Brief Review

Neural mechanism of rapid eye movement sleep generation: Cessation of locus coeruleus neurons is a necessity

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Abstract: Two types of neurons are involved in the regulation of rapid eye movement (REM) sleep, the REM-ON and the REM-OFF neurons. As the name suggests, the REM-OFF neurons cease firing during REM sleep and they are norepinephrinergic. It has been shown that cessation of these neurons is a pre-requisite for the generation of REM sleep and GABA shuts them off. Further, if these neurons do not shut off, there is increased levels of norepinephrine in the brain and loss of REM sleep. The REM sleep deprivation induced increase in norepinephrine is responsible for mediating at least REM sleep loss induced increase in Na+-K+ ATPase activity, which is likely to be the primary factor for causing REM sleep deprivation induced effects.

Key words: GABA; locus coeruleus; Na-K ATPase; norepinephrine; REM sleep generating mechanism; REM sleep loss

Introduction

Sleep and wakefulness are spontaneous cyclic changes in behavior and associated levels of consciousness in higher living beings. To avoid subjective bias, electrophysiological signals from the brain, the electroencephalogram (EEG), the muscles, the electromyogram (EMG) and the eye movements, the electrooculogram (EOG) have been used for classification and quantification of sleep and wakefulness objectively in higher species including humans. While analyzing sleep using the electrophysiological parameters, Aserinsky and Kleitman [1] observed that electrophysiologically sleep was not a homogenous state. After a minimum time was spent in deep sleep, the EEG and EOG expressed signs apparently resembling wakefulness, although the EMG did not show signs associated to wakefulness. Since that appeared to be a paradox, i.e. presence of signs of wakefulness in EEG and EOG during a phase of sleep, it was termed as paradoxical sleep. Also, since it appeared to be an active state of the brain with desynchronization of the EEG within sleep, it was termed as active sleep or desynchronized sleep. Since dreams are associated with
this state of sleep, it has been termed as dream sleep. Additionally, as rapid eye movements during sleep formed a characteristic feature of this state, this state has also been termed as rapid eye movement (REM) sleep. The sleep state was thus classified into non-REM sleep and REM sleep.

The REM sleep is present in species higher in the evolutionary ladder, viz. birds and mammals, and has been classically identified by the simultaneous presence of desynchronization (low voltage high frequency waves) of the EEG, frequent eye movements, muscle atonia and hippocampal theta rhythm. Several other characteristic features, e.g. ponto-geniculo-occipital waves, irregular respiration as well as heart rate, body temperature fluctuation, etc. also are associated with this state. Though all the REM sleep signs may not be present in all the species, some of these signs may be expressed in some lower species suggesting that REM sleep-like state may be present in lower species as well[2-4]. Hence, it is debatable if REM sleep evolved in lower species or it is of relatively later origin in evolution. REM sleep loss affects several physiological processes necessary for normal routine behavior[5-7] and it is also essential for life to the extent that accumulated effect of its loss may be fatal[8]. REM sleep is regulated by the brainstem though other brain areas may modulate it as well. The role of specific area(s) and group of neurons in the brainstem that play key role in the regulation of REM sleep would be discussed. Briefly, the neurons in the locus coeruleus (LC) cease firing during REM sleep and if an experimental animal was not allowed to have REM sleep, these neurons in the LC continued firing incessantly leading to disturbance or loss of REM sleep. Alternatively, if these neurons were not allowed to cease firing either by continuous electrical stimulation[9] or by applying antagonist of the neurotransmitter that keep these neurons inhibited[10,11], REM sleep did not continue resulting in its reduction. Thus, the neurons in the LC must cease firing (as if it is a pre-requisite) for the generation of REM sleep and non-cessation of these neurons caused reduction of REM sleep associated with increased levels of norepinephrine (NE) in the brain which ultimately induces the effects associated to REM sleep deprivation/loss.

Localization of area(s) in brainstem responsible for the regulation of REM sleep

Initial studies to localize anatomical structure(s) in the brain responsible for the regulation of REM sleep generation started with transection and lesion experiments. The premise behind such studies is that if any normal manifestation, behavioral or otherwise, of a living organism continues to be expressed even after the destruction of certain brain area(s), the damaged area of the brain is possibly not essential for normal manifestation of the function under consideration. Work on cats with spinal transection and in humans with spinal injury showed that the spinal cord makes no essential contribution to the brainstem signs of REM sleep. The transection studies suggested that structures caudal to the midbrain and rostral to the spinal cord are necessary for REM sleep regulation. Further, when the pons was connected to midbrain and forebrain structures, most of the defining signs of REM sleep were seen in the rostral structures, whereas, if the pons was connected to the medulla and spinal cord, most of the identifying signs of REM sleep were seen in caudal structures. A transection through the middle of the pontine region abolished the major defining characteristic signs of REM sleep. Thus, based on the results of transection studies it was concluded that the pontine region in the brainstem is both necessary and sufficient to generate the basic phenomenon of REM sleep[12,13].

Pontine region and regulation of REM sleep

The pontine region contains noradrenergic, cholinergic as well as GABAergic neurons. The noradrenergic neurons are clustered in the LC, which is the primary site for supplying NE in the brain. The functional characteristic of these NE-ergic neurons in the LC is that they cease firing during REM sleep[14,15] and hence they have been termed as REM-OFF neurons. On the other hand, the cholinergic neurons in the laterodorsal tegmentum (LDT) and pedunculo pontine tegmentum (PPT) in the brainstem increase firing during REM sleep and they have been termed as REM-ON neurons[16]. The present knowledge indicates that interactions between the neurons located in these nuclei in the pontine region are responsible for the generation and regulation of REM sleep.

Locus coeruleus: An anatomical description

The LC is a small cluster of neurons situated in the pontine region near the wall of the fourth ventricle and is one of the few pigmented structures in the brain. Depending on the size of the cells and their organization, the LC has further been subdivided into LC-principal, LC-α, peri-LC-α and sub-coeruleus by some sleep researchers[17]. The LC or its analage, projecting to the forebrain is not found until reptiles[18] and avians[19], though some catecholaminergic neu-
rons projecting to the cerebellum and nearby tegmentum has been reported in teleosts and amphibians. Therefore, it was proposed that the development of LC is in tandem with the appearance of its cortical target areas. The number of neurons in LC increases from 200 in parakeet to 1,600 in rats and 20,000 in humans. Projections from these neurons divide into ascending and descending branches and innervate almost all the areas in the brain, spinal cord, and brainstem. The LC receives cholinergic as well as GABA-ergic projections from other parts of the brain and it also has GABA-ergic interneurons. Galanin-ergic and GABA-ergic neurons from ventrolateral preoptic area also project to the LC.

**Locus coeruleus and REM sleep**

There is ample evidence that the brain noradrenergic system plays a significant role in the regulation of REM sleep. Several techniques including electrical as well as chemical lesion, stimulation and microinjection have been extensively used to explore the role of LC in regulating REM sleep. Although these studies gathered a large volume of knowledge, much remains to be known in terms of the relationship of neurons in LC with other nuclei in the brain and the exact role that it plays in the generation and regulation of REM sleep. Electrical destruction of the dorsal part of LC did not suppress the occurrence of REM sleep. Similarly, destruction of ventral part of the LC (LCα and peri-LCα) was followed by irreducible disappearance of REM sleep atonia. However, destruction of LCp and LCα along with peri-LCα suppressed REM sleep completely during the two post lesion months. Electrolytic lesions of the dorsal noradrenergic bundle that ascends from the LCp resulted in increase in both non-REM sleep and REM sleep. The firing rate of the neurons in the LCp is maximum during wakefulness, decreases during non-REM sleep and almost ceases during REM sleep. While that of the neurons located ventrally increase their firing rate (almost exclusively) during REM sleep. The activity of the NE-ergic neurons in the LC has been positively correlated with activation of the sympathetic nervous system. Sympathetic activation is normally accompanied by EEG desynchronization and according to Reiner, the activity of the LC-NE-ergic neurons increases with an increase in discharge in the sympathetic nervous system. Reversible inactivation of the LCp by localized cooling (+10°C) induced non-REM sleep followed by REM sleep. On the other hand, it was found that continuous activation of the LC neurons inhibited REM sleep by reducing the frequency of generation of REM sleep although the duration per episode remained unaffected. Thus, the results suggested that activation of LC neurons did not allow REM sleep occurrence while inactivation of those neurons allowed REM sleep to continue.

**Norepinephrine in REM sleep**

The concentration of NE increased in the brain after REM sleep deprivation. An increase in NE concentration in serum was also reported after REM sleep deprivation. There was an increase in the activity of tyrosine hydroxylase, the enzyme involved in the first rate-limiting step of synthesis of NE and mRNA levels of tyrosine hydroxylase, whereas there was a decrease in the activity of the NE degrading enzyme, monoamine oxidase-A after REM sleep deprivation. The above findings may be supported by a recent study that there was increased tyrosine hydroxylase activity within the neurons located in the LC. These results suggested that there would be increased NE in the brain after REM sleep deprivation. An inhibitory role of NE on REM sleep may be supported by the fact that NE levels decreased during REM sleep. Since most of the supply of NE in the brain comes from the neurons in LC, it is likely that the activity of those neurons must be getting modulated during normal REM sleep and/or upon REM sleep deprivation. This view may be confirmed by the fact that the REM-OFF neurons in the LC cease firing during REM sleep, and they continue firing incessantly during REM sleep. Also, their activation by electrical stimulation or disinhibition by GABA-antagonist, picrotoxin did not allow REM sleep to continue whereas GABA in LC caused an increase in REM sleep. A comparative effect on the REM sleep upon electrical stimulation of LC neurons and microinjections of GABA as well as picrotoxin in LC are shown in Fig. 1A–E.

The studies mentioned above support the involvement of LC in the regulation of REM sleep. Those studies also suggest that continuous activity of the neurons in the LC possibly prevented generation of REM sleep and cessation of activity of those neurons induced REM sleep possibly through withdrawal of inhibition. However, the mechanism of cessation of activities of the LC neurons was not known. The presence of adrenergic receptors in the brain was shown long ago. Since the LC neurons are noradrenergic, agonists and antagonists of NE were used to study the role and mechanism of action of NE released by the LC-neurons in REM sleep regulation. REM sleep
Fig. 1. Percent changes in REM sleep under various experimental conditions. The original reference is shown beneath each pie diagram. The numbers in parenthesis show respective references in the reference list. The pie chart shows sleep-wakefulness recordings (8 h except where mentioned otherwise) under the following conditions: A: Without any treatment (control study). B: Single bilateral microinjection (250 nl) of normal saline into LC (control study). C: Low frequency, low amplitude electrical stimulation of bilateral LC for 8 h. D: Single bilateral microinjection (250 nl) of picrotoxin into LC. E: Single bilateral microinjection (250 nl) of GABA into LC. F: Continuous low frequency, low amplitude electrical stimulation of bilateral PrH. G: Continuous low frequency, low amplitude electrical stimulation of bilateral PrH in presence of single injection of picrotoxin into LC. H: Repeated intermittent microinjections (250 nl) of picrotoxin into bilateral LC for 48 h at an interval of 6 h. The sleep-wakefulness recording was also done for 48 h continuously.

was facilitated by systemic injection of drugs that stimulated β-adrenoceptors\textsuperscript{[51,52]} and by blocking α\textsubscript{1}-adrenoceptors\textsuperscript{[53-55]}. On the other hand, REM sleep was inhibited by blocking β-adrenoceptors\textsuperscript{[51,52]} and stimulation
of $\alpha_1$-adrenoceptors\cite{56}. Oral administration of prazosin in rats was found to shorten quiet waking and REM sleep while it increased active waking and slow wave sleep\cite{53}. $\alpha_2$-agonist, clonidine, when injected intraperitoneally, reduced REM sleep in rats and cats\cite{57,58}. A similar decrease in REM sleep was observed in man with a dose roughly five times smaller than that used in the rat\cite{59}. Yohimbine, $\alpha_2$-antagonist, increased active wakefulness immediately after administration but did not affect REM sleep. Though systemic injections advanced our understanding of the LC mediated regulation of REM sleep, localized injections of adrenergic agonist and antagonist provided a more robust evidence for the role of LC in the regulation of REM sleep.

The REM sleep was decreased when methoxamine, $\alpha$-1-agonist, was injected into the dorsal pontine tegmentum of cats. The decrease in total REM sleep was found to be due to both, an increased REM sleep latency and a reduced number as well as duration of REM sleep episodes\cite{60}. Bilateral injection of $\alpha_2$-agonist, clonidine, in the dorsal pontine tegmentum of cat produced an almost complete suppression of REM sleep\cite{61}, $\beta$-agonist isoproterenol almost suppressed REM sleep, while $\beta$-antagonist propranolol consistently enhanced it, mainly through an increase in the number of REM sleep episodes\cite{62}. Microinjection of $\beta$-agonist isoproterenol into medial septal region of basal forebrain significantly increased the time spent awake and a near complete suppression of REM sleep\cite{63}. Norepinephrine in peri-LC-$\alpha$ caused a dose-dependent inhibition of REM sleep and induction of REM sleep without atonia. These effects were also produced by clonidine, an alpha-$\beta$-agonist, whereas alpha-$\beta$-antagonists were found to block the effect of norepinephrine. When co-applied with carbachol into the caudal peri-LC-$\alpha$, clonidine completely blocked the marked REM sleep inducing effect of carbachol\cite{64}. Thus, the interaction of various networks of neurons having different adrenoceptors plays a crucial role for the generation and regulation of REM sleep. Although systemic and local injection studies advanced the knowledge about the role of NE in REM sleep regulation, they were unable to elucidate the role of NE released from the LC in such regulation. Based on our earlier studies\cite{65} and the results obtained in a recent study where LC stimulation was carried out in presence of adrenergic agonists and antagonists, we have proposed a model showing the possible mechanism of action of NE release from the LC-neurons and its role in REM sleep regulation\cite{66}.

The studies mentioned above suggest that although some NE may be needed, excess NE is inhibitory for the generation of REM sleep. The brain receives most of NE from the LC-neurons, which cease firing during REM sleep and they continue firing during REM sleep deprivation. Thus, cessation of firing of the LC-neurons is likely to be at least one of the key factors for the generation of REM sleep. Further, increased NE in the brain during REM sleep deprivation due to non-cessation of firing of the LC-neurons is likely to be the primary factor for REM sleep deprivation induced effects.

**How are the LC neurons kept active through wakefulness**

Normally REM sleep does not appear during wakefulness or immediately after going to sleep. It appears after certain period of non-rapid eye movement (NREM) sleep. At least in humans, the duration and number of REM sleep episodes increase with progress and depth of sleep through the night. Although it was known that the REM sleep cannot be initiated as long as the LC noradrenergic REM-OFF neurons continue firing\cite{64,67}, the cellular mechanism(s) of sleep-wake state dependent changes in the LC neuronal firing from highly active state during wakefulness to slowing down during NREM sleep and finally cessation of firing during REM sleep was not known. Mallick’s group proposed that the wakefulness inducing area, the midbrain reticular formation (MRF), possibly exerted opposite influence on REM-OFF and REM-ON neurons and hypothesized that the MRF would excite REM-OFF and inhibit REM-ON neurons during wakefulness. In a combined single unit recording and MRF stimulation study carried out in freely moving normally behaving cats it was observed that a majority of the neurons whose firing rate increased during spontaneous wakefulness, including the REM-OFF neurons, were excited, while the REM-ON neurons were inhibited\cite{67} by the MRF wakefulness inducing area. These results supported our hypothesis and suggested that the wake active neurons in MRF continuously excite the NE-ergic REM-OFF neurons in the LC and inhibit the REM-ON neurons throughout the waking period\cite{67,68}. This view may also be supported by the fact that activation of the REM-OFF neurons is reported to prevent REM sleep\cite{70} and is likely to increase the level of NE in the brain causing cortical activation and desynchronization of the EEG\cite{55,69-71}. Therefore, it is likely that continuous activation of the noradrenergic REM-OFF neurons contributes to EEG desynchronization associated with wakefulness, but not with that of REM sleep when the effect of cholinergic REM-ON neurons is pronounced.
This view may be supported by the power spectrum analysis study in the freely moving cats that the adrenergic and cholinergic antagonists affected different higher frequency bands of the desynchronized EEG. Additionally, as mentioned above, MRF wakefulness-inducing area inhibited the REM-ON neurons and this may be the cause for non-activation of the REM-ON neurons during waking period. It may be supported by the fact that activation of the area containing the REM-ON neurons increases REM sleep[72].

It has also been reported that activation of the REM-ON neurons in the peri-LC during REM sleep is responsible for muscle atonia during REM sleep[73,74]. All these results considered together provide possible explanation for neural mechanism as to why does muscle atonia, associated with REM sleep, not appear during wakefulness although the EEG is desynchronized during both those stages. Moreover, logical extrapolation of these observations is that in case of narcolepsy possibly there occurs an error in this neural pathway resulting in appearance of muscle atonia during wakefulness.

Thus, the following is likely to be the working model of neuronal mechanism of REM sleep generation. The wake active neurons in the MRF are active during wakefulness. Activity of these neurons keeps activating the REM-OFF neurons and inhibiting the REM-ON neurons, which do not allow REM sleep signs to appear during wakefulness. Experimentally, we found that the REM-OFF neurons are normally active during all the stages except during REM sleep, while the REM-ON neurons behave in an opposite manner. As a mechanism of action one or more of the following possibilities may exist. One, that MRF neurons exert independent excitatory and inhibitory effects on the REM-OFF and the REM-ON neurons, respectively; two, that the MRF neurons exert an excitatory effect on the REM-OFF neurons that in turn (may be through GABAergic neurons) inhibit the REM-ON neurons[68]; and three, that the MRF neurons exert an inhibitory effect on the REM-ON neurons and that in turn (may be through withdrawal of GABAergic inhibition) exert an excitatory effect on the REM-OFF neurons[48]. It was known that neurons in the wake (MRF) and sleep (caudal brainstem) areas are mutually inhibitory to each other[75,76] and at the onset of sleep the activity of the wake active neurons in the MRF is significantly reduced[77]. This reduction in the activity of wake active neurons in MRF gradually withdraws the excitatory and the inhibitory effects from the REM-OFF and the REM-ON neurons, respectively[67]. Gradually NREM sleep sets in when the sleep inducing neurons in the caudal brainstem[75,78] and basal forebrain further increase firing[79,81]. At some point when certain (yet unknown) conditions are satisfied, the sleep active neurons stimulate the REM-ON neurons which in turn actively inhibit and cease firing of the REM-OFF neurons (directly or indirectly) and initiate REM sleep[67,68]. Also, recently it was reported from our laboratory that picrotoxin, a GABA-A antagonist, in PPT, site of REM-ON neurons decreases REM sleep[82]. We expect that GABA from interneurons within the PPT[83] or from sleep area (caudal brainstem) dis-facilitates the inhibitory influence of NE-ergic inputs from LC onto PPT neurons resulting in an increase in REM sleep. These findings have been summarized in Fig.2.

How do the LC neurons cease firing during REM sleep

Reciprocal interaction model

The reciprocal interaction model was proposed by Hobson et al[87]. The model hypothesized that the REM-OFF neurons are inhibitory to REM-ON neurons and to themselves, but the latter are excitatory to the former and to themselves. Electrophysiologically it has been shown that presumably noradrenergic LC neurons and serotonergic raphe neurons are REM-OFF while the cholinergic FTG neurons are REM-ON. Thus, according to the hypothesis, inactivation of putative monoaminergic REM-OFF neurons plays a critical role in the generation and maintenance of REM sleep.

Mutual inhibitory model

The mutual inhibitory model between the NE-ergic REM-OFF and cholinergic REM-ON neurons was proposed by Sakai[84]. It was based on the hypothesis that cessation of firing of the REM-OFF neurons excites the REM-ON neurons by disinhibition while the excitation of the REM-ON neurons inhibits the REM-OFF neurons. Therefore, REM sleep can appear either by excitation of the REM-ON neurons or by inhibition of the REM-OFF neurons. This hypothesis seems to imply that for the generation of REM sleep, cholinergic neurons directly inhibit NE-ergic REM-OFF neurons.

Lacuna in the above mentioned models

The two hypotheses mentioned above did not consider the type and role of neurotransmitters involved in mediating such actions. As mentioned earlier, during REM sleep the cholinergic REM-ON neurons increased firing, the REM-OFF neurons in the LC ceased firing and continuous activation of the LC neurons by electrical or by chemical means prevented generation of REM sleep. Since NE inhibits cholinergic tegmental neurons[85], it is reasonable to understand
that projections from the monoaminergic REM-OFF neurons in LC would tonically inhibit the cholinergic REM-ON neurons during waking and NREM sleep states. However, since acetylcholine is reported to depolarise and excite the noradrenergic neurons in LC\[86\], it is not possible that the increased activity of the cholinergic REM-ON neurons, by releasing acetylcholine, would inhibit the REM-OFF neurons in the LC for the generation and regulation of REM sleep. These facts taken together strongly suggest presence of an inhibitory input that would transduce the cholinergic input from the REM-ON neurons on the REM-OFF neurons into an inhibitory one. Therefore, Mallick and his group hypothesised that an intervening inhibitory interneuron, possibly GABA-ergic, could be converting the excitatory cholinergic effect into an inhibitory effect on the REM-OFF neurons for the regulation of REM sleep\[87,88\].

**GABA-ergic interneuron based model**

The possibility of a role of GABA in LC for the regulation of REM sleep was supported by the facts that GABA-ergic interneurons and terminals are present in LC\[29\], GABA receptors are present on the neurons in LC\[89,90\] and GABA levels increase in LC during REM sleep\[91\]. Therefore, Mallick and his group hypothesised that an intervening inhibitory interneuron, possibly GABA-ergic, could be converting the excitatory cholinergic effect into an inhibitory effect on the REM-OFF neurons for the regulation of REM sleep\[87,88\].

**Fig. 2. Schematic diagram of a model for REM sleep regulation. The numbers in parenthesis represent the reference number. Abb: PrH, Prepositus hypoglossus.**

![Schematic diagram of a model for REM sleep regulation](image_url)
antagonist were micro-injected into the LC bilaterally either individually or in sequence one after the other in selected combinations. The microinjection study revealed that individual injection of the cholinergic agonist and antagonist, carbachol and scopolamine, respectively, affected the frequency of REM sleep; the agonist increased while the antagonist decreased the frequency of generation of REM sleep. On the other hand, GABA and picrotoxin individually affected the mean duration of REM sleep per episode; the former increased while the latter decreased the duration of REM sleep. These results suggested that GABA-ergic input to the LC modulated the duration of REM sleep per episode, while that of the cholinergic input modulated the frequency of generation of REM sleep. These results also indicated that GABA acted after the cholinergic system had initiated the action because maintenance of any process would be required only after the process had initiated. Thus, individual injection studies confirmed the role of cholinergic as well as GABA-ergic inputs into the LC for the regulation of REM sleep; however, the sequence of connections between those neurons could not be understood from those results.

To confirm, LC was microinjected with any of the following three combinations (a) GABA-ergic antagonist, picrotoxin, followed by cholinergic agonist, carbachol or (b) cholinergic antagonist, scopolamine, followed by GABA or (c) cholinergic antagonist, scopolamine, followed by GABA-ergic antagonist, picrotoxin. These microinjections were done with the assumption that if cholinergic input acted on the GABA-ergic neurons, carbachol in presence of picrotoxin would induce an effect similar to that of picrotoxin alone, while GABA in presence of scopolamine would show an effect similar to that of the GABA alone. The results from the combination injection studies showed that picrotoxin followed by carbachol microinjection caused significant reduction in REM sleep by decreasing mean REM sleep duration per episode, whereas scopolamine followed by GABA microinjection significantly increased REM sleep by increasing mean REM sleep duration per episode. Thus, when cholinergic and GABA-ergic agonist or antagonist (as the case may be) were injected into the LC in any combination, the effect of the GABAergic agonist/antagonist prevailed over that of the cholinergic. The most likely explanation for such a result is that the cholinergic input in the LC was acting on the GABA-ergic neurons to mediate its effects[49]. Thus, the observation supported our hypothesis that cholinergic influence in LC is mediated through GABA[47,48] and that acetylcholine-sensitive GABA-ergic neurons are present in and immediately around the LC[49].

In the above mentioned studies a complete suppression of REM sleep was not observed after blocking either the cholinergic or the GABA-ergic receptor. However, there was an almost complete suppression of REM sleep when scopolamine and picrotoxin were injected together. This supports mutually permissive and co-operative role between cholinergic and GABA-ergic systems in LC. Nevertheless, since cholinergic antagonist alone into the LC could not completely prevent occurrence of REM sleep, possibility of additional GABA-ergic input to LC from any other source that was triggered by the cholinergic REM-ON neurons, could not be ruled out. Since it was known that there was GABA-ergic input to the LC from the prepositus hypoglossus (PrH)[27] and that the latter receives input from PPT [49], the site of REM-ON neurons, it was hypothesized that the cholinergic REM-ON neurons could also inhibit the REM-OFF neurons in the LC by triggering the GABA-ergic neurons in PrH. To confirm the hypothesis, a combined study involving electrical stimulation of PrH neurons along with simultaneous picrotoxin microinjection in LC in freely moving normally behaving rats was carried out (Fig. 3). It was shown that PrH stimulation increased REM sleep. However, if the PrH stimulation was carried along with simultaneously blocking GABA-ergic transmission using picrotoxin in LC, the increased REM sleep was prevented (Fig.1F~G). The results showed that in addition to the local GABA-ergic input in LC, GABA-ergic input from PrH to LC may also modulate REM sleep[50]. Thus, the cholinergic REM-ON neurons may excite the GABA-ergic neurons either in PrH or locally in the LC, which in turn inhibit the REM-OFF neurons in the LC for the generation of REM sleep (Fig. 2).

Functional implications
The studies reviewed above show that the NE-ergic REM-OFF neurons in the LC must cease firing for the generation of REM sleep and if those neurons do not cease firing, REM sleep is significantly reduced. Alternatively, if REM sleep is not allowed to express, those neurons do not cease firing and they continue to remain active. Further, if those NE-ergic neurons in the LC continue firing incessantly there will be increased NE in the brain. The increase in NE levels in the brain because of incessant firing of LC neurons may also be indirectly supported by the fact that after increased waking (i.e. reduced NREM and REM sleep) there was increased expression of several transcription factors. This
increased expression was found to be regulated by the LC, the primary site for providing NE in the brain\textsuperscript{[97]}. Furthermore, Cirelli\textsuperscript{[98]} in her recent review put forward the argument that role of sleep (including REM sleep) is to interrupt continuous activity of the catecholaminergic system in the brain, which also supports our contention\textsuperscript{[5]}. These results also indirectly suggest that REM sleep deprivation caused increased levels of NE in the brain, which may be responsible for altered function(s) associated with REM sleep loss. In order to confirm the same it was hypothesised that if those neurons in the LC were not allowed to cease firing or kept continuously active, there would be loss of REM sleep leading to REM sleep deprivation induced changes. Further, these changes would be prevented by blocking the action of NE.

REM sleep deprivation is associated with reduced concentration, body weight, memory consolidation and increased irritability, food intake, aggressiveness, hypersexuality, etc.\textsuperscript{[5-7]}. At the cellular level it was shown that after REM sleep deprivation the inhibition of neurons induced by auditory stimulation was lost\textsuperscript{[99]}. Therefore, it was proposed by Mallick \textit{et al.} that one of the functions of REM sleep is to maintain neuronal excitability\textsuperscript{[5]}, however the mechanism of action was not known. It was hypothesized that after REM sleep deprivation there might be an increase in Na-K ATPase activity in neurons in the brain and that might be responsible for increased neuronal excitability. To confirm, Na-K ATPase activity was estimated in the normal (control) and REM sleep deprived rat brains. The results confirmed that there was increased Na-K ATPase activity after REM sleep deprivation while the enzyme was not significantly affected in the control animals\textsuperscript{[100]}. Further, it was confirmed by \textit{in vivo} and \textit{in vitro} studies using various adrenoceptor agonist and antagonist that the increased Na-K ATPase activity after REM sleep deprivation was due to increased levels of NE acting through $\alpha_1$ adrenoceptors\textsuperscript{[101]}. The role and mechanism of action of NE on Na-K ATPase activity was later studied in detail. Such studies revealed that NE acted on $\alpha_1$ adrenoceptor that activated phospholipase C pathway. In addition, NE released membrane bound calcium, activated calmodulin and dephosphorylated Na-K ATPase resulting in an increase in its activity\textsuperscript{[102-104]}. Thus, so far it has been shown that LC-neurons must stop firing for the generation of REM sleep and if they do not cease firing, there would be decrease or loss of REM sleep. Furthermore, during
REM sleep deprivation there is increased Na-K ATPase activity and this increase is due to increased NE in the brain.

Finally, it needed to be confirmed whether the REM sleep deprivation induced increase in NE level in the brain was due to non-cessation of the LC-neurons and that this increase in NE, as a result of non-cessation of LC-neurons, was accompanied with a simultaneous increase in Na-K ATPase activity in the brain. In order to do so, rats were surgically implanted with bilateral chemitrodes in the LC for microinjection and other electrodes for recording EEG, EOG and EMG. The rats were recovered with adequate post-operative care. It was shown earlier that the LC neurons ceased firing due to presence of GABA. Hence, after recovery from the surgical trauma, the LC of the normally behaving freely moving rats were infused with GABA-A antagonist, picrotoxin, every 6 h for 48 h and sleep-wakefulness was recorded continuously. At the end of the study, the brain was removed after decapitation and Na-K ATPase activity estimated. The sleep-wakefulness was also scored. The results showed that picrotoxin significantly reduced REM sleep in the rats (Fig.1/H) and there was significant increase in Na-K ATPase activity in the brain, which was comparable to that of REM sleep deprived rats[11]. Thus, it may be said with certainty that GABA is responsible for cessation of the noradrenergic REM-OFF neurons in the LC for generation of REM sleep. If those neurons do not cease firing there is loss of REM sleep and vice versa. Non-cessation of those neurons during REM sleep deprivation increases levels of NE in the brain and that is at least one of the primary factors for REM sleep deprivation induced behavioral and other effects[65].

Summary and conclusion
The primary site in the brain where there is maximum concentration of NE-ergic neurons is the LC. The neurons in the LC cease firing during REM sleep and hence are called as REM-OFF neurons. During REM sleep REM-OFF neurons are inhibited through GABA. These neurons fire incessantly during REM sleep deprivation and conversely, if they continue firing incessantly, REM sleep will not appear. Continuous activity of these neurons results in increased levels of NE in the brain. This increased level of NE is one of the major factors for mediating REM sleep deprivation induced effects.

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