Discussion
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REM-OFF and REM-ON neurons interact to regulate rapid eye movement (REM) sleep (Steriade and McCarley, 1990), however, the neuropharmacological basis and mechanism(s) of such regulation are matter of intense investigations. The cholinergic REM-ON neurons located in the laterodorsal/pedunculopontine tegmentum (LDT/PPT) remains shut throughout the sleep-wake cycle except during REM sleep when they are active, while the NA-ergic REM-OFF neurons located in the locus coeruleus (LC) are active all through but shut off only during REM sleep (Jacobs, 1986; Steriade and McCarley, 1990). The cholinergic neurons in the PPT receive NA-ergic inputs from LC (Rye et al., 1987). The PPT also possesses GABA-ergic neurons (Ford et al., 1995) as well as terminals (Rye et al., 1987). Thus, the cholinergic neurons in the PPT receive two types of inhibitory inputs, NA-ergic and GABA-ergic. Independent isolated studies have shown that both NA (Pal et al., 2005) and GABA (Gottesmann et al., 2002) affect REM sleep. It was hypothesized that GABA modulates those REM sleep related neurons for REM sleep regulation (Alam et al., 1993; Mallick et al., 1999). The hypothesis was confirmed for neurons located in LC through a series of in vivo microinjection studies (Kaur et al., 2001, 2004; Mallick et al., 2001). The role of GABA in PPT for REM sleep regulation as proposed earlier (Mallick et al., 2001) has been investigated in this study. Although the inhibition of REM-ON cholinergic neurons in PPT by NA forms the central paradigm of the current knowledge on REM sleep physiology, a direct involvement of NA in PPT of normally behaving freely moving animals for the regulation of REM sleep has not been shown till now. Further, the knowledge of the receptor subtypes of NA and GABA in PPT and the interactions, if any, between NA and GABA in PPT for the regulation of REM sleep was also lacking. The present study in freely moving normally behaving rats was conducted to investigate the role of GABA and NA as well as their interaction in PPT for REM sleep generation/maintenance. Also, attempt was made to better understand the GABA-ergic and NA-ergic receptor subtypes in PPT involved in REM sleep regulation.
I Role of GABA-ergic inputs in PPT for REM sleep regulation: The results showed that picrotoxin injection into PPT significantly decreased REM sleep (Pal and Mallick, 2004a). The decrease in REM sleep was due to significant reduction in the frequency of occurrence of REM sleep (number of REM sleep episodes) while there was no effect on the mean duration of REM sleep per episode. The effect of picrotoxin lasted for six post-injection hours (Pal and Mallick, 2004a), which is in conformity with the temporal extent of the effect of picrotoxin injection into LC (Kaur et al., 1997; Mallick et al., 2001). Microinjection of muscimol, GABA-A agonist, into PPT significantly enhanced the total time spent in REM sleep by increasing the number of REM sleep episodes (Pal and Mallick, 2004b). There was no effect on the mean duration of REM sleep per episode. The results of this study may be supported by a previous study in cats where muscimol (GABA-A agonist) and bicuculline (GABA-A antagonist) injection into the PPT increased and decreased REM sleep, respectively (Torterolo et al., 2002). However, in that study the number of REM sleep episodes as well as the duration of REM sleep per episode was affected unlike the present study where only a change in the frequency of REM sleep generation was observed. In addition, in the above-mentioned study in cat the injections were done unilaterally and manually, whereas in the present study in rats, the injections were made bilaterally using an automatic remote controlled pump. Simultaneous bilateral microinjection using an automatic remote controlled pump, as was done in this study, is advantageous over manual unilateral microinjection because the former is closer to normal physiological conditions. In unilateral injections, the effects of drugs in one side of the brain may be compensated by the corresponding target neurons on the other side of the brain. Besides the variations in species, the difference in anatomical locations and in neuron as well as receptor density might be responsible for the differences in results between cats and rats.

Since picrotoxin and muscimol decreased and increased the frequency of REM sleep generation, respectively, it suggests that GABA in PPT modulates the frequency of generation of REM sleep unlike in LC, where it maintains the duration of REM sleep (Kaur et al., 1997; Mallick et al., 2001). The role of GABA in PPT in REM sleep generation/regulation is also supported by the study, which showed increased c-Fos expression (marker of cellular activity) in GABA-ergic neurons in PPT during recovery from REM sleep deprivation (Maloney et al., 1999). It is known that GABA is an inhibitory
neurotransmitter. Therefore, agonist of GABA should inhibit and antagonist of GABA should disinhibit/increase the activity of target neurons. The presumed target of GABA in PPT is REM-ON neurons (Mallick et al., 2001). Thus, hypothetically withdrawal/blocking of inhibitory influence of GABA by microinjecting picrotoxin should result in increased activity of REM-ON neurons and consequent increase in REM sleep. However, in the present study, injection of GABA-ergic antagonist decreased REM sleep whereas injection of GABA agonist (muscimol) increased REM sleep. Considered along with the reported increase in the activity of GABA-ergic neurons in PPT (Maloney et al., 1999), the results obtained in this study strongly support a REM sleep promoting role for GABA in PPT. One of the possibilities explaining REM sleep promoting action and hence indirectly an excitatory role of GABA is that GABA is acting on an inhibitory presynaptic terminal that synapse onto REM-ON neurons. Hence, the withdrawal of GABA-ergic inhibition by picrotoxin microinjection facilitates the effect of inhibitory inputs onto the REM-ON neurons resulting in decreased REM sleep. In contrast, GABA agonist, muscimol, kept the activity of inhibitory inputs on REM-ON neurons in check resulting in increased firing of REM-ON neurons and consequently increase in REM sleep. Although the detailed cellular and synaptic mechanism of action of GABA cannot be confirmed from this study, in the light of the above-mentioned arguments it may be postulated that GABA is acting on presynaptic NA-ergic inhibitory terminals from LC, which keep the PPT REM-ON neurons inhibited during wakefulness and NREM sleep (Rye et al., 1987; Steriade and McCarley, 1990). The source of presynaptic GABA-ergic projections on the NA-ergic inhibitory terminals may be either the local GABA-ergic interneurons (Ford et al., 1995) or the GABA-ergic neurons in SNrpr (Inglis and Winn, 1995; Mena-Segovia et al., 2004).

II. Role of NA-ergic inputs into PPT for the regulation of REM sleep: The results showed that REM sleep was increased either by reducing the release of NA by applying NA-ergic α2-adrenoceptor agonist, clonidine, or by preventing spontaneously released NA to act on its receptors by applying α1- adrenoceptor antagonist, prazosin, or β- adrenoceptor antagonist, propranolol, into PPT. These findings suggest that: i) normally NA is available and tonically inhibits the PPT REM-ON neurons preventing the expression of REM sleep and ii) if such tonic inhibitory effect of NA on PPT neurons was
withdrawn, REM sleep was expressed. The NA-ergic neurons from LC project onto the cholinergic neurons in LDT/PPT (Rye et al., 1987; Honda and Semba, 1995). The NA-ergic neurons in LC are known to be active during all stages of sleep-wake cycle except during REM sleep (Jacobs, 1986). These LC neurons are also active during REM sleep deprivation (Mallick et al., 1990). Therefore, there would be relatively increased levels of NA in PPT during prolonged wakefulness and REM sleep deprivation. NA has been shown to modulate the activity of cholinergic PPT neurons in vitro (Muhlethaler et al., 1990). Systemic (Leppavuori and Putkonen, 1980) as well as local injections of either NA-ergic agonist (Tononi et al., 1991; Cirelli et al., 1992) or NA reuptake blocker (Hilakivi et al., 1987; Ross et al., 1990) decreased REM sleep. Also, the adrenoceptor antagonist prevented the physiological effect of REM sleep deprivation on Na⁺-K⁺ ATPase activity (Gulyani and Mallick, 1995). The role of NA in REM sleep regulation is also strengthened by the reports showing that non-cessation of LC neurons either by electrical (Singh and Mallick, 1996) or chemical (Kaur et al., 2004) means significantly reduced REM sleep.

1. Effect of clonidine microinjection into PPT on REM sleep: It was observed that clonidine injection into the PPT enhanced REM sleep by increasing the mean duration of REM sleep per episode. Since, clonidine acts on α₂- presynaptic autoreceptors and reduce NA release (Tecott, 2005), it suggests that normally NA is tonically released in PPT thereby preventing the generation of REM sleep and when NA release in PPT is reduced due to the cessation of LC neurons, REM sleep is initiated. Thus, clonidine in PPT seems to reduce the release of NA in PPT by acting on α₂- presynaptic autoreceptors resulting in withdrawal of NA-ergic inhibition and increased expression of REM sleep.

2. Effect of propranolol microinjection into PPT on REM sleep: The propranolol induced increase in REM sleep suggests that β-adrenoceptor in PPT is involved in spontaneous regulation of REM sleep. This supports an earlier in vivo study in cats, which reported increase in number of REM sleep episodes after bilateral propranolol injection into dorsal tegmental area (Tononi et al., 1991). However, the study was conducted in cats, the site of microinjection covered wider area including peri-LCα and pontine reticular formation and it was not confirmed if the injections were located...
within an area rich in cholinergic neurons. Nevertheless, these two studies broadly tend to agree that in PPT β-adrenoceptor mediated regulation of REM sleep is present across species. The involvement of β-adrenoceptors in PPT for REM sleep regulation was also proposed recently by systemic β-antagonist injection study (Mallick et al., 2005), though the presence of such receptors in PPT needs to be confirmed. Besides being a potent antagonist of β-adrenoceptor (Tecott, 2005), propranolol is also reported to have some 5-HT1A antagonistic properties. Since PPT receives serotonergic projections (Honda and Semba, 1995), it may be argued that propranolol-induced effect could partly be a result of synergistic action on β-adrenoceptor and serotonergic receptors.

3. Effect of prazosin microinjection into PPT on REM sleep: Increase in REM sleep after prazosin injection suggests that NA in PPT also acts on α1-adrenoceptors for the regulation of REM sleep. Isolated studies reported that there are two distinct populations of cholinergic neurons in PPT, one of which possess α1-adrenoceptors (Takakusaki et al., 1997; Hou et al., 2003) and NA excites a distinct population of cholinergic PPT neurons in vitro (Muhlethaler et al., 1990), presumably through α1-adrenoceptors (Fort et al., 1995; Szabadi and Bradshaw, 1998; Hou et al., 2003). There are also wake-active neurons in PPT (Garcia-Rill, 1991) and chemical stimulation of PPT neurons increased both wakefulness as well as REM sleep (Datta and Siwek, 1997). Further, PPT has strong inputs to thalamus and acetylcholine concentration is reported to be high in the thalamus during both wakefulness (Phillis et al., 1968) and REM sleep (Williams et al., 1994), as compared to slow wave sleep. These findings strongly support that separate populations of cholinergic neurons in PPT are involved in the regulation of REM sleep and wakefulness. Therefore, in the light of the above-mentioned evidence, it can be proposed that normally NA activates wake-active neurons through α1-adrenoceptors, which on inhibition by antagonist (in this study) reduced wakefulness. The reduction in wakefulness in turn indirectly increased REM sleep by disinhibiting the REM-ON neurons because wakefulness inducing area inhibits REM-ON neurons (Thankachan et al., 2001). This explanation may be supported by the observation of the present study that prazosin in PPT reduced quiet wakefulness in addition to increasing REM sleep. The differential role of adrenoceptors in maintaining REM sleep and wakefulness, as observed in this study, is strongly supported by a recent report that both
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REM sleep as well as waking were reduced in genetically modified mice lacking the ability to synthesize NA (Ouyang et al., 2004).

The results of this study however, do not agree with an earlier report that local injection of NA into PPT did not modulate sleep-wakefulness (Datta et al., 2003). The differences in the results could be due to any or all of the following factors: i) in that study NA as such was injected, which in addition to being a readily oxidizable compound also has a short half life (a few minutes in plasma), ii) the injected NA would have simultaneously acted on both the α and β subtypes of adrenoceptors evoking an opposite response (as observed in this study) resulting in apparently no significant change in REM sleep, iii) NA was unilaterally injected, whereas bilateral and simultaneous injection in the present study is closer to normal physiological conditions; effects of unilateral injection might be compensated by the neurons in the normal contralateral side; iii) the animals were disturbed by handling for inserting and removal of the cannula a few minutes before the injection unlike in our study where the cannulae were inserted long before and the injections were made using a remote controlled pump; and iv) it is also possible that normally there were enough NA in the system reaching a ceiling effect and hence additional NA injected from external source did not bring about any significant change in the response i.e., REM sleep. The injection of antagonist in this study withdrew the tonic effects of NA and thus was free from such drawbacks.

III. Co-injection of picrotoxin and clonidine into PPT: It was observed that the overall increase in REM sleep after injection of clonidine into PPT, was neutralized by simultaneous co-injection of GABA-A antagonist, picrotoxin. Picrotoxin in PPT decreased REM sleep by reducing the frequency of REM sleep generation while clonidine increased REM sleep by increasing the duration of REM sleep per episode. The co-injection expressed the effects specific to both clonidine and picrotoxin (when injected individually), thereby suggesting that the clonidine and picrotoxin must have acted independently and in parallel to induce respective effects on REM sleep. This is in contrast to the injection of cholinergic and GABA-ergic agonists/antagonists into LC, where the effects of one of the neurotransmitters prevailed when combination of those
receptor agonists and antagonists was microinjected suggesting that the former input acts on the latter neurons (in series) (Mallick et al., 2001).

IV. **Source of GABA-ergic inputs into PPT:**

1. **Electrical stimulation of SNrpr:** Anatomical link between GABA-ergic neurons in SNrpr and cholinergic neurons in PPT is well established (Inglis and Winn, 1995; Mena-Segovia et al., 2004), although a functional link showing the contribution of GABA-ergic neurons in SNrpr for the generation/maintenance of REM sleep, if any, was not shown. In order to investigate the role of SNrpr in REM sleep regulation, SNrpr was electrically stimulated. Further, SNrpr was electrically stimulated along with microinjection of picrotoxin in the PPT of the same animal. The premise behind these experiments was that if the GABA-ergic neurons in SNrpr were projecting to REM-ON neurons in PPT then the excitation of SNrpr GABA-ergic neurons by continuous electrical stimulation would release more GABA in PPT, which in turn would inhibit the NAergic projections presynaptically as discussed above. Hence, the excitation of SNrpr should result in an increase in REM sleep. However, the results showed that electrical stimulation of SNrpr alone as well as in the presence of picrotoxin in PPT did not cause significant change in any of the stages of sleep-wakefulness. Further, analysis in the bins of two-hours showed that electrical stimulation of SNrpr in the presence of picrotoxin in PPT significantly decreased the mean number of REM sleep episodes in the initial bin of two-hour. The decrease in the number of REM sleep episodes can be attributed to the effect of picrotoxin as was shown previously (Pal and Mallick 2004a). As compared to microinjection of agonists, electrical stimulation confers the advantage that the neurons in the nucleus of interest can be excited for a long duration. However, electrical stimulation excites the cell bodies as well as the fibers that pass through the area of stimulation. The confounding of the effects because of the stimulation of the cell bodies and the fibers could be one of the explanations for absence of any change in sleep-wakefulness after electrical stimulation of SNrpr. In addition, proximity of stimulation electrodes (implanted in SNrpr) to the EEG recording electrodes restricted the strength and duration of the stimulation pulse that could be used for stimulation. Increasing the strength, duration and frequency of the stimulation pulse
beyond those used in this study caused the appearance of excessive artifacts in the EEG records making it difficult to analyze sleep-wakefulness.

2. **Chemical stimulation of SNrpr:** In order to segregate the stimulation of cell bodies in SNrpr from a combined stimulation of cell bodies and fibers, glutamate (monosodium glutamate) was injected bilaterally into SNrpr. The results showed that bilateral injection of glutamate into SNrpr significantly enhanced the total time spent in REM sleep by increasing the mean number of REM sleep episodes. There was no effect on mean duration of REM sleep per episode. Analysis of the post-injection data in the bins of 2h showed that there was a statistically significant increase in the mean number of REM sleep episodes in bin 2 and bin 3. Although there was an increase of 45.31% in the mean number of REM sleep episodes in the first bin as compared to both baseline and saline injection group, the increase was not found to be significant primarily because of a high standard error of mean. It has been reported that the GABA-ergic neurons in SNrpr increase their firing rate exclusively during REM sleep (Datta et al., 1992). In addition, the GABA-ergic neurons in SNrpr show an increased c-fos expression (as a marker of neural activity), during recovery from REM sleep indicating that those GABA-ergic neurons are active during REM sleep (Maloney et al., 2002). These reports complement the observation in the present study that the chemical excitation of the presumed GABA-ergic neurons in SNrpr increased REM sleep. Thus, GABA-ergic neurons in SNrpr are one of the sources of GABA in PPT for REM sleep regulation.

V. **Interplay of NA and GABA in PPT for the regulation of REM sleep:**

The results of this study confirm that NA and GABA in PPT regulate spontaneous REM sleep. It is also evident that there is interplay of α1-, α2-, β-adrenoceptors and GABA-A receptors in modulating generation and maintenance of spontaneous REM sleep. Although the presence of adrenoceptor subtype on the REM-ON and wake-active neurons cannot be commented with certainty, it is unlikely that different subtypes of adrenoceptors are present on the same neuron. Thus, together with the existing knowledge, the present findings may be explained as follows (Fig. D1): REM-ON and wake-active neurons have been reported in PPT (Datta et al., 2001). It has also been reported that NA exerts excitatory and inhibitory effects through α1- and β-adrenoceptors,
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respectively (Fort et al., 1995; Szabadi and Bradshaw, 1998). Thus, it is being proposed that the β-receptors are present on the REM-ON neurons, while the wake-active neurons possess α1-adrenoceptors. NA in PPT simultaneously excites and inhibits the wake-active and REM-ON neurons through β- and α1- adrenoceptors, respectively. During wakefulness, sustained activity of NA-ergic REM-OFF neurons in LC increases the levels of NA in PPT. With prolonged wakefulness, the NA concentration in the PPT gradually increases to a level that activates presynaptic α2-adrenergic autoreceptors, which leads to reduction but not complete inhibition of NA release. The reduction in the levels of NA withdraws excitation from the wake-active neurons resulting in a gradual shift from wakefulness to sleep. Simultaneously, the reduced level of NA tends to withdraw the inhibition and prime the REM-ON neurons towards depolarization. However, the withdrawal of inhibition from REM-ON neurons does not appear to be sufficient to allow those neurons to be active. Under these conditions, although the precise mechanism is unknown, GABA from presumably non-cholinergic REM-ON neurons reaches the PPT and acts on the GABA-ergic heteroreceptors on the NA-ergic terminals. This action of GABA completely shuts off NA release resulting in dis-inhibition and thus activation of REM-ON neurons, which results in initiation of REM sleep. The picrotoxin prevented the action of GABA causing enhanced release of NA and therefore showed an effect opposite to that of prazosin and propranolol. The GABA-ergic projections onto the NA-ergic terminals in PPT may be reaching either from the local GABA-ergic interneurons (Ford et al., 1995) which are active during REM sleep (Maloney et al. 1999) and/or from the GABA-ergic neurons in substantia nigra that project to the PPT (Rye et al., 1987) as has been discussed above.

This model may be supported by previous findings that the wake and sleep inducing areas have excitatory and inhibitory effects on the REM-OFF neurons (Thankachan et al., 2001) and opposite effects on REM-ON neurons (Mallick et al., 2004). Thus, interplay of GABA and NA on respective receptors on NA terminals from REM-OFF neurons plays a significant role in regulating the levels of NA, which then acts on the α1- and β- adrenoceptors present on wake-active and REM-ON neurons respectively, for expression of respective behaviours. The levels of such neurotransmitters and their affinity on respective receptors are crucial factors for regulating the dynamic states.
VI. **Physiological Significance:**

This study provides a holistic understanding unlike most other isolated anatomical or pharmacological or physiological single chemical injection studies. In addition to being a key regulator of REM sleep, PPT modulates motivation, reward, cognitive and stimulus processing, which are affected by REM sleep loss; however, their neurochemical regulation was not known. REM sleep plays an important role in the pathophysiology of depression and its amelioration (Benca et al., 1997; Holsboer-Trachsler and Seifritz, 2000). The cholinergic-aminergic imbalance has been hypothesized for mood disorders, depression and other sleep related abnormalities like narcolepsy, REM sleep behavioral disorders and akathisia (Gillin et al., 2000). On the other hand, such psychiatric and behavioral disorders have been associated with disturbance in NA-ergic (Berridge and Waterhouse, 2003) and GABA-ergic systems, though the precise knowledge about interaction between these neurotransmitters is lacking. The present findings help in understanding the neurochemical mechanism and relationships between depression and psychosomatic disorders especially in association with REM sleep disorder due to modulation of NA-GABA-cholinergic axis and vice versa.
Noradrenergic REM-OFF Neurons (Locus coeruleus)

Pedunculopontine tegmentum

α1-, α2-, β- adrenoceptors; G_A - GABA-A receptor

(+): Excitatory; (-): Inhibitory

1. Rye et al., 1987
2. Tecott, 2000
4. Torterolo et al., 2002
5. Mena-Segovia et al., 2004
6. Honda and Semba 1994

Figure D1
Conclusion

The following conclusions can be drawn on the basis of the results obtained:

1. GABA plays a REM sleep promoting role in PPT.
2. NA in PPT inhibits the expression of REM sleep.
3. The NA-ergic α1-, α2 and β- adrenergic receptors in PPT are involved in REM sleep regulation. The presynaptic α2 adrenergic receptors are involved in the maintenance of REM sleep whereas postsynaptic α1- and β- adrenoceptors are involved in the generation of REM sleep.
4. GABA and NA in PPT interact with each other for the regulation of REM sleep. GABA inhibits the release of NA in PPT possibly by acting presynaptically on α2 adrenergic autoreceptors present on NA-ergic terminals in PPT.