DISCUSSION
Sleep has a significant evolutionary role in that it has evolved itself along with the evolutionary tree. It is a state of unresponsiveness to the external environment and plays an important role in the maintenance of normal cellular and physiological homeostasis. Disturbance in the normal sleep wake cycle creates several behavioral abnormalities like increased irritability, excitability, aggressiveness, depression, loss in concentration, etc. Thus it is essential for the proper functioning of our nervous system. Sleep deprivation makes a person drowsy and unable to concentrate the next day. It also leads to impairment of memory and physical performance and reduced ability to carry out mathematical calculations. If sleep deprivation continues, hallucinations and mood swings may develop. Further many of the neurodegenerative diseases show a manifestation of sleep loss (in particular REM sleep). Hence tremendous work is going on to find out the molecular mechanism of REM sleep generation and maintenance which is essential to find a cure for various REM sleep disorders including associated neurodegenerative diseases.

In this study rats were REM sleep deprived by the classical flower-pot method, the most preferred method of choice globally for such studies. Nevertheless, to rule out the effects of non-specific factors including non-definable stress-induced effects, in addition to FMC control we carried out standard LPC and REC control experiments. Although there may be some loss of non-REM sleep on the small platform, it is comparable to the LPC rats, at least after more than 48 h deprivation (Mendelson et al., 1974). Electrophysiological recordings showed that this method indeed induced selective REM sleep loss and the effects were unlikely due to stress (Porkka-Heiskanen et al., 1995).

REM sleep deprivation increases NA level (reviewed in Mallick et al., 2002; Pal et al., 2005) as a combinatorial effect of the following reasons: (i) continued activity of the NA-ergic REM-OFF neurons (Mallick et al., 1989); (ii) increased synthesis (Sinha et al., 1973; Majumdar and Mallick, 2003) and decreased breakdown (Thakkar and Mallick, 1993b) of the NA synthesizing (tyrosine hydroxylase) and hydrolyzing (monoamine oxidase) enzymes, respectively; (iii) reduction of REM sleep by activation of the REM-OFF neurons either by electrical stimulation (Singh and Mallick, 1996) or by withdrawing the inhibitory effect chemically (Kaur et al., 2004) and finally, (iv) NA levels increase after REMSD (Porkka-Heiskanen et al., 1995). Increase in NA level causes various cellular changes including alterations in biochemical, physiological and molecular processes, resulting in disturbed homeostasis, which has deleterious effects on the behavior of a subject (Kopp et al., 1982; Heaney et al., 1999).
Studies from our lab have shown that the REM sleep loss induced enhanced NA that in turn increased Na-K-ATPase activity (Gulyani and Mallick, 1995; Mallick et al., 2000). Isolated studies have shown that NA decreases LP (Schaefer et al., 1974). Thus, we wanted to study whether the REMSD-induced elevated NA would also decrease membrane LP. Moreover, REMSD decreased intrasynaptosomal calcium (Mallick and Gulyani, 1996). Further, LP (Morel et al., 1998; Rauchova et al., 1999; Malfatti et al., 2003) and calcium (Davis and Vincenzi, 1971; Mallick and Adya, 1999) are known to inhibit the activity of the integral membrane bound protein Na-K-ATPase. Although increased calcium inhibits the enzyme activity, surprisingly some calcium is still needed to activate Na-K-ATPase by bringing about its dephosphorylation (Aperia et al., 1992; Mallick et al., 2000). Thus, the study attempted to find out the relationship between Na-K-ATPase activity and neuronal membrane LP with particular reference to NA and calcium after REM sleep loss. The synaptosome was used as a model membrane for all the studies. The observations of the study are discussed under the following heads:

(A) Noradrenaline Acting on α1-Adrenoceptor Mediates REM Sleep Deprivation Induced Increased Membrane Potential in Rat Brain Synaptosomes.

(B) Stimulatory Role of Calcium in Rapid Eye Movement Sleep Deprivation Induced Noradrenaline Mediated Increase in Na-K-ATPase Activity in Rat Brain.

(C) REM Sleep Loss associated Iron Deficiency and Related Symptoms are due to its chelation actions by Noradrenaline in Rat Brain.

(D) Mechanism of REM sleep deprivation induced Noradrenaline mediated protein oxidation in rat brain synaptosomes
Noradrenaline Acting on α1-Adrenoceptor Mediates REM Sleep Deprivation Induced Increased Membrane potential in rat brain synaptosomes


Transmembrane potential provides an overview of the state of affairs of a neuron, which reflects its physiological condition. It may modulate directly or indirectly a variety of neuronal functions including signaling, neurotransmitter release and neurogenesis (Choi, 1988; Hochner et al., 1989; Deisseroth et al., 2004). The depolarization of neurons brought about either by an increased influx of positive ions or efflux of the negative ions, is associated with increased neuronal excitability (Deisseroth et al., 2004). It is known that abnormal alteration in the ionic flux may release apoptotic factors like cytochrome c thereby activating the caspase cascade which ultimately may lead to neuronal death (Moon et al., 2005). The Na-K-ATPase plays a key role in maintaining the transmembrane potential by an efflux of 3 Na⁺ in exchange for 2 K⁺ influx (Trachtenberg et al., 1981; Horisberger et al., 1991). Various neurotransmitters also modulate the neuronal transmembrane potential by regulating the cellular ion homeostasis either directly by activating ion channels or indirectly through various signaling molecules. NA is one such neurotransmitter that has been shown to modulate membrane depolarization in neurons (Pan et al., 1994).

REM sleep loss has been reported to affect mood, behavior and threshold for electroconvulsive shock in both animal and human subjects (Kushida et al., 1989; Gulyani et al., 2000; Clark, 2005). Recent studies have shown that longer duration of REM sleep deprivation (REMSD) results in morphological changes in neurons (Majumdar and Mallick, 2005) and neuronal death (Biswas et al., 2006; Cordova et al., 2006). Based on these altered behavioral and physiological changes we hypothesized that REMSD alters brain excitability and as a corollary we proposed that one of the functions of REM sleep is to maintain the threshold of neuronal and brain excitability and responsiveness (Mallick et al., 1994). A reciprocal relationship exists between the level of excitability and membrane potential in neurons, where the latter is the cause and an estimate of the former. The higher the positivity of the intracellular potential, greater is the excitability level and lower is the threshold of responsiveness of the neuron. Earlier studies from our lab have reported that NA increased Na-K-ATPase activity (Gulyani and Mallick, 1993; Mallick et al., 2000). Thus, we studied the relationship between the REM sleep loss induced increase in Na-K-ATPase activity with synaptosomal potential and the modulatory role of NA in such a phenomena.
Relationship between REMSD induced NA mediated increase in Na-K-ATPase activity and synaptosomal membrane potential:

The results of this study showed that upon REMSD there was increased DiSC2-fluorescence intensity compared to that of the FMC. This suggested that after REMSD the neurons tended to remain in a relative depolarized state, thereby reducing its threshold for excitation. Intraperitoneal injection of PRZ and CLN, which block the NA action and release, respectively, prevented the REMSD-induced synaptosomal depolarization in-vivo. Further, incubation of the synaptosomes with NA in-vitro increased the DiSC2-fluorescence, which however, was prevented by PRZ but not by PRN. Based on these observations we showed that the REMSD-induced increased depolarization was mediated by NA acting through the α1-adrenoceptor (Das and Mallick, 2007). Simultaneously, in the same synaptosomal sample we observed that REMSD increased Na-K-ATPase activity and the effect was also mediated by NA acting on α1-adrenoceptor as reported earlier (Gulyani and Mallick, 1995; Mallick et al., 2000).

Possible mechanism of synaptosomal depolarization after REMSD:

The fact that both Na-K-ATPase activity and synaptosomal potential were increased after REMSD in the same sample and both were mediated by NA acting on α1-adrenoceptor, suggest that the two processes are linked phenomena. Independent studies have shown that NA depolarizes a membrane due to a net efflux of the chloride anions (Lamb and Barna, 1998), thereby activating the voltage dependent sodium channels (Takahashi et al., 1999). Thus, REMSD-induced elevated NA increases intracellular positivity possibly by an efflux of negative ions resulting in membrane depolarization. Previous studies from our lab have shown under similar conditions that REMSD stimulated the chloride-sensitive Mg-ATPase pump activity (Mallick and Gulyani, 1993), which is known to extrude the negative chloride ions (Shiroya et al., 1989), support this finding. The REMSD-induced NA mediated membrane depolarization then activates the voltage dependent sodium channels (Catterall, 1992) to cause a net influx of sodium ions, which also adds to the increase in excitable state of the neuron. As the intracellular level of Na⁺ increases, the neuron tries to compensate and balance the Na⁺ overload inside the cell (Takahashi et al., 1999) by activating the Na-K-ATPase, which extrudes 3 Na⁺ in lieu for 2 K⁺ influx (Trachtenberg et al., 1981; Horisberger et al., 1991). Notwithstanding, both in-vivo and in-vitro experiments have shown that the
REMSD-induced increased NA increases the Na-K-ATPase activity (Gulyani and Mallick, 1995; Mallick et al., 2000). Thus, the NA-induced stimulation of Na-K-ATPase may be a direct effect or secondary to efflux of anions. Although it is difficult to confirm if one mechanism follows the other, both the mechanisms might exist and they may be activated under different conditions including various diseases. Therefore, the etiology and progression of various diseases associated to REMSD may be different, though the symptoms expressed may be similar. Notwithstanding, the increased Na-K-ATPase activity alters the release of various neurotransmitters (Vizi et al., 1982) which may be the cause of symptoms expressed during REMSD and associated disorders. Although it was known that REMSD increases NA that stimulates Na-K-ATPase activity, the mechanism of enzyme activation was not known. It was also known that intracellular positivity stimulates the enzyme. In this study, we observed that indeed the intracellular positivity is increased after REMSD and this is mediated by NA acting through \( \alpha_1 \)-adrenoceptor. Thus, the results of this study confirm and provide direct evidence in support of our hypothesis that \textit{"REMSD increases brain excitability"} and that is mediated by an increased level of NA (Das and Mallick, 2007).
(B) Stimulatory Role of Calcium in Rapid Eye Movement Sleep Deprivation Induced Noradrenaline Mediated Increase in Na-K-ATPase activity in rat brain

REM sleep is a unique state expressed at least in almost all mammals through evolution (Siegel, 1995, 1999; Frank, 1999). Its physiological significance cannot be ignored because several psycho-somato-physio-logical conditions have been associated to it (Dement, 1960; Vogel, 1975; Rechtschaffen et al., 1989; Miyamoto, 2001; Biswas et al., 2006; Cordova et al., 2006). We hypothesized that one of the functions of REM sleep is "to maintain brain excitability" and that is achieved by modulation of the neuronal membrane bound enzyme Na-K-ATPase (Gulyani and Mallick, 1993; Mallick et al., 1994), the key enzyme that maintains neuronal transmembrane potential (Trachtenberg et al., 1982; Horisberger et al., 1991). The Na-K-ATPase is an integral membrane protein (Moller et al., 1996; Knap et al., 2000; Skou, 2000), which is activated by Ca\textsuperscript{2+}-dependent dephosphorylation (Marcaida et al., 1996; Blanco and Mercer, 1997; Lecuona et al., 2006). It has been reported that the activity of Na-K-ATPase is modulated by changes in membrane LP (Rauchova et al., 1999; Morel et al., 1998; Malfatti et al., 2003), Ca\textsuperscript{2+} (Davis and Vincenzi, 1971; Mallick and Adya, 1999) and NA (Gulyani and Mallick, 1995; Mallick et al., 2000; Das and Mallick, 2007) as they share an inverse relationship with each other (Morel et al., 1998; Rauchova et al., 1999; Malfatti et al., 2003). An increase in LP is known to decrease Na-K-ATPase activity. This is explained by the fact that Na-K-ATPase is an integral

**Effect of REMSD on synaptosomal LP:**

REMSD caused a significant reduction in neuronal membrane LP as compared to normal FMC. This reduction in LP returned to the normal baseline level after REC of REM sleep loss, thus proving that the observed effect was specifically due to REM sleep deprivation and not due to change in any other non-specific factor. This reduction in LP was observed in both whole brain as well as in different brain areas synaptosomal preparation.

Thus, this decrease in LP might be one of the factors regulating the deprivation induced increase in Na-K-ATPase activity (Gulyani and Mallick, 1995; Mallick et al., 2000; Das and Mallick, 2007) as they share an inverse relationship with each other (Morel et al., 1998; Rauchova et al., 1999; Malfatti et al., 2003). An increase in LP is known to decrease Na-K-ATPase activity. This is explained by the fact that Na-K-ATPase is an integral
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membrane spanning protein (Moller et al., 1996; Knapp et al., 2000; Skou, 2000) and alterations in the membrane lipids as an effect of LP alters the Na-K-ATPase structure (Subramaniam et al., 1997; Zhu et al., 2005) and function (Morel et al., 1998; Rauchova et al., 1999; Malfatti et al., 2003) resulting in reduced Na-K-ATPase activity. Thus, from the observations, it can be inferred that the increase in Na-K-ATPase activity after REMSD (Gulyani and Mallick, 1993, 1995; Mallick et al., 2000) is facilitated by the NA-induced decrease in membrane LP.

**NA and Ca\(^{2+}\) modulation of synaptosomal lipid peroxidation:**

Recent studies have shown the modulatory role of NA and calcium on membrane LP. NA has been shown to decrease (Schaefer et al., 1974) while calcium increases (Lu et al., 2002; Obata, 2003) LP. Further, REMSD increases the NA level as stated above. Moreover, previous studies have shown that REMSD decreases intrasynaptosomal calcium levels (Mallick and Gulyani, 1996). Recent studies have shown the stimulatory role of the divalent calcium ion on LP (Braughler et al., 1985; Lu et al., 2002). Therefore, we wanted to study whether the REMSD-induced increase in NA and decrease in Ca\(^{2+}\) might be modulating the deprivation induced decrease in synaptosomal LP.

We observed a decrease in synaptosomal LP by NA pretreatment. NA decreased LP through the \(\alpha\)-adrenoceptor as observed by both *in-vivo* and *in-vitro* study. Intraperitoneal injection of PRZ, \(\alpha_1\)-antagonist and CLN, \(\alpha_2\)-agonist that prevents NA action and release respectively, attenuated the REMSD-induced decrease in LP. Moreover, *in-vitro* incubation with NA decreased LP that was prevented by preincubation of the preparation with PRZ but not by PRN, \(\beta\)-antagonist. Thus, the observations suggest that NA reduced LP through the \(\alpha_1\)-adrenoceptor.

Chelation of calcium by EDTA also decreased neuronal membrane LP in all the preparation of experimental animals. *In-vitro* incubation with NA caused a further reduction in LP in EDTA pretreated synaptosomes. This NA-induced decrease in membrane LP was prevented by PRZ but not PRN in both the EDTA-treated and non-treated synaptosomes. Hence, the observations further support the role of NA in reducing membrane LP through the \(\alpha_1\)-adrenoceptor. Moreover, the fact that NA decreased membrane LP in synaptosomes treated with EDTA that chelates divalent calcium ions, strongly suggest that the NA-induced effect (decreased LP) is modulated by chelation of calcium levels further.
We also observed in *in-vitro* experiments that NA as well as Nif both inhibited the \( \text{H}_2\text{O}_2 \) induced \( \text{Ca}^{2+} \)-influx in synaptosomes. Further CyA increased the \( \text{H}_2\text{O}_2 \) induced increase in intra-synaptosomal \( \text{Ca}^{2+} \)-influx. The effect of Nif and CyA may be explained by the fact that Nif is a blocker of DHP non-resistant (L-type) \( \text{Ca}^{2+} \)-channels and consequently decreases \( \text{Ca}^{2+} \)-influx (Mulvaney et al., 1999), while CyA increases intracellular \( \text{Ca}^{2+} \)-concentration by its release from internal stores (Ng and Gurney, 2001). The CyA data shows that LP enhances intrasynaptosomal calcium levels. Thus, the observations suggest that NA probably acted through the \( \alpha_1 \)-adrenoceptor linked to the L-type calcium channels to modulate calcium and membrane LP levels.

Thus, the observations clearly suggest a relationship between REMSD induced increased NA level and decreased \( \text{Ca}^{2+} \) concentration with that of LP. Since NA decreased neuronal membrane LP even in the presence of EDTA, which chelated extracellular \( \text{Ca}^{2+} \), NA must have manipulated the membrane-bound \( \text{Ca}^{2+} \) as proposed earlier (Mallick and Adya, 1999). Thus, the REMSD-induced elevated NA decreased LP by acting on the \( \alpha_1 \)-adrenoceptor that is linked to L-type \( \text{Ca}^{2+} \)-channel by chelating the membrane bound calcium ions as well as reducing calcium influx to increase Na-K-ATPase activity.

**Relationship between synaptosomal LP and Na-K-ATPase activity: Modulation by REMSD, NA and \( \text{Ca}^{2+} \)**

REMSD caused a significant decrease in synaptosomal LP while increasing both Na-K-ATPase activity as well as its dephosphorylation (the active form) at both the serine as well as threonine residues and NA mediated the effects through the \( \alpha_1 \)-adrenoceptors. Intraperitoneal injection of PRZ and CLN prevented the deprivation-induced increase in Na-K-ATPase activity and decrease in LP. Moreover, we observed a significant dephosphorylation of Na-K-ATPase at its serine and threonine residues in both EGTA and non-EGTA pretreated synaptosomes upon NA incubation. This NA induced dephosphorylation was prevented by PRZ but not by PRN.

The Na-K-ATPase activity is known to be modulated by changes in membrane fluidity (Lebel and Schatz, 1990), membrane LP (Morel et al., 1998; Malfatti et al., 2003), \( \text{Ca}^{2+} \) ions (Davis and Vincenzi, 1971) and NA (Gulyani and Mallick, 1995; Mallick et al., 2000). On the other hand, changes in the membrane environment affect various ion channels like calcium and regulate intracellular \([\text{Ca}^{2+}]_i\) concentration. Isolated studies have shown that \([\text{Ca}^{2+}]_i\) modulates Na-K-ATPase activity apparently in two opposite ways.
First, it inhibits the enzyme (Davis and Vincenzi, 1971) and secondly, some Ca$^{2+}$ is necessary for its dephosphorylation to the active form (Aperia et al., 1992; Mallick et al., 2000). Brown and Lew (1983) have proposed that Ca-ATP inhibits Na-K-ATPase by displacing Mg-ATP from a low affinity site in Na-K-ATPase for which it has high affinity. Shainskaya et al. (2000) has postulated the binding of divalent Mg$^{2+}$/Ca$^{2+}$ ions at the cytoplasmic loop between the transmembrane loops M6/M7 of the α-subunit. Further, Schneeberger and Apell (2001) have shown that the binding affinity of calcium is higher than that of magnesium, which provides support to the competitive inhibition mechanism of Ca-ATP on Na-K-ATPase activity. Secondly, some amount of calcium is essential to stimulate Na-K-ATPase activity by inducing its dephosphorylation through calcineurin (Aperia et al., 1992; Mallick et al., 2000). The crucial point deciding whether calcium will act as inhibitory or stimulatory will depend on the concentration of intracellular calcium levels. As shown by Brown and Lee (1983), low levels of Ca$^{2+}$ produced a marked stimulation of Na-K-ATPase activity, which was maximal between 10-60μM Ca$^{2+}$ and then decreased as Ca$^{2+}$ was decreased.

The involvement of these factors in regulating Na-K-ATPase activity is supported by the findings in this study that H$_2$O$_2$, which increased synaptosomal membrane LP, simultaneously increased intracellular Ca$^{2+}$-level and inhibited Na-K-ATPase activity; further, both these processes were prevented by NA acting on α1-adrenoceptor blocker.

We also observed in in-vitro experiments that NA as well as Nif both inhibited the H$_2$O$_2$ induced Ca$^{2+}$-influx in synaptosomes; NA increased while H$_2$O$_2$ decreased Na-K-ATPase activity and the NA action on Na-K-ATPase activity as well as on LP was mediated through the α1-adrenoceptor. Therefore, it is likely that the NA actions were mediated through α1-adrenoceptor with the participation of the membrane bound Ca$^{2+}$ and Nif blocked the actions. Further CyA increased the H$_2$O$_2$ induced increase in intra-synaptosomal Ca$^{2+}$-influx. Nif blocks the DHP non-resistant Ca$^{2+}$-channels and consequently decreases Ca$^{2+}$-influx (Mulvaney et al., 1999), while CyA increases intracellular Ca$^{2+}$-concentration by releasing Ca$^{2+}$ from internal stores (Ng and Gurney, 2001).

It is known that Ca$^{2+}$ ion is required for the activation of calcineurin (Stemmer and Klee, 1994) that dephosphorylates and activates Na-K-ATPase (Fisone et al., 1994; Therien and Blostein, 2000). However, since this Ca$^{2+}$ is neither present inside nor outside the synaptosomes and intracellular increase in Ca$^{2+}$ inhibits the enzyme (Davis and Vincenzi, 1971; Lingrel and Kuntzweiler, 1994), we hypothesized that the Ca$^{2+}$ required for Ca$^{2+}$-calmodulin dependent dephosphorylation of the enzyme must be membrane bound, possibly
Fig. 30

Schematic presentation of the proposed model of the REMSD-induced elevated NA mediated increase in Na-K-ATPase activity in the rat brain. This is based on the findings of this study and that of earlier studies referred in the figure.
associated with the transmembrane region of Na-K-ATPase. Further, this Ca\(^{2+}\) might be linked to the \(\alpha_1\)-adrenoceptor and NA caused its release to stimulate Na-K-ATPase activity; thus the entire sequence of events must be a tightly coupled cascading process. It was shown earlier from this lab that upon REMSD elevated NA acts on \(\alpha_1\)-adrenoceptors, releases membrane-bound Ca\(^{2+}\) and stimulates Na-K-ATPase activity by a Ca\(^{2+}\)-calmodulin dependent process (Mallick and Adya, 1999; Mallick et al., 2000). In this in-vivo and in-vitro study we confirmed that REMSD and NA indeed increased the activity of Na-K-ATPase by its dephosphorylation. The NA mediated dephosphorylation of Na-K-ATPase, the active form (Lecuona et al., 2006), was comparable (Fig. 19a, Fig. 19b, Fig. 20a, Fig. 20b) in both the media containing or without EGTA, which is known to chelate Ca\(^{2+}\) (Cohen et al., 1979). On the other hand, dephosphorylation of Na-K-ATPase (Aperia et al., 1992; Marcaida et al., 1996; Klee et al., 1998) and its stimulation by NA (Mallick et al., 2000) are Ca\(^{2+}\)-calmodulin dependent processes. These findings taken together suggest that although Ca\(^{2+}\) was necessary for the NA induced stimulation of Na-K-ATPase activity, it was not coming from the extracellular or intra-synaptosomal medium. The only possibility of availability of Ca\(^{2+}\) which increased Na-K-ATPase activity must have remained bound to the synaptosomal membrane, which could not be chelated by EGTA, however, got released by the treatment of NA as shown previously (Mallick and Adya, 1999). This is supported by our observation that the decrease in neuronal membrane LP by NA even in the presence of EDTA, which chelated extracellular Ca\(^{2+}\), suggest that NA must have manipulated the membrane-bound Ca\(^{2+}\), which however, may or may not be associated with Na-K-ATPase as proposed earlier (Mallick and Adya, 1999).

Further, it is known that there is increased NA in the brain after REMSD (reviewed in Mallick et al., 2002; Pal et al., 2005). Thus, based on the findings of this study along with earlier reports (from various labs including ours) we propose the following mechanism of action. REMSD increases NA in the brain that on one hand reduces Ca\(^{2+}\)-influx into neurons by decreasing neuronal membrane LP (Lu et al., 2002), while on the other hand, by acting on \(\alpha_1\)-adrenoceptor activates PLC and releases membrane-bound Ca\(^{2+}\), which may or may not be linked to the transmembrane region of Na-K-ATPase. This released Ca\(^{2+}\) then activates calcineurin (Son and Brinton, 2001), which dephosphorylates Na-K-ATPase to its active form resulting in its increased activity (Mallick et al., 2000; 2002). All these steps must be tightly coupled cascading biochemical processes, which have been diagrammatically presented in a simplified model (Fig. 30).
Schematic presentation of the possible mechanism of REMSD-induced Increase in Na-K-ATPase activity (A) Cyclopiazonic acid (CyA) induced increased calcium causes Na-K-ATPase inactivation. NA by reducing calcium levels prevents this inactivation ("+" denotes stimulation, "-" denote inhibition). (B) Calcium ion required for Na-K-ATPase dephosphorylation might be associated in a membrane bound form (?) associated to Na-K-ATPase (?). (C) Earlier study showing the pathway of calcineurin activation following REMSD.

REM Sleep Deprivation

Pal et al., 2005

Pal and Mallick, 2007

Increased NA

(A) EXTRACELLULAR

Active

Inactive

Neuronal Membrane

Reduced L.P.

Lu et al., 2002

(Mg^{2+} + ATP)

(-)

(+) CyA induced Ca^{2+}

(B) EXTRACELLULAR

Na-K-ATPase Phospho-Form (Inactive)

Na-K-ATPase Dephospho-Form (Active)

(Mallick et al., 1999, 2000)

Mallick & Adya, 1999

(C) INTRACELLULAR

Ca^{2+}

Calcineurin (Active)

Calcineurin (Inactive)

Mallick et al., 2000

Fig. 31
Studies have shown that REMSD resulted in both decrease in intrasynaptosomal calcium levels (Mallick and Gulyani, 1996) as well as phosphorylation of Na-K-ATPase (present study), that is known to increase or activate enzyme activity (Davus and Vincenzi, 1971; Aperia et al., 1992; Mallick et al., 2000). Thus, an attempt has been made to understand the mechanism of regulation of enzyme activity by calcium after REMSD as shown in Fig 31.
(C) REM Sleep Loss Associated Iron Deficiency and Related Symptoms are due to its Chelation actions by Noradrenaline in rat brain.

The LP is a process initiated and propagated by free radicals generated both endogenously (Seaver and Imlay, 2004) and exogenously (Vallyathan and Shi, 1997), which causes significant damage to the membrane structure and function by altering its integrity. ROS is continuously formed in the tissues as a byproduct of normal physiological processes (Seaver and Imlay, 2004) and alterations in ROS concentration is the key underlying factor in several diseases (Götz et al., 1990; Multhaup et al., 1997). They are transient chemical species with high reactivity that often trigger cascading effects thereby causing massive lipid, protein and DNA oxidation (Galli et al., 2005; Valko et al., 2006). The neurons in the brain are relatively more vulnerable to such oxidative processes, as they possess very few antioxidants and are rich in polyunsaturated fatty acids, the substrate for ROS generation. However, the brain possesses the neurotransmitter, NA that has been sometimes reported to behave as an antioxidant (Troadec et al., 2001; Traver et al., 2005) and hence may reduce such free radical mediated oxidative damage.

Iron generates ROS through the Fenton reaction. On the other hand, iron deficiency is reported to be common among several diseases where REMS is also disturbed eg., alzheimer's, multiple sclerosis, schizophrenia, restless leg syndrome, depression and epilepsy (Parks and Wharton, 1989; Rooney et al., 1999; Holsboer-Trachsler and Seifritz, 2000; Daoud et al., 2002; Sadrzadeh and Saffari, 2004; Allen and Earley, 2007; McCann and Ames, 2007; Shariatpanaahi, 2007); some of these diseases are also associated with altered neuronal excitation (Leist and Nicotera, 1998) that is maintained by Na-K-ATPase (Trachtenberg et al., 1981). LP is directly correlated to the level of ROS (Dröge and Schipper, 2007) while Na-K-ATPase activity is known to be modulated by membrane LP (Malfatti et al., 2003). Thus, we studied the relationship between the REMS loss induced elevated NA mediated increase in Na-K-ATPase activity with that of ROS and LP. Further, the modulatory role of NA in such a phenomenon was also studied.

**Relationship between ROS and LP: Modulation by REMSD and NA**

In the present study we observed that upon REMSD there was a decrease in both LP and ROS production in neuronal membrane *in-vivo*. The former effect was mediated by NA through the α1-adrenoceptor and the latter is through α2-adrenoceptor, respectively as the
Discussion

effects were prevented by i.p. injection of PRZ that prevents NA action and CLN, which reduces the release of NA (Curet et al., 1987). This NA-mediated reduction in ROS formation was further confirmed *in-vitro* by treating synaptosomes with NA. It was observed that NA decreased ROS generation even in the presence of the α1 and β-adrenoceptor antagonists, PRZ and PRN, respectively. Further, although the NA induced attenuation of LP was mediated by its action on the α1-adrenoceptor, the ROS production was independent of receptor mediated action. Hence, the observation suggests that the REMSD-induced decrease in LP and ROS might be an interrelated phenomenon.

**Mechanism of action of NA on ROS and LP:**

ROS induces LP (Dröge and Schipper, 2007) while H$_2$O$_2$ treatment is known to induce both (Ullrich and Kissner, 2006). Hence, to understand the mechanism of action, we used H$_2$O$_2$ to simulate the effect. The H$_2$O$_2$ was used as it is metabolized *in-vivo*, is membrane permeable, possesses longer half-life than most other biological radicals, is a relatively stronger oxidant and a highly stable molecule (Antunes and Cadenas, 2000). The level of H$_2$O$_2$ plays a significant role in cellular membrane LP that affects membrane integrity, protein activity and cellular functioning (Schnitzer et al., 2007; Stark, 2005). Although H$_2$O$_2$ caused an increase in both ROS and LP, these effects were prevented by pre-treatment of the sample with NA.

To elucidate whether NA acted as an antioxidant to diminish ROS as well as LP and to understand its mechanism of action, we compared its effect on ROS and LP with that of the effect of Vit E, Vit C and DFO in the presence and absence of H$_2$O$_2$. Vit E is a potent antioxidant (Singh et al., 2005); Vit C is reported to function as both pro- and anti-oxidant (Halliwell, 1996; Carr and Frei, 1999), while DFO is a specific chelator of the Fe$^{3+}$ form of iron (Goodwin and Whitten, 1965; Blake et al., 1983) that induces the generation of ROS (Halleen et al., 1999). We observed that in the absence of H$_2$O$_2$, Vit E decreased both ROS and LP levels in a manner similar to that of NA. However, opposite effects were induced by Vit C; it decreased ROS while simultaneously increased LP. Further, both NA and Vit E prevented the H$_2$O$_2$-induced increase in ROS and LP, while Vit C potentiated the H$_2$O$_2$ effects. These findings suggest that NA functions by a mechanism similar to that of Vit E for decreasing ROS and LP, however, the dual nature of Vit C needed further elaborate study.

DFO, a known ferric ion (Fe$^{3+}$) chelator, is reported to inhibit iron-mediated hydroxyl radical formation from superoxide and hydrogen peroxide thereby decreasing LP (Sergent et
When the Fe$^{3+}$ ions are not converted into the Fe$^{2+}$ (ferrous) form, the substrate for
the action of H$_2$O$_2$, the Fenton reaction is inhibited (Ullrich and Kissner, 2006) resulting in
decrease in the level of ROS. In this study DFO treatment reduced the availability of the Fe$^{3+}$
form of iron and not only reduced the ROS level in comparison to the Ctl, but also prevented
the potentiation of ROS formation by both the H$_2$O$_2$ as well as Vit C. However, NA and Vit E
when added in samples preincubated with DFO, they further decreased the ROS level in
the presence of H$_2$O$_2$ but there was no difference between the effect of DFO alone or in
presence of NA and H$_2$O$_2$. The latter event may be explained by the divalent ion chelating
property of NA (Wu, 1986) resulting in chelation of ferrous (Fe$^{2+}$) form of iron which is
essential for the Fenton reaction as shown below:

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot \text{OH} + \text{OH}^-$$

However, it is also known that the ferric form of iron-ions (Fe$^{3+}$) are converted to the
Fe$^{2+}$ form (by Haber-Weiss and Udenfriend reaction) which then takes the Fenton reaction
forward. Thus, chelation of the Fe$^{3+}$ ion by DFO reduced ROS, however, it could not prevent
Fe$^{2+}$-induced generation of ROS through the Fenton reaction, which was existing in the
system or formed by some other reaction. Hence, reduction in ROS levels by NA in the
presence of DFO suggest that addition of NA chelated the Fe$^{2+}$ ion thus blocking the final
step to carry forward the Fenton reaction even in the presence of H$_2$O$_2$. Therefore, in our
study although DFO chelated Fe$^{3+}$, it could not block the action of H$_2$O$_2$ on Fe$^{2+}$ ion to
initiate Fenton reaction, which was further prevented by NA (Fig. 32).

Further, although DFO prevented the Vit C induced potentiation of the H$_2$O$_2$ effect on
ROS generation, preincubation of the sample with NA caused a further reduction in ROS
levels. Thus, from these observations, it may be inferred that NA chelated the transition metal
Fe$^{2+}$, to block the Fenton reaction and decreased formation of ROS. Further as levels of ROS
and LP are proportional to each other (Dröge and Schipper, 2007), NA-induced decrease in
ROS formation resulted in decreased LP.

Based on these observations it may be said that once NA level was elevated after
REMSD, it decreased the ROS levels by decreasing the availability of iron (Fe$^{2+}$) (Beard et
al., 1988; Troadece et al., 2001). Unavailability of free iron blocked the Fenton reaction
(Ullrich and Kissner, 2006) and thereby decreased LP. Thus this study showed the
mechanism of REMSD-induced NA mediated decrease in LP and functional iron deficiency especially during REMSD associated disorders, which remained unexplained so far.

**Effect of NA, Vit E, Vit C on Na-K-ATPase activity: Modulation by H2O2**

We observed that the activity of integral membrane protein Na-K-ATPase, which maintains the neuronal transmembrane potential (Trachtenberg, 1981), was stimulated by NA; Vit E was ineffective while Vit C reduced its activity. Increased LP is known to inhibit Na-K-ATPase activity (Malfatti et al., 2003) and as observed in the present study Vit C augmented LP. Therefore, it appears that the Vit C-induced reduction of Na-K-ATPase activity is likely to be due to elevated level of LP. However, we have shown that REMSD-induced increased Na-K-ATPase activity was due to increased NA mediated release of membrane bound Ca^{2+} (Mallick and Adya, 1999) and dephosphorylation of Na-K-ATPase (Mallick et al., 2000).

REM sleep loss has been reported to be associated to various diseases including the restless leg syndrome, alzheimer’s, parkinson’s, schizophrenia, epilepsy, multiple sclerosis, narcolepsy and sleep apnea (Parks and Wharton, 1989; Mamelak, 1991; Rooney et al., 1999; Daoud et al., 2002; Sadrzadeh and Saffari, 2004; Hoy et al., 2004; Gagnon et al., 2006; McCann and Ames, 2007). Two important symptoms common among these disorders are that they are characterized by an increase in neuronal excitation (Leist and Nicotera, 1998) and deficiency of iron in the system (Rooney et al., 1999; Daoud et al., 2002; Sadrzadeh and Saffari, 2004; Merlino et al., 2007). Iron deficiency is associated with several neurodegenerative diseases characterized by severe impairment in cognitive ability and development of the central nervous system with the concomitant appearance of mitochondrial malfunction (Parks and Wharton, 1989; McCann and Ames, 2007). Further, decreased iron levels are associated with narcolepsy, multiple sclerosis, restless leg syndrome and sleep apnea where REM sleep is also affected (Mamelak, 1991; Rooney et al., 1999; Hoy et al., 2004). Also, as mentioned above NA is elevated after REMSD (Mallick et al., 1989; Thakkar and Mallick, 1993; Majumdar and Mallick, 2003; Pal and Mallick, 2007) and in related studies it has been shown that NA level increases in iron deficiency (Beard et al., 1988). Thus, we propose that the REM sleep loss induced elevated NA is likely to be chelating iron resulting in apparent iron deficiency and associated pathological symptoms.

Many of the REMSD associated diseases are also exemplified by an increased neuronal excitation (Leist and Nicotera, 1998). Earlier studies have shown that REMSD-
Schematic representation of REM sleep loss induced associated increase in neuronal excitability and iron deficiency. NA mediates the effects of increased Na-K-ATPase activity, decreased LP & ROS following REM sleep loss; all of which acts in a cumulative manner to cause various disorders.

**Fig. 32**

The number in this figure denote the reference number as below:

1. Udenfriend et al., 1954
2. Wu, 1986
3. Mallick et al., 1999
4. Gulyani and Mallick, 1995
5. Rooney et al., 1999
6. Mallick et al., 2000
7. Troade et al., 2001
8. Daoud et al., 2002
9. Liochev and Fridovich, 2002
10. Malfatti et al., 2003
11. Sadrazadeh and Saffari, 2004
15. Merlino et al., 2007
16. Singh and Mallick, 1996
17. Kaur et al., 2004
Discussion

elevated NA acted on the $\alpha1$-adrenoceptor to increase Na-K-ATPase activity (Gulyani and Mallick, 1995; Mallick et al., 2000) thus increasing neuronal excitation. The Na-K-ATPase activity is directly correlated to neuronal excitability; greater the enzyme activity higher is the excitability level (Trachtenberg et al., 1981). This increased Na-K-ATPase activity may be the cause of changes in neuronal morphology, release of apoptotic factors like cytochrome c to activate the caspase cascade (Majumdar and Mallick, 2005; Moon et al., 2005) and ultimately apoptosis (Biswas et al., 2006; Panayiotidis et al., 2006) as evident in neurodegenerative diseases and other REM-sleep loss associated disorders (Leist and Nicotera, 1998).

Thus, the findings of this study revealed the relationship between increased neuronal excitability and iron deficiency associated to REM sleep loss related disorders. This highly complex phenomenon has been schematically represented as a model (Fig. 32). The findings are of great significance that opens up several aspects for further studies to understand the cellular as well as molecular level of actions and further to design therapeutics for REM sleep loss associated disorders.
(D) Mechanism of REM Sleep Deprivation Induced Noradrenaline Mediated Protein Oxidation in rat brain synaptosomes

Oxidation of lipids and proteins are some of the important causal factors underlying various neurodegenerative diseases. Different endogenous and exogenous factors like the reactive oxygen species, reactive nitrogen species, etc initiate the process of LP. Lipid radicals generated by oxidation of lipids induce protein oxidation either directly or through the induction of various signaling mechanisms like the LOXs and COXs (Uchida, 2003; Sayre et al., 2006). The brain is vulnerable to such oxidative insults as on one hand it has an abundance of the polyunsaturated fatty acids (PUFAs), the substrate for such oxidative processes and on the other hand it possess very few antioxidants.

Neurons of the CNS are rich in arachidonic acid, which is synthesized in the brain from the fatty acid linoleic acid via chain elongation and desaturation. The enzymes 5-Lipoxygenase or 1-Cyclooxygenase catalyze the oxidation of linoleic (18:2) or arachidonic (20:4) acids to generate the highly reactive secondary aldehyde, 4-HNE (Warner and Mitchell, 2004). Generation of 4-HNE causes oxidations of both cellular as well as membrane proteins associated with various neurodegenerative diseases like alzheimer’s, parkinson’s, (Uchida, 2003; Sayre et al., 2006) etc. Of the three types of LOXs (5, 12 and 15), only 5-LOX is concentrated in the brain (Manev et al., 2000). On the other hand, 1-COX is involved in maintaining normal homeostasis (Warner and Mitchell, 2004). Moreover, many of the neurodegenerative diseases in which these enzymes are activated like alzheimers, parkinsons etc are characterized by loss of REM sleep (Hoy et al., 2004; Gagnon et al., 2006). Thus, we wanted to study whether the deprivation associated neuronal cell death (Biswas et al., 2006) is modulated by protein oxidation and the role of NA in such a phenomena.

Relationship between REMSD induced increased NA level and levels of 4-HNE, 1-COX, 5-LOX:

In the present study we observed that REM sleep loss increased protein oxidation and also elevated the levels of the inflammatory enzymes, viz., 5-LOX and 1-COX. Intraperitoneal injection of PRZ could not prevent the REMSD induced increase in the level of 45kDa protein oxidation by 4-HNE while it blocked the deprivation-induced increase in the levels of 5-LOX but not 1-COX. On the other hand intraperitoneal injection of CLN that prevent the release of NA decreased the level of the 45kDa protein adduction by 4-HNE in
Overview of the Study

REM Sleep Deprivation

Increased NA

Reduced ROS

Neuronal Membrane

Na-K-ATPase Dephospho-Form (Active)

Na-K-ATPase Phospho-Form (Inactive)

Calcineurin (Active)

Calcineurin (Inactive)

CaM (Active)

CaM (Inactive)

Increased Intrasynaptosomal Potential

Psycho-Somato-Patho-Physiological Disorders

Overview of the Study

REM Sleep Deprivation

Increased NA

Reduced ROS

Neuronal Membrane

Na-K-ATPase Dephospho-Form (Active)

Na-K-ATPase Phospho-Form (Inactive)

Calcineurin (Active)

Calcineurin (Inactive)

CaM (Active)

CaM (Inactive)

Increased Intrasynaptosomal Potential

Psycho-Somato-Patho-Physiological Disorders

Fig. 33

Schematic diagram of the mechanism of REM sleep loss associated intracellular changes leading to expression of various symptoms.
both FMC and REMSD rat. Injection of CLN also reduced the level of 5-LOX induced by REMSD while there was an increase in the level of 1-COX in both FMC as well as REMSD rat. Thus, though NA acted on α1-adrenoceptor to activate the 5-LOX signaling mechanism, it modulated the levels of 4-HNE induced protein oxidation in a manner independent of the α1-adrenoceptor pathway. On the other hand, the increase in the level of 1-COX after REMSD was not due to NA. This is because if the deprivation-induced effect on 1-COX was due to NA then intraperitoneal injection of PRZ and CLN should have brought the level of 1-COX to the baseline level or at least have reduced it. But it was not so and surprisingly, there were increased 1-COX levels in the PRZ and CLN treated FMC rats. Moreover, CLN caused a significant reduction in the HNE adduct formation even in the control rat. Thus, deprivation induced elevated NA increased protein oxidation either directly or it could be activating the enzyme 5-LOX to generate 4-HNE which then modified proteins by forming adducts with them.

This is the first report of the effect of REM sleep loss induced increased NA on synaptosomal 4-HNE protein oxidation and 5-LOX levels. Moreover, as 1-COX is an enzyme involved in maintaining normal cellular homeostasis, the upregulation of this enzyme after REMSD will most probably cause havoc in normal cellular and physiological processes. In a nutshell it can be hypothesized that REMSD-induced increased NA elevated the level of the inflammatory enzyme, 5-LOX to increase 4-HNE-induced modification of membrane proteins thereby disturbing the intracellular homeostasis to cause an imbalance of the normal physiological processes resulting in neuronal cell loss and apoptosis symptomized in different diseased conditions. As REM sleep loss is associated to many neurodegenerative diseases like the alzheimers, parkinsons, etc (Leist and Nicotera, 1998; Hoy et al., 2004; Gagnon et al., 2006), the NA induced modulation of protein oxidations might be the likely pathway of neuronal cell death in such diseases (Fig. 33).
SIGNIFICANCE OF STUDY
This study has made a significant contribution towards elucidating the complex mechanism of REMSD induced increase in neuronal Na-K-ATPase activity and associated brain excitability (Fig. 33). The studies suggest that although initially the deprivation-induced increased NA might protect neurons by lowering LP and ROS; continued deprivation resulted in iron deficiency associated to various REM sleep disorders like restless leg syndrome, sleep apnea, parkinson’s, etc. These pathologies are epitomized by increased neuronal depolarization and excitability brought about by the increased Na-K-ATPase activity. Further, it has elucidated the mechanism of deprivation induced increase in neuronal excitability through the NA mediated modulation of membrane calcium to dephosphorylate and activate Na-K-ATPase through the calcineurin pathway (Fig 31). Further, the study clearly showed modulation of membrane calcium by NA in enhancing Na-K-ATPase activity. Moreover as a mechanism the study has shown that the observed increase in neuronal excitability after REMSD is due to NA-induced increase in neuronal depolarization.