and mPOA.
6.5.1 *Electron microscopy*:

Electron microscopy was done in brain areas of FMC, REMSD, LPC and REC rats. In the electron micrographs of all the three areas studied viz. LC, LDT/PPT as well as the mPOA the neurons showed characteristic features of apoptosis like chromatin condensation and accumulation near the nuclear membrane, membrane blebbing and disorganization of the cell organelles after REM sleep deprivation. In FMC and LPC the neurons showed clearly demarcated nuclei with nucleoli having no chromatin condensation or any other apparent signs of degeneration. In REC, some of the neurons were normal while others showed signs of degeneration.

As discussed before, in the neurons in LC, LDT/PPT and mPOA the neuronal size was seen to alter after REM sleep deprivation. The increased levels of NE in the LC and in the projected areas like LDT/PPT and mPOA after deprivation may be responsible for the changes in firing rate of the neurons in these areas. It was reported earlier that cell death is preceded by changes in cell size (Wyllie et al., 1980). Probably, when the cell passes through certain conditions that are detrimental to its survival, it tries to compensate by altering its size. However, in case of extreme insults, the compensatory mechanisms fail and the cell undergoes degeneration. In this study as discussed before, the ionic balance is altered in both REM-OFF neurons that are continuously active as well as in the REM-ON and in mPOA neurons that are inhibited. This alteration in ionic balance may lead to change in cell size in the initial stages and subsequently cell death. In the electron micrographs, after deprivation, most of the neurons observed showed signs of degeneration. However after recovery, some neurons were degenerated while others were normal. It is possible that during the deprivation process, some of the neurons try to compensate for the changes in their firing rates as discussed earlier and try to recover. If the animal was allowed to recover, such neurons also recovered. However, other neurons
that could not withstand the detrimental effects, died and were observed as damaged neurons.

Electron microscopy is an effective tool to study the internal structures of cells. However, it is not possible to estimate the percentage of normal or degenerated cells using this technique. For this reason, other techniques that stain degenerated neurons were applied to study neuronal degeneration after deprivation.

6.5.2 Amino-cupric-silver technique:

This technique is based on the principle that degenerating neurons have increased affinity for binding with silver or to become argyrophillic (de Olmos et al., 1994). Moreover it stains neurons that undergo cellular reactions rather than cellular degeneration, hence some of the neurons might recover back. Using this technique, it was found that in all three areas viz. LC, LDT/PPT and mPOA a higher percentage of degenerated neurons was present after REMSD as compared to FMC and LPC. However, in the lateral septum there was no significant increase in percentage of degeneration as compared to FMC. After recovery, the percentage of degenerated neurons was not found to be significantly high as compared to FMC.

This technique has a disadvantage that some of the normal cells particularly the nuclei also stained along with the degenerated ones (de Olmos et al., 1994). Hence, as compared to the FMC of other staining procedures, many neurons in FMC stained after amino-cupric-silver technique. However, after REM sleep deprivation, the percentage of amino-cupric-silver stained neurons were significantly higher than FMC. The percentage of degenerated neurons after REC was comparable to FMC indicating that some of the neurons still had the capacity to recover. As discussed earlier, the REM-OFF neurons in LC continuously fire and REM-ON neurons in LDT/PPT and sleep active neurons in
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mPOA are inhibited during deprivation. This might lead to excess activity and synthesis of neurotransmitter in one group and forced inactivity in the other. Both the conditions are detrimental for the survival of neurons. Hence, in the initial stages, there might be change in cell size probably as a compensatory mechanism, which may lead to cell death in the later stages.

However, amino-cupric-silver does not distinguish between apoptosis and necrosis. There is hardly any technique to study necrosis as compared to apoptosis for which numerous techniques are available. Therefore, it was necessary to study apoptotic specific techniques and confirm if the neurons after deprivation are undergoing apoptosis. For this, immunostaining with bcl-2/bax and TUNEL technique was utilized.

6.5.3 bcl-2/bax immunostaining:

Bcl-2 is an anti-apoptotic protein that is present on the mitochondrial and nuclear membranes and prevents the activation of caspases and the apoptotic process. Bax is a pro-apoptotic protein that is normally present in the cytoplasm. In the event of cellular damage, bax is translocated into the mitochondrial membrane and allows the release of cytochrome-c from the mitochondria that activates caspases leading to cell death. By studying the relative percentage of bcl-2 and bax expressing neurons, the extent of degeneration can be ascertained.

In this study, the percentage of neurons staining for bcl-2 were found to be higher in FMC while the percentages of bax stained neurons were higher after deprivation in all three areas. In FMC 77% of the neurons were bcl-2 positive while only 23% were bax positive. After deprivation, only 41% were bcl-2 positive while 58% were bax positive. Hence, the ratio of bcl-2 and bax was reversed after deprivation. Since bax is a pro-apoptotic protein, increased percentage of bax positive neurons may be interpreted as
increased incidence of apoptosis after REM sleep deprivation in all the areas studied. However, both bcl-2 and bax are present in the normal cells. Only their relative expression changes during apoptosis i.e. bcl-2 expression decreases and bax expression increases. Therefore, there was the need to further confirm our results using a technique that stains only apoptotic cells and not normal cells. Hence, TUNEL technique was used.

6.5.4 TUNEL technique:

The DNA in the nuclei of apoptotic cells develops nicks resulting in break points. These nicks may result in formation of overhangings (short sequences which do not have a complementary strand) at both the 3' end and the 5' end. In the TUNEL technique, synthetic nucleotides are attached to the 3' overhangs by using the enzyme TdT. These nucleotides can be labelled and the TUNEL positive (apoptotic) nuclei detected in the tissue.

After REM sleep deprivation, the percentage of TUNEL positive nuclei increased in all the three areas as compared to FMC. A significant percentage of the nuclei were also labelled after recovery but it was less than deprivation. They probably represent the population of neurons that have degenerated beyond repair. TUNEL being a sensitive technique was able to detect these degenerated neurons present after recovery. The advantage of this technique is that it does not label normal cells. Hence the number of TUNEL positive cells in FMC was found to be very low. The increase in TUNEL positive nuclei observed after REM sleep deprivation further confirm our previous observations that there is increased neuronal damage after REM sleep deprivation.
6.5.5 Correlation between REM sleep deprivation, neurodegenerative diseases and cell death:

In several degenerative diseases like Alzheimer's disease, a decrease in the amount of REM sleep is seen in the patients (Christos 1993). Alzheimer's disease is also associated with severe memory loss that is coupled with degeneration of cholinergic neurons and the formation of neurofibrillary tangles or senile plaques (Sheng et al., 1998). Since memory loss is also seen in REMSD, it can be hypothesized that the loss of REM sleep may lead to the degeneration of neurons in the brain especially in those areas that are related to REM sleep generation. In contrast to the observations in the present study, Cirelli et al. (1999) have reported that there is no cell death after total sleep deprivation. However, using amino-cupric-silver technique, Siegel and his coworkers had found the presence of neuronal degeneration in the basal forebrain in narcolepsy, a REM sleep disorder in cats (Siegel et al., 1999). Recently their group also reported an increase in amino cupric silver stained degenerated neurons in the supraoptic nucleus after total sleep deprivation (Eiland et al., 2002). The previous study by Cirelli's group had performed total sleep deprivation ranging from a few hours to days and studied the expression of stress related genes and neuronal degeneration using TUNEL technique. As discussed earlier, no significant degeneration was observed in our study after 4 days of deprivation. Probably, the duration of total sleep deprivation in Cirelli's study was not sufficient to induce any significant amount of neuronal degeneration. In Siegel's study, degeneration was observed in basal forebrain in narcolepsy and in supraoptic area after total sleep deprivation using amino-cupric silver technique. However, none of these studies had taken into consideration the effect of deprivation on REM sleep generating areas. As discussed earlier, due to continuous firing of LC neurons and long term inhibition of LDT/PPT and mPOA neurons, these neurons are likely to undergo degeneration. In the present study, the
neuronal degeneration was studied in the REM sleep related areas using electron microscopy and amino-cupric-silver technique, which was further confirmed using bcl-2/bax and TUNEL. The observations made in this study further confirm that REM sleep deprivation has degenerative effects on the brain areas involved in REM sleep regulation.

6.5.6 Role of norepinephrine in neuronal death:

It was known that increased activity of noradrenergic neurons as a result of long term stress may lead to increased transcription of TH enzyme gene leading to increase in TH enzyme production (Kandel 2000). This would in turn lead to more of norepinephrine synthesis and release. Norepinephrine, at higher concentration has been reported to increase apoptosis by acting through β-adrenergic pathway (Communal et al., 1998). In our study, we found that there is an increase in percentage of apoptotic neurons in LC, LDT/PPT and mPOA after REMSD. It has been previously reported that there is an increase in NE concentration in different brain areas after REMSD (Porkka-Heiskanen et al., 1995). This high level of NE may be responsible for the neuronal degeneration observed in our study. After REMSD, percentage of apoptotic cells increased as compared to FMC and LPC sets. However, after recovery, though the percentage of apoptotic cells was less than deprivation, it was still high enough to be significantly different from FMC (as revealed in TUNEL staining). This indicates that even after 3 days of recovery, a significant percentage of neurons could not recover from the detrimental effects of REMSD. Thus, if REMSD was allowed to continue more neurons were likely to undergo degeneration. This significant percentage of apoptotic neurons after recovery was only detected by the TUNEL technique. This may be because TUNEL stains only degenerated cells; consequently, the percentage of degenerated neurons after FMC was very low and the recovery values were significantly higher than FMC. A decrease in cell size was also
reported in apoptosis (Wyllie et al., 1980), so a higher percentage of apoptotic neurons after REMSD may explain a decrease in size of neurons in certain areas.

In the lateral septum, no change in either the size of neurons or the number of degenerated neurons was observed after REMSD. Lateral septum has not been implicated in the REM sleep generating mechanism though other parts of basal forebrain are reported to be involved. This supports our proposition that the effects of REMSD on the neuronal structure are not generalized, rather they are more pronounced on the areas that are involved in generation of REM sleep since the firing rate of the neurons are altered in these areas.

6.6 EFFECT OF REM SLEEP DEPRIVATION ON STRUCTURAL PROTEINS:

Structural proteins play an important role in maintaining the size and volume of cells. The effect of REM sleep deprivation was investigated on actin content. Actin has been reported to play a role in cell volume regulation (Henson, 1999). Disruption of actin organization has been reported to trigger the process of apoptosis. Disorganization of actin inside a cell leads to changes in position and distribution of the mitochondria, leading to attenuation of the anti-apoptotic activity of bcl-2 present on the mitochondrial membrane (Domnina et al., 2002; Kim et al., 2002).

The concentration of actin was estimated densitometrically in LC, LDT/PPT and mPOA. The mean density of immunostain per unit area was found to be proportional to the amount of actin in the cells. The mean density was divided by area to negate the effect of difference in the size of neurons between deprived and controls as discussed earlier. In all the three areas, there was a decrease in actin concentration after REM sleep deprivation. This may be a consequence of the increase in apoptosis after REM sleep.
deprivation reported earlier. It has been reported that caspases that are responsible for the disruptive changes associated with apoptosis target the cellular cytoskeleton. Caspases act on gelsolin that is known to sever actin filaments in a regulated manner (Yin and Stull, 1999). This may explain the decrease in actin concentration after REM sleep deprivation as some actin might have been cleaved in the apoptotic process by caspases. Since in any mechanism there is a balance between synthesis and breakdown, during recovery the synthesis of actin might increase which would counteract the loss of actin during deprivation. Thus, the actin concentrations were observed to be comparable to FMC levels after recovery. This may also be one of the possible explanations of the earlier observations that after recovery cell size comes back to FMC levels. It is possible that after recovery as the actin levels come back to normal and the cell is able to regain its lost shape and size.

6.7 SIGNIFICANCE OF THIS STUDY:

The significance of the above discussed results is immense and the consequences far reaching. These results confirm that REM sleep deprivation not only affects the brain physiology but also the morphology of the neurons in the brain. It has important implications for the nighttime workers like hospital staff, long distance heavy-vehicle drivers etc who spend a large part of their sleep time being awake. Though these studies are on rats, the results are still valid in the human context. In humans, the effects of sleep loss may become cumulative and in the long term prove fatal for the neurons in the brain. These may lead to several neurodegenerative diseases where the neurons in selected areas of the brains are damaged leading to loss of several vital functions like memory and motor coordination. Presently, the reason for degeneration and subsequent loss of neurons in neurodegenerative diseases is not known. This study proposes that loss of REM sleep
observed in some of these diseases may be a probable cause for neuronal death. Further, it can be ascertained from this study that REM sleep deprivation in rats is an excellent model for studying the long term and far reaching consequences of loss of REM sleep especially in relation to neurodegenerative diseases and memory loss.