Comparison of Three Muscarinic Agonists Injected into the Medial Pontine Reticular Formation of Rats to Enhance REM Sleep

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To gain insight into the subtypes of muscarinic receptors mediating the long lasting increase in REM sleep following microinjection of muscarinic agonists into the medial pontine reticular formation of the rat, multiple 60 nl injections were made utilizing three different muscarinic agonists. All agonists, in a concentration range of $10^{-6}$ to $10^{-2}$ Molar, increased REM sleep in a dose-related manner with an inverted “U” relationship. The order of potency to increase REM sleep was McN-A-343>oxotremorine-M=carbachol. Based on each agonist’s subtype-selective potency in functional assays, oxotremorine-M is the most potent agonist at every subtype. It was therefore concluded that the observed order of potency is not consistent with activation of a single muscarinic receptor. Inasmuch as McN-A-343 acts as a weak, partial agonist and the other ligands full agonists at m3 receptors, we hypothesize that activation of m3 receptors by oxotremorine-M and carbachol antagonizes REM sleep increases and results in their lowered potency. This hypothesis is consistent with preliminary evidence demonstrating that injecting a relatively selective m3 antagonist, p-F-HHSiD, can increase the REM-inducing potency of a low and formerly ineffective dose of oxotremorine-M. An m3 muscarinic receptor mechanism in the pons antagonistic to increasing REM sleep, present in the rat but absent in the cat, may underlie some of the species differences observed in the cholinergic induction of REM sleep.

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METHODS

Male Long-Evans Hooded rats (Harlan) weighing between 220 and 488 gms were anesthetized (ketamine/acepromazine, 80/2.5 mg/kg) and implanted with an array of electrodes for chronic sleep recording, which included the cortical electroencephalogram (EEG), the hippocampal EEG and the nuchal

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electromyogram. In addition, two guide-cannulae (Plastic One), with sealed cannulae inserted as stylettes (28-gauge), were implanted bilaterally and aimed at medial sites in the caudal, oral pontine reticular formation that were previously shown to support REM sleep increases in the rat after carbachol microinjections (bregma -8.3 to -8.8, L 2.9, 7.7 mm from skull, 14° lateral to medial angle). After one week recovery from surgery, animals were placed individually in our chronic recording environment (12 h light/12 h dark) connected to the recording equipment, where they remained for the duration of the experiment except when removed for adaptation to handling (each day for the first week) or during the injection procedures. As described previously (Marks and Birabil, 1998), injections were performed within 1 h after lights-on by inserting a cannula (28-gauge), which was back-filled with drug solution, connected to a 1.0-µl syringe through double-walled polyethylene tubing (Plastic One, Roanoke). The volume injected was held constant at 60 nl; 20 nl were injected every 45 secs followed by a 3-min period before the cannula was removed and stylet reinserted. Dose was adjusted by altering the molar concentration of active agent.

A total of 15 microinjections were given unilaterally to each of three pontine sites, one site per animal. At least four days elapsed between injections. The three muscarinic agonists (RBI) used were carbachol (CARB), oxotremorine-M (OXO-M) and McN-A-343 (McN) (concentrations in the range of 10^-2 - 10^-6 M). Microinjections of CARB at 10^-3 then 10^-4 M were administered first followed by the remaining concentrations of the three ligands in random order. Each animal also received four saline vehicle injections distributed through the course of the injection series for determination of baseline, control values. Following each injection, eight-hours of electrographic recordings were obtained and scored as wake, slow wave (SW) and REM sleep in 15-sec epochs. For each eight-hour recording, the measures of time in stage, stage-period frequency, stage-period duration, and latency to SW and REM sleep onset were computed. These measures were expressed as a percentage of the animal’s mean baseline recordings. Group means were determined different from baseline by the t-test for dependent means (α=0.05, two-tail).

An additional animal received five paired, consecutive microinjections to explore the sleep-related effects of OXO-M when preceded by a relatively selective m3 muscarinic antagonist, the p-fluoro analog of hexahydro-sila-difenidol hydrochloride (p-F-HHSiD) (RBI) (3 x 10^-4 M) (Lambrecht et al., 1989). Except for the unilateral, double injections, the procedures followed were the same as described above.

Following the last injection of the series, animals were killed by decapitation under deep pentobarbital anesthesia (80 mg/kg). Brains were removed, frozen, sectioned on a cryostat (40 µm), and stained with cresyl violet for identification of the injection sites.

RESULTS

Time in Stage (Figure 1)

All muscarinic agonists studied produced inverted "U"-shaped dose-response curves for increasing REM sleep. The 10^-4 M doses resulted in significant REM sleep increases for all agents, but only McN at the lower 10^-5 M dose significantly increased REM sleep over control values. The grouped data show a leftward shift in the curve of concentration-dependent responses for McN compared to both OXO-M and CARB, indicating an increased efficacy of McN to elevate REM sleep amounts when injected in the pons of the rat. In general, increased REM sleep was accompanied by moderate increases in SW sleep and significant decreases in wake. An exception to this occurred at the 10^-3 M dose of CARB, which reduced SW sleep with little effect on wake. In addition, CARB significantly increased SW sleep and decreased wake at the 10^-5 M dose while having only a small effect on REM sleep.
Significant increases in REM sleep period frequency paralleled the changes in REM sleep amount. Mean REM sleep percentages of baseline accounted for 74, 70 and 62 percent of the variation in REM period frequency across doses of CARB, OXO-M and McN, respectively ($R^2$, linear regression). No significant effects were detected for SW sleep or wake period frequencies and changes in these parameters appeared little related to agonists effects on sleep/wake amounts.

Period Duration (Figure 3)
Only McN had significant effects on REM sleep period duration, which also correlated well with effects on REM sleep amounts, accounting for 82% of the variation. Thus, the elevated REM sleep resulting from McN administration was due to a combination of increase in REM sleep period frequency and duration, while CARB and OXO-M elevated REM sleep predominantly through augmented period frequency alone. SW sleep period duration tended to be
increased by all agonist-ligands through the entire dose-range except for CARB at $10^{-3}$ M. No wake period durations significantly differed from baseline but there was a strong tendency towards decrease for all agonists at the $10^{-4}$ M dose, a dose at which all increased REM sleep.

**Latency (Data Not Shown)**

Both SW and REM sleep latency are highly variable measures in these studies. Mean values for latency to REM sleep onset for CARB and OXO-M never fell below 90% of baseline for any dose given. Mean latency was significantly reduced only after McN at the $10^{-3}$ M dose (60% of baseline). Mean values for latency to SW sleep onset tended to be decreased at all doses for all agents. Significant reductions were observed for CARB at the $10^{-4}$ and $10^{-5}$ M doses (62% and 69% of baseline) and for McN at the $10^{-3}$ M dose (45% of baseline). Reductions in latency for SW or REM sleep onset did not appear related to the muscarinic agonists’ actions on any other measure.

**p-F-HHSiD (Figure 4)**

Preliminary data were sought to test the hypothesis that m3 activation may be antagonistic to increasing REM sleep and that the lower efficacy of OXO-M compared to McN to increase REM sleep may be due to the activation of m3 muscarinic receptors by OXO-M (see Discussion). Consistent with results obtained in the previously described group, OXO-M at the $10^{-4}$ M concentration, in this case preceded by a saline injection, elevated REM sleep amounts in the following eight hours. This increase was 8.4 standard errors of the mean (SEMs) above the mean for this animal’s four baseline recordings. Also consistent with the ineffectiveness of lower doses was the minor increase in REM sleep that resulted from OXO-M at the $10^{-3}$ M concentration when preceded by saline (0.9 SEMs). Injecting the relatively selective m3 antagonist p-F-HHSiD ($3 \times 10^{-4}$ M) preceding the low and ineffective dose of OXO-M ($10^{-3}$ M) resulted in an enhancement of its REM sleep-elevating action to 2.7 SEMs above the baseline mean. This elevated amount of REM sleep does not appear to be the additive effects of these two agents. Injection of p-F-HHSiD ($3 \times 10^{-4}$ M) followed by saline resulted in a decrease in REM sleep (-3.5 SEMs). Neither does it appear that all muscarinic receptor antagonists can produce these interactive effects. The potent, but non-selective, muscarinic antagonist atropine ($10^{-4}$ M) preceding OXO-M ($10^{-3}$ M) resulted in REM sleep amounts not different from this dose of OXO-M preceded by saline (1.0 vs 0.9 SEMs above the baseline mean).

**Injection-Site Localization (Figure 5)**

All four injection sites utilized in this study were located in the caudal aspects of the nucleus pontis oralis (PnO), a region of the pontine reticular formation. The sites were encompassed by the anterior-posterior coordinates of Paxinos and Watson (1986), Bregma -8.3 to -8.8, and distributed through the dorsal-ventral extent of the reticular formation.

**DISCUSSION**

In evaluating the outcome of the current study, it is important to consider that the method of intracerebral injection does not permit a knowledge of the absolute concentration of ligand at the receptors mediating the behavioral response. Drugs are typically injected at high concentrations in small volumes, and following injection, concentrations probably decline rapidly as drugs diffuse away from the injection site. It is, however, reasonable to
assume that agents with similar chemical properties will have similar diffusion rates when injected with the same volume and concentration. Thus, comparisons can be made among agents injected at known concentrations having reasonable certainty that their relative concentration at the site of action will be the same. Bourgin et al. (1995) have reported the dose-response relationship for CARB utilizing intracerebral injection in the pontine region of the rat under study here with very similar results on sleep/wake parameters. This further indicates that these methods can yield reproducible results.

All three muscarinic agonists used in this study had qualitatively similar effects on the time spent in the different sleep/wake stages. Within the effective dosage ranges, the predominant result was a long-lasting elevation of REM sleep. This increase in REM sleep was almost always at the expense of wakefulness while SW sleep showed small and variable increases. Elevated REM sleep amounts were accompanied by significant increases in the frequency of REM sleep periods. Only with McN were REM sleep period durations also increased. Little systematic effect was observed on the latency to REM or SW sleep onset. Very similar effects on sleep/wake behavior result from the injection of a variety of agents into this region of brain including agonists of adenosine, vasointestinal polypeptide, and an inhibitor of adenylyl cyclase (Bourgin et al., 1997; Marks and Birabil, 1998, 2000). These findings indicate that a REM sleep modulating mechanism resides in the caudal aspects of the PnO of the rat.

In that the muscarinic antagonist atropine can block the REM sleep-inducing effect of CARB injection (Bourgin et al., 1995; Marks and Birabil, 1998), muscarinic receptors appear to be one of the mechanisms in the pons subserving the REM sleep modulation. All of the muscarinic agonists used in this study were capable of increasing REM sleep in a dose-related manner (inverted "U"). The increased potency, by at least an order of magnitude, of McN over CARB and OXO-M can shed some light on the muscarinic receptor subtypes mediating these effects. Interpretation of the current data relies on the subtype selectivity of the muscarinic ligands studied. That is, different ligands have different dose-response profiles when the responses are mediated by different receptor subtypes. This is usually reflected by a range of receptor binding affinities a ligand has for the different receptor subtypes. The degree of variance in these specific binding affinities determines a ligand’s selectivity. When interpreting dose-response curves, however, a ligand’s binding affinity may be poorly related to its ability to activate the receptor it binds to. A ligand with high affinity for a receptor and moderate ability to activate is referred to as a mixed or partial agonist, while an antagonist is unable to activate the receptor. A ligand may possess different properties of affinity and activation at different receptors. A functional assay of an agonist-ligand that is dependent upon the ability to activate a specific receptor provides a better basis for the interpretation of dose-response relationships. This is especially so for the currently available muscarinic ligands that express relatively low selectivity for the various muscarinic receptor subtypes. It was fortuitous in this study that a measurable difference among ligands was observed and surprising that McN exhibited the highest potency, since, based on receptor subtype binding affinities, McN is the least selective of the three ligands (Richards and van Giersbergen, 1995).

The following discussion largely relies on data reported by Richards and van Giersbergen (1995) that combines, in a single study, comparisons of receptor subtype activation for the muscarinic agonists examined here. Binding and functional assays were conducted on Chinese hamster ovary (CHO) cells transfected with genes encoding one of the five subtypes of human muscarinic receptors. Utilization of this expression system permits the comparison of agonists that is not confounded by the occurrence of multiple receptor subtypes found in tissues. Agonist receptor activation, however, is dependent upon many variables and some differences may arise with respect to tissue-expressed receptors.

Based on receptor subtype binding affinity, the m3 receptor is the only subtype at which McN has a higher affinity than OXO-M and CARB (approximately 2 and 14.5 fold, respectively). One might conclude that the higher binding affinity for the m3 receptor accounts for McN’s increased potency. A functional assay, however, reveals that McN has little or no agonist activity at m3 receptors. While OXO-M and CARB are effective in stimulating m3 coupled formation of inositol monophosphate in transfected CHO cells (EC$_{50}$ 0.22 and 0.9 mM, respectively), a response could not be obtained with McN until 1 mM concentrations were reached. McN has been observed to act as a partial agonist at m3 receptors in other expression systems as well (Hu and el-Fakahany, 1990). To the extent that these findings apply to cells expressing m3 receptors in the rat pons, activation of m3 receptors cannot mediate McN’s increased potency to induce REM sleep. On the contrary, the action to bind and not activate m3 receptors may be a key to its potency if activation of this receptor is antagonistic to increasing REM sleep.

Comparing potencies (EC$_{50}$) of the ligands in functional assays (formation of inositol monophosphate coupled to m1, m3 and m5 receptors, and inhibition of forskolin stimulated cAMP coupled to m2 and m4 receptors) reveals that McN is considerably less efficacious than OXO-M at all receptor subtypes and less than CARB at all but two (m1 and m4), where they are similar. Based on these data, there is no one muscarinic receptor subtype whose activation could result in elevated REM sleep that is consistent with the observed increased potency of McN over OXO-M and CARB. If activation of a single muscarinic receptor subtype mediated the REM sleep-inducing effects of ligand injection, OXO-M should be the most potent because EC$_{50}$ values are lowest for OXO-M in functional assays when mediated by any single muscarinic receptor subtype.

A preliminary experiment was performed to test the hypothesis that activation of m3 receptors antagonizes the effectiveness of low doses of OXO-M to increase REM sleep. In confirmation, we found that injecting the relatively selective m3 antagonist p-F-HHISiD preceding OXO-M resulted in increasing the potency of a formerly ineffective dose of OXO-M (10$^{-5}$ M) from 0.9 SEMs to 2.7 SEMs above the mean,
baseline, REM sleep amount. The non-selective, muscarinic antagonist atropine did not produce this effect and p-F-HHSiD in the absence of OXO-M resulted in a decrease in REM sleep. It is additionally interesting that this low dose of p-F-HHSiD (3 x 10^-4 M) was so effective in that the more potent antagonist atropine (1 x 10^-4 M) did not block the REM sleep-inducing effect at this low dose. Higher doses of atropine are required to block the effect of CARB (Bourgin et al., 1995; Marks and Birabil, 1998). This effect of p-F-HHSiD may be highly dependent on the dose and site at which it is applied in that injection of a much higher dose (3 x 10^-2 M) of p-F-HHSiD at a more caudal site in the reticular formation of the rat has been reported to be without effect on sleep/wake parameters (Imeri et al., 1994). Intracerebroventricular injection of p-F-HHSiD in rats resulted in decreased wake and increased SW sleep (Imeri et al., 1992). These latter effects were also observed here with injection of p-F-HHSiD alone. The currently observed decrease in REM sleep by a m3 antagonist could be interpreted as inconsistent with our proposed role for m3 receptors mediating reduction of REM sleep increases. This would not be so if m3 receptors were affecting alterations in SW sleep or wake mechanisms and indirectly influencing REM sleep. Thus, we do not conclude that m3 receptors in the pons of the rat mediate a direct inhibition of REM sleep, but rather are involved with mechanisms of SW sleep and wakefulness. Dependence in expression among the different states of arousal results in m3 activation limiting REM sleep amounts that would be increased further by activation of other muscarinic receptors. This may be accomplished through a mechanism controlling the duration of SW sleep periods. SW sleep period duration was greatly increased (over 30 SEMs from control) in the combination of the low dose of OXO-M and p-F-HHSiD (data not shown). In addition, the most potent agonist to increase REM sleep, McN, also was the most potent to increase SW sleep period duration (see Figure 3). The natural dependence of REM sleep on the preceding SW sleep may involve the mechanism by which activation of m3 receptors limits REM sleep expression.

The hypothetical data of Figure 6 illustrates our interpretation of the current results. Shown are two curves labeled m3 and mx. One curve represents the binding of OXO-M to m3 receptors producing concentration-dependent effects that are antagonistic to REM sleep increases. The other curve represents the binding of OXO-M to an unidentified receptor subtype, mx, that mediates induction of REM sleep. The nature of the OXO-M influence on REM sleep is dependent on activation of both receptor subtypes. Note that we predict the slope of the m3 curve to be shallower than that for mx. This difference in slope is critical to producing a range of concentrations of OXO-M at which mx receptors are activated to a greater degree than m3 receptors. This concentration range, labeled the REM Induction Zone for OXO-M, is where the activation of m3 is not sufficient to antagonize the REM sleep-inducing effects of mx activation. At slightly lower concentrations, the curves are closer together, so that in addition to OXO-M having less effect on either receptor, activation of m3 relative to mx is greater than in the REM Induction Zone. In this concentration range, blockade of m3 receptors can unmask the REM sleep-inducing action of OXO-M on mx receptors. At even lower concentrations, OXO-M would be ineffective due to its inability to sufficiently activate either receptor subtype.

If the role of m3 receptors is antagonistic to REM sleep induction, what receptor subtype or subtypes, mx, are activated following injection of muscarinic agonists into the pontine reticular formation of the rat to subserve the increased REM sleep? We predict from the current data that the EC50 in functional assays would be lower, more potent, for mx than m3 to produce the curves shown in Figure 6. Although receptor coupling to effectors and the number of receptors expressed may be additional factors, data indicate much lower EC50 s for the effect of OXO-M at m2 and m4 receptors when compared to m3 in their respective functional assays of cAMP inhibition and PI turnover (Richards and van Giersbergen, 1995). This differential relationship in potency of OXO-M to activate muscarinic receptor subtypes is also found in transfected CHO cells when a common assay is used reflecting guanine nucleotide exchange (Lazareno et al., 1993). Therefore, based on our model, mx should be m2, m4, or both receptor subtypes. This is consistent with our previous finding that an adenylyl cyclase inhibitor, SQ22.536 injected into pontine sites effective for CARB induction of REM sleep also produces a long-lasting increase in this sleep-state (Marks and Birabil, 2000). The m2 and m4 receptors are the subtypes that couple to G-proteins mediating the inhibition of adenylyl cyclase (Caulfield, 1993). We conclude that the increased potency of McN over CARB and OXO-M to induce REM sleep in the rat is due to the ability of McN to activate m2 or m4 receptors while not activating m3

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**Figure 6. Graph of a Hypothetical Relationship Between Concentration and Activation by OXO-M for Two Muscarinic Receptor Subtypes, m3 and mx.** The large rectangle indicates the concentration range of the REM Induction Zone. The smaller, dotted rectangle to the left indicates the concentration range where p-F-HHSiD can unmask the REM sleep inducing property of low doses of OXO-M. The small squares on each trace represent the concentration at half-maximal receptor activation (EC50). See text for discussion of model.
receptors. Both CARB and OXO-M may be more potent in activating m2 and m4 receptors, but their simultaneous activation of m3 receptors antagonizes their ability to increase REM sleep.

Selective mediation by different muscarinic receptor subtypes has been most intensely investigated utilizing the cat model of cholinergic induction of REM sleep. Although in the cat there is general support for the role of m4 and particularly m2 in this process (discussed in Baghdoyan and Lydic, 1999), no evidence for the antagonistic role of m3 receptors has been described. Utilizing a single and high dose (10^2 M) of agonists injected into the medial pontine reticular formation, Valazquez-Moctezuma et al. (1989) reported McN to be without effect on REM sleep amounts in the cat while OXO-M was equally effective as CARB in significantly increasing REM sleep. A study utilizing microdialysis in the cat, conducted by Sakai and Onoe (1997), in the region of the peri-locus coeruleus alpha report data implicating m3 receptors in mediating CARB induction of REM sleep. We believe there may be fundamental species differences between the cat and rat in the receptors mediating pontine induction of REM sleep. The presence of a mechanism that is antagonistic to increasing REM sleep in the rat and possibly absent in the cat may be related to the many species differences observed in the nature of REM sleep induced by muscarinic agonists (Vivaldi et al., 1980; Deurveilher et al., 1997). Among these differences in the cat compared to rat are: greater magnitude increases in REM sleep; significant reduction versus small or no reduction in REM latency; and a broad, effective, dose-range versus a narrow, effective, dose-range producing an inverted "U" dose-response relationship. Simultaneous activation of an antagonistic mechanism with pontine injections of muscarinic agonists may limit the amount of REM sleep expressed, prevent the hastening of the appearance of REM sleep, and permit REM sleep increases only within dose-ranges where the REM-promoting effects are high and the antagonism relatively low. The role implicated in the present study for the m3 muscarinic receptor subtype supports such an antagonistic mechanism in the pontine reticular formation of the rat in the modulation of REM sleep.

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