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GABA_A Receptors Implicated in REM Sleep Control Express a Benzodiazepine Binding Site

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Abstract

It has been reported that non-subtype-selective GABA_A receptor antagonists injected into the nucleus pontis oralis (PnO) of rats induced long-lasting increases in REM sleep. Characteristics of these REM sleep increases were identical to those resulting from injection of muscarinic cholinergic agonists. Both actions were blocked by the muscarinic antagonist, atropine. Microdialysis of GABA_A receptor antagonists into the PnO resulted in increased acetylcholine levels. These findings were consistent with GABA_A receptor antagonists disinhibiting acetylcholine release in the PnO to result in an acetylcholine-mediated REM sleep induction. Direct evidence has been lacking for localization in the PnO of the specific GABA_A receptor-subtypes mediating the REM sleep effects. Here, we demonstrated a dose-related, long-lasting increase in REM sleep following injection (60 nl) in the PnO of the inverse benzodiazepine agonist, methyl-6,7-dimethoxy-4-ethyl- α -carboline (DMCM, 10⁻² M). REM sleep increases were greater and more consistently produced than with the non-selective antagonist gabazine, and both were blocked by atropine. Fluorescence immunohistochemistry and laser scanning confocal microscopy, colocalized in PnO vesicular acetylcholine transporter, a presynaptic marker of cholinergic boutons, with the $\alpha 2$ subunit of the GABA_A receptor. These data provide support for the direct action of GABA on mechanisms of acetylcholine release in the PnO. The presence of the $\alpha 2$ subunit at this locus and the REM sleep induction by DMCM are consistent with binding of benzodiazepines by a GABA_A receptor-subtype in control of REM sleep.

Keywords

DMCM; Gabazine; nucleus pontis oralis; rat; GABA_A receptor $\alpha 2$ subunit; muscarinic receptor

1. Introduction

The brainstem reticular formation has long been recognized as a locus mediating control over sleep/wake behavior (Moruzzi and Magoun, 1949). Despite the application of diverse neuroscience methodologies, specific mechanisms of the reticular formation and their

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interactions to achieve state-control are incompletely understood. Here, we address one specific mechanism of interaction between the GABAergic and cholinergic systems in the oral pontine reticular formation (PnO) in control of REM sleep in the rat.

The PnO of rat is a site in which local injection of non-subtype-selective GABA_A receptor (GABA_AR) antagonists results in a long-lasting increase in REM sleep (Marks et al., 2008; Sanford et al., 2003). We hypothesized a mechanism by which antagonizing GABA disinhibits acetylcholine (ACh) release from cholinergic axons in the PnO. Support for this mechanism came from studies of in vivo microdialysis of the GABA_AR antagonist, bicuculline, in the PnO of cat and mouse that resulted in significantly increased levels of microdialyzed ACh (Flint et al., 2010; Vazquez and Baghdoyan, 2004). REM sleep increases produced by injecting the GABA_AR antagonist, gabazine (GBZ), into rat PnO were blocked by a preceding local injection of the muscarinic antagonist, atropine (Marks et al., 2008). These findings support a mechanism of disinhibition of ACh release in PnO and result in the well documented induction of atropine sensitive, cholinergic-mediated REM sleep (Bourgin et al., 1995; Marks and Birabil, 1998).

Local injection of selectively acting agents to antagonize GABA synaptic transmission in PnO could identify specific GABA_AR subtypes mediating REM sleep increases, and immunohistochemistry could anatomically localize the expression of these GABA_ARs. This line of investigation would delineate the pharmacological properties and location in PnO of the subset of GABA_ARs mediating REM sleep control.

GABA_ARs are ligand-gated Cl⁻ ion channels structurally assembled from five individual protein subunits, which include α 1– α 6, β 1– β 3, γ 1– γ 3, δ , and ϵ (reviewed in Olsen and Sieghart, 2009). The interface between the α 2 and certain alpha subunits (α 1, α 2, α 3 and α 5) form the benzodiazepine (BZ) binding site (reviewed in Sieghart, 1995; Sigel and Steinmann, 2012). In the current studies, a BZ inverse agonist was injected into PnO to determine whether the GABA_AR-subtype subserving REM sleep induction expresses a functional BZ binding site.

The α -carboline, methyl-6,7-dimethoxy-4-ethyl- α -carboline (DMCM), has a high affinity for the BZ binding site (Sieghart, 1995), and it acts at this site as an inverse agonist (Honore et al., 1983). In a heterologous expression system, the action of DMCM (10⁻⁶ M) on α 1, α 2, α 3 GABA_ARs increased the EC₅₀ for GABA greater than five-fold (Campo-Soria et al., 2006), indicating an allosteric alteration of GABA_ARs and antagonism of GABA activity. Thus, its action is the inverse of a BZ-site agonist. If the action of DMCM in PnO is to produce an atropine-sensitive REM sleep induction, then it likely would be acting at the same receptors subserving the action of GBZ in PnO to increase REM sleep.

Localization of GABA_ARs within cholinergic terminals in PnO would facilitate a direct action of GABA to inhibit ACh release, and for GABA_AR antagonists to block this inhibition and promote increased release. Immunohistochemistry can be used to determine if cholinergic terminals express GABA_ARs and whether their subunit composition is consistent with possessing a BZ binding site. Preliminary to a rigorous quantitative investigation, we sought to determine if α 2 GABA_AR-subunits colocalize to cholinergic axon fiber varicosities in PnO.

In the results presented below, we show DMCM was capable of inducing REM sleep when injected into the PnO of rat and that it was blocked by a preceding injection of atropine. Further, we observed α 2 GABA_AR-subunits in some of the cholinergic varicosities. Unexpectedly, we found sites in PnO where DMCM was effective at increasing REM sleep but GBZ was not.

2. Results

2.1 DMCM injected into PnO altered states of arousal

DMCM (10^{-2} M, 60 nl) produced a significant increase in REM sleep compared to mean control values for the eight-hours following injection into the PnO (see sites injected fig.1). For the entire group of eight sites investigated, REM sleep was significantly increased to a mean $173 \pm 13.7\%$ of control. To a lesser extent, DMCM also significantly increased non-REM (NREM) sleep ($126 \pm 7.5\%$) and decreased wake ($88 \pm 2.0\%$). The time course plotted in two-hour intervals indicates the long-lasting duration of these effects on states of arousal (fig. 2).

2.2 Dose-dependent action of DMCM on REM sleep

Group means ($N=5$), as a percentage of mean control, for different doses of DMCM are shown in Figure 3 for indices of REM sleep. DMCM significantly increased REM sleep amounts to $184 \pm 20.1\%$ for the 10^{-2} M dose whereas it did not significantly alter REM sleep for the 10^{-3} or 10^{-1} M dose (fig. 3A). This outcome was mirrored for REM period frequency (fig. 3B) with significant increases observed only for the 10^{-2} M dose ($183 \pm 24.7\%$). At no dose tested was REM period duration or REM sleep onset latency significantly altered (not shown). Thus, REM sleep was increased due to increased period frequency and not period duration. The dose-response relationship for DMCM increased REM sleep is that of an inverted-AU@, indicating reduced effectiveness at high doses.

2.3 DMCM action on REM sleep was atropine sensitive

For determination of dependency on muscarinic cholinergic receptors, five-sites were tested with an injection of atropine (4×10^{-3} M) that preceded a second injection of DMCM (10^{-2} M) or normal saline, in random order (fig. 4). For the group ($N=5$), DMCM significantly increased REM sleep as a percentage of mean control ($148 \pm 8.1\%$). Neither atropine preceding saline ($90 \pm 5.5\%$) nor atropine preceding DMCM ($78 \pm 9.8\%$) significantly differed from control, or from each other, on total REM sleep amount in the 8-hours following injection. REM sleep amounts were significantly greater following DMCM alone compared to saline or DMCM preceded by atropine. Pre-injection of a dose of atropine, which alone is not sufficient to reduce REM sleep, blocked the DMCM-increased REM sleep.

2.4 Actions of DMCM and GBZ injected into PnO were not identical

It was highly unexpected to find the action of DMCM on REM sleep at sites in PnO not to be accompanied by similar REM sleep increases by GBZ. There was a total lack of linear relationship between the two GABA-antagonizing drugs to increase REM sleep ($r=0.09$). DMCM (10^{-2} M) injections, at all eight sites, resulted in REM sleep totals for the eight hours following injection being greater than the 95% confidence interval of the four mean control injections. GBZ, at these same sites, resulted in only 5 of 8 sites out of the 95% confidence interval and a significantly lower mean increase than DMCM for the five sites ($t=2.68$; $P < 0.05$). As shown in Figure 5, there is an apparent independence between the effects of DMCM and GBZ when data were grouped according to the effect of GBZ on REM sleep.

In a previous study, we had shown atropine not only blocked the REM sleep increase by GBZ in PnO, but it also unmasked an action of GBZ to reduce REM sleep (Marks et al., 2008). Here we found that atropine blocked all tested REM sleep increases by either GBZ or DMCM. Atropine reduced REM sleep further when preceding DMCM, but it was highly variable, not statistically significant (see above), and there was no relationship between the DMCM increase and atropine-coupled decrease. In a smaller sample ($N=3$), atropine further reduced REM sleep when injected prior to GBZ (77 ± 12.6), and this also was not statistically

significant for similar reasons and no relationship between the increase and decrease was observed. Three sites respectively received atropine in combination with both DMCM and GBZ (fig.6). The data indicated the lack of a relationship between the magnitudes of the REM sleep increase and the atropine-related decrease. Although variable, atropine interacting with GBZ and DMCM did result in a sizable decrease in REM sleep at some sites in PnO (fig. 6).

GBZ injection into the PnO previously was shown to induce compulsive ipsilateral turning behavior (Marks et al., 2008). In the current study, we found induction of turning by GBZ (10^{-4} M) in 3 of the 8 cases. The mean time from injection to the first contraversive turn was 38 min. Although all three sites without significant REM sleep increases following GBZ did not express compulsive turning, there was no relationship in the remaining five cases between the magnitude of the REM sleep increase and induction of turning. No cases of turning behavior were observed following DMCM at any given dose, including the three sites in which GBZ induced turning was observed.

2.5 GABA_ARs were observed in Cholinergic varicosities in PnO

Vesicular acetylcholine transporter (VACHT) and vesicular GABA transporter (VGAT) are specific presynaptic markers for cholinergic, and GABAergic or glycinergic boutons, respectively (Gilmor et al., 1996; Chaudhry et al., 1998). Our preliminary investigation identified an estimated 10% of VACHT-labeled varicosities in PnO colocalized the $\alpha 2$ GABA_AR subunit. Often observed was a VGAT-labeled varicosity apposed to such VACHT/ $\alpha 2$ colabeled varicosities (fig. 7). It appeared that some cholinergic boutons in PnO expressed GABA_ARs and they are likely involved in a synapse with a GABAergic axon fiber. The GABA_AR subtype contained a $\alpha 2$ -subunit, which is consistent with expression of a BZ binding site (Sigel and Steinmann, 2012).

3. Discussion

We have shown that the BZ inverse agonist, DMCM, was an effective inducing agent of a long-lasting increase in REM sleep when injected into the PnO of the rat. These results confirm previous findings of antagonizing action of GABA at GABA_ARs in PnO of rat utilizing the receptor antagonists, bicuculline and GBZ (Marks et al., 2008; Sanford et al., 2003). Action by DMCM strongly implicates a GABA_AR-subtype expressing a BZ binding site mediating these effects in PnO.

3.1 Dose-dependent action of DMCM on REM sleep

DMCM (10^{-2} M) induced increases in REM sleep through increased REM period frequency and had little effect upon the duration of REM episodes. This is a characteristic shared by other agents injected into PnO including GBZ and cholinergic agonists (Bourgin et al., 1995; Marks et al., 2008). Another common feature of drug-induced REM sleep in rat was a lack of very short REM sleep latencies that were observed in cat following injection into PnO (Baghdoyan et al., 1987; Xi et al., 1999). The basis of the species differences remains to be understood. It is yet unclear what results obtained in rat will apply to other species such as cat.

The shape of the dose-response relationship for DMCM to induce REM sleep was an inverted AU@. This was a feature shared with other REM sleep inducing agents including cholinergic agonists (Marks and Birabil, 2001). Of the three doses tested (10^{-3} , 10^{-2} , 10^{-1} M), only the 10^{-2} M dose significantly increased REM sleep. It is possible the high dose resulted in ACh levels being too high to produce an optimal response, however, a previously described mechanism can account for the observed dose-response relationship. DMCM has

been shown to bind to a low-affinity site on the GABA_AR complex located in a binding pocket at the interface of the α and β subunits, BZ binding site is at the interface of α and γ subunits (Stevenson et al., 1995). This low-affinity site for DMCM is the binding site for the anticonvulsant drug, loreclezole. Both DMCM and loreclezole act as agonists at this site producing allosteric changes resulting in potentiating efficacy of GABA (Stevenson et al., 1995). Action at this binding site would occur only at high concentrations due to the low affinity of DMCM. This can explain how the highest dose of DMCM (10^{-1} M) failed to increase REM sleep. Action to potentiate GABA would antagonize the DMCM-BZ-site-effect to lower the efficacy of GABA. This interpretation is most straight forward if these opposing actions were to occur at the same GABA_ARs. Binding at the low affinity site may delay onset of REM sleep even at the 10^{-2} M dose as one would expect initial concentrations at receptors following injection to be highest. This may be responsible for not having found a reduction in latency.

3.2 DMCM action on REM sleep is dependent on functional muscarinic receptors

We showed a dose of the muscarinic antagonist atropine (4×10^{-3} M), which did not significantly alter REM sleep preceding a vehicle injection in PnO, resulted in complete block of the REM sleep increase when preceding a DMCM (10^{-2} M) injection. As with GBZ (10^{-4} M), this confirms the REM sleep increases resulting from antagonizing GABA to be dependent upon local, functioning, muscarinic receptors. Inasmuch as the GABA_AR-subtype acted upon by DMCM is also subject to antagonism by GBZ, it is likely both these agents acted upon the same GABA_AR-subtypes subserving the REM sleep increase. This would be a subtype expressing a BZ binding site. Further support for this interpretation comes from similarities in the nature of the REM sleep increases produced by DMCM and GBZ (see Marks et al., 2008). These include: induction of a long-lasting increase in REM sleep (~8 hrs) through increased REM period frequency not duration; small effects on REM sleep latency; trends to increase NREM sleep and decrease wake (significant for DMCM); and the action of atropine to completely block the REM sleep increases induced by either DMCM or GBZ. The shared sensitivity to atropine is perhaps most convincing as to a common mechanism dependent upon functioning muscarinic receptors.

3.3 Cholinergic boutons in PnO express GABA_ARs

Utilizing fluorescence confocal microscopy in a descriptive examination, we found colocalization of VAcHT labeled varicosities and the $\alpha 2$ GABA_AR-subunit in the PnO. This is consistent with direct presynaptic control over ACh release by GABA. $\alpha 2$ subunit immunoreactivity has been reported in processes in PnO with moderate density and absent from somata (Pirker et al., 2000). This mechanism is supported further by the finding of local blockade of GABA_ARs in PnO of cat and mouse that resulted in increased levels of ACh (Flint et al., 2010; Vazquez and Baghdoyan, 2004). Cholinergic agonists, GABA_AR antagonists and a BZ inverse agonist injected into the PnO of rat induced increases in REM sleep with very similar characteristics and these drug-inductions were blocked by atropine (Bourgin et al., 1995; Marks et al., 2008). Thus, the mechanism of GABA antagonism is dependent upon the function of local cholinergic receptors. The presence of GABA_ARs on cholinergic boutons in PnO favors the direct action of GABA_AR blockade on these presynaptic terminals to increase ACh release and result in a cholinergic REM sleep induction mediated through muscarinic receptors. GABA_ARs are ubiquitously expressed in the PnO (Pirker et al., 2000). Data presented here support the conclusion that the specific GABA_ARs mediating REM sleep-induction by GABA_AR antagonists are selectively located on boutons of cholinergic axon fibers and, at least in part, include a subtype containing the $\alpha 2$ subunit. Presence of the $\alpha 2$ subunit is consistent with expression of a BZ-binding site (Sigel and Steinmann, 2012) and further supports the action of DMCM to directly produce a

disinhibition of ACh release from cholinergic terminals in PnO resulting in increased REM sleep.

Not all VACHT immuno-labeled varicosities in PnO colabeled for the $\alpha 2$ -subunit. We roughly estimate about 10% contain detectable $\alpha 2$ subunit label. Work is in progress performing a more rigorous, quantitative evaluation of this percentage. Efforts also are directed towards determining whether these double-labeled varicosities arise from a specific cholinergic neuronal population, and/or are apposed to a specific neuronal type or muscarinic receptor-subtype in PnO. Many double-labeled cholinergic varicosities in PnO were observed apposed by VGAT immuno-labeled varicosities. This is consistent with the GABA_ARs on cholinergic boutons in rat PnO being postsynaptic to a specific GABAergic innervation. Identifying the source, or sources, of this GABAergic innervation presynaptic to cholinergic terminals in PnO could reveal a critical, up-stream mechanism of REM sleep control.

It has been recently reported that the GABA analog 4,5,6,7-tetrahydroisoxazolo (5,4-c)pyridin-3(-ol) (THIP) injected into rat PnO at low concentrations resulted in significant increases in wake and significant decreases in NREM and REM sleep (Vanini and Baghdoyan, 2013). Similar results also were reported for injections of the non-selective GABA_AR agonist muscimol (Sanford et al., 2003), however, THIP potentiates the action of GABA with high selectivity for certain $\alpha 2$ -subunit expressing GABA_AR subtypes and has low affinity for $\alpha 2$ expressing GABA_AR subtypes (Meera et al., 2011). In addition, while the distribution of $\alpha 2$ -subunits in PnO was localized to processes, $\alpha 1$ -subunits were localized to somata (Pirker et al., 2000). $\alpha 1$ -subunits are associated with extrasynaptic GABA_ARs mediating tonic inhibition (Belelli et al., 2009), while $\alpha 2$ -subunits are associated with GABA_ARs clustered postsynaptically mediating phasic transmission (Thomson and Jovanovic, 2010). This indicates a highly complex role for GABA in PnO in the control of states of arousal. It appears that tonic inhibition of at least some local neurons in PnO promotes wakefulness and disinhibition through specific axo-axonic GABAergic synapses on cholinergic terminals promotes sleep, particularly REM sleep.

3.4 Actions of DMCM and GBZ injected into PnO are not identical

We have presented the case for DMCM and GBZ to induce REM sleep following injection in PnO by acting upon the same GABA_ARs and through the same cholinergic mechanism. Consistent with this argument, we posit site-specific differences in these GABA_AR-ligands to be caused by their differential actions on other GABA_AR-subtypes distributed in the PnO.

3.4.1 DMCM—DMCM acting as an agonist at its low-affinity site differs in valence from the action of GBZ. In that the non-selective, GABA_AR agonist muscimol injected in PnO resulted in increased wakefulness and reduced REM sleep (Sanford et al., 2003; Vanini and Baghdoyan, 2013), this can account for the ineffectiveness of the 10^{-1} M dose of DMCM to increase REM sleep and possibly the tendency to reduced REM sleep when atropine preceded the 10^{-2} M dose. These actions, presumably, would include, but not be limited to GABA_ARs controlling ACh release. There also may be an involvement of GABA_CRs where loreclezole inhibited GABA induced currents (Thomet et al., 2000) and DMCM may bind to this site. The efficacy of DMCM at the 10^{-2} M dose indicates its dominant action to increase REM sleep is through its high affinity BZ-binding site acting as an inverse agonist and yet, at this dose, site-differences with GBZ were observed.

3.4.2 GBZ—GBZ is a non-selective GABA_AR antagonist. DMCM acts as an inverse agonist only at a subset of GABA_ARs expressing the BZ binding site. One consequence of this may be seen in the ability of GBZ injected into PnO to induce stereotyped, ipsiversive

turning behavior. We had shown this response to be independent of REM sleep increases and insensitive to atropine (Marks et al., 2008). Here we found DMCM at 10^{-2} M, or any dose tested, incapable of inducing turning at PnO sites at which GBZ induced turning behavior. This supports an interpretation of the GABA_AR mediating the turning-response not being one expressing a BZ-binding site as well as illustrating that GBZ acts upon mechanisms in the PnO that DMCM does not.

Based on the eight, PnO sites investigated here, there was an apparent lack of consistent relationship in the magnitude of REM sleep increases produced by DMCM (10^{-2} M) and GBZ (10^{-4} M) at each site. While DMCM was effective at all sites in PnO, GBZ was highly variable and failed to increase REM sleep at three sites. Compared to a previous study of GBZ (10^{-4} M) injected in PnO of rat that demonstrated greater efficacy to increase REM sleep (Marks et al., 2008), sites in the current study were located more dorsally in the pons. This indicates there may be a mechanism in the dorsal pons, not interacting with DMCM, but resulting in GBZ antagonizing its own action to increase REM sleep. Two sites at which GBZ was ineffective were very close to or impinging upon the pedunculo-pontine tegmental nucleus (PPT) located just dorsal to the PnO (see fig. 1). DMCM (10^{-2} M) increased REM sleep greater than 200% at both these sites. Pal and Mallick (2004) have shown GABA_AR antagonists injected into PPT of rat resulted in a significant decrease in REM sleep. Thus, GBZ at these dorsal injection sites could have antagonized REM sleep increases by action in the PPT. Similar diffusion of DMCM may not result in the same antagonism of REM sleep because the GABA_ARs mediating this effect in PPT to reduce REM sleep do not have a BZ binding site. A third site without GBZ effect on REM sleep was ventral, but at the lateral boarder of the PnO. Data about this site are insufficient to speculate on a mechanism to account for variance in the actions of GBZ and DMCM. A local effect by GBZ to antagonize REM sleep increases is plausible. More needs to be known about the mechanisms antagonizing REM sleep increases in PnO with GBZ.

We previously demonstrated atropine not only to block REM sleep increases by GBZ, but also to unmask a GBZ-induced decrease in REM sleep (Marks et al., 2008). Low statistical power and a small size of effect prevented a replication here for the group, but the reduction was observed in individual cases. This unmasking-effect indicates the presence of GABA_ARs in PnO, that when GABA is antagonized, mediates a REM sleep decrease independent of muscarinic receptor function. Such a mechanism could account for variability in GBZ=s effects on REM sleep if expression of the REM-antagonism also were variable across PnO sites. In addition, if these GABA_ARs did not express a BZ-binding site, then it would not operate with DMCM-inductions of REM sleep. One aspect of the data, however, failed to support this hypothesis to the extent we found no relationship between the magnitude of REM sleep increase by GBZ and the magnitude of the decrease when following atropine. Relationships among GBZ=s ability in PnO to both increase and decrease REM sleep may be dependent on several, yet unknown, mechanisms involving multiple GABA_AR-subtypes.

3.5 Unexpected reported results

A recent study measuring levels of ACh reported on the microdialysis of three BZ-site agonists into the PnO of anesthetized rat (Hambrecht-Wiedbusch et al., 2010). Diazepam was without effect, zolpidem produced only a small, but significant increase in ACh at the highest dose, and eszopiclone (ESZ) produced a dose-related increase in ACh reaching more than 400% of control levels at the 10^{-3} M concentration. To the extent these agonists would potentiate efficacy of GABA, we would predict a decrease in ACh release in PnO based on previous and current findings. We can identify two factors, in this study, possibly contributing to these unexpected results. One was the use of isoflurane, known to interact with GABA_ARs allosterically potentiating GABA (Hall et al., 1994). Based on previous

reports, one would predict a potentiation of the ESZ-agonist effect (Sieghart, 1995). There may, however, be a specific interaction of isoflurane with ESZ in their simultaneous binding to the same GABA_AR. A single cysteine substitution in the $\alpha 1$ subunit (F99C) resulted in ESZ acting as an inverse agonist (Morlock and Czajkowski, 2011). It appears to us, the results of the microdialysis study are consistent with a negative modulation of GABA_ARs by ESZ. Allosteric alteration of the BZ-binding site by isoflurane may make certain binding residues inaccessible to ESZ altering its action. This is a testable hypothesis.

A second factor was the use of 1% dimethyl sulfoxide (DMSO) as a solvent. The wide spread use of DMSO for this purpose at 1% or less reflects an acceptance of this procedure. It has been shown in brain slices, however, that DMSO perfused through the bath accumulated in the tissue (Nasrallah et al., 2008). This phenomena also would be expected to occur with continuous perfusion during in vivo microdialysis. In the study under discussion, the PnO was perfused for 62.5min with Ringer's solution containing 1% DMSO followed by drug solution for 62.5 min also with DMSO (Hambrecht-Wiedbusch et al., 2010). The tissue concentrations during drug infusion and ACh dialysate collection could have been considerably higher than 1%. It is generally agreed that high concentrations of DMSO can produce many varied effects upon cellular physiology and here may have influenced the release of ACh in interaction with ESZ. Use of anesthetized rats precludes knowledge of the effects on sleep. DMCM is water soluble and we would predict increased ACh levels when the appropriate dose is dialyzed in the unanesthetized rat PnO without use of DMSO. This remains to be shown.

3.6 Conclusion

DMCM at a dose likely to selectively act as an inverse agonist at the BZ binding site at GABA_ARs, reliably induced a long-lasting increase in REM sleep when injected into the PnO of rat. Sensitivity to atropine indicates mediation through the local cholinergic system. GABA_ARs were found in cholinergic boutons in the PnO expressing a GABA_AR-subunit, $\alpha 2$, consistent with the presence of a BZ binding site. These finding support the conclusion of a GABA_AR with a BZ binding site directly disinhibiting ACh release from axon terminals in PnO in the control of REM sleep. Multiple, GABA_AR-dependent mechanisms reside in the PnO in control of states of arousal. Additional investigation is required to identify these mechanisms and determine how they may interact.

4. Experimental Procedures

4.1 Animal preparation

All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the local Institutional Animal Care and Use Committee at the Dallas VA. Male, Long-Evans Hooded rats (Harlan, Indianapolis, IN, USA) weighing between 255 and 446 gms were instrumented for chronic sleep-recording under isoflurane anesthesia. As previously described (Marks et al., 2008), this consisted of electrodes for recording: cortical electroencephalogram, two screws tapped ipsilaterally into skull respectively over somatosensory and motor cortex; hippocampal electroencephalogram, a stainless steel wire (200 μ m dia.), insulated except at the tip, implanted in the dorsal hippocampus referenced to a cortical screw over the cerebellum; and the nuchal electromyogram, two spring electrodes sutured under the trapezius muscle. In addition, guide cannulas, with stylettes inserted (Plastics One, Inc., Roanoke, VA, USA) were stereotaxically, bilaterally aimed at sites in the PnO (bregma -7.8 to -8.8 , L 1.0, 8.6 mm from skull). Lateral to medial angles of approach were used (14° from vertical). A total of 8 PnO sites were investigated in 7 rats.

After one week recovery from surgery, animals were placed individually in sound-attenuated, video-monitored, light- (12 h light/12 h dark) and temperature-($22.0 \pm 1.5^\circ\text{C}$) controlled enclosures. A cable connected to an electrical commutator tethered each rat to the recording equipment while allowing unrestricted movement in the cage. Animals remained in the recording environment except when removed for adaptation to handling (each day for the first week).

4.2 Intracerebral injection

Injections were performed unilaterally within 30 min of the start of the dark period by inserting a cannula (28-gauge, extending 1mm from the guide-tube), back-filled with drug solution, connected to a 1.0- μl syringe (Hamilton, Reno, NV, USA) through, oil-filled, polyethylene tubing (Plastics One). All solutions were filter sterilized (0.2 μm , pore size) before injection. The volume injected was held constant at 60 nl, of which 20 nl were injected every 45 s followed by a 3-min wait before cannula was removed. Under conditions in which two injections were carried out consecutively at the same site (double injection), the cannula was purged, refilled with oil, and back-filled with second drug solution. The second injection followed the procedure of the first ending with stylette reinsertion. The rat was then returned to its cage. Each pontine site received multiple single or double injections with a minimum of seven days between injection-procedures.

All chemicals were dissolved in normal saline. Each rat received four normal saline-vehicle injections as control distributed randomly throughout the course of the drug injection series. The drugs and concentrations used were: GBZ (SR-95531, Tocris Bioscience, Ellisville, MO, USA), 10^{-4} M; DMCM (Tocris), 10^{-3} , 10^{-2} , 10^{-1} M; atropine sulfate (Sigma-Aldrich, St. Louis, MO, USA), 4×10^{-3} M. All 8 PnO sites received GBZ and DMCM 10^{-2} M, 5 sites received all three doses of DMCM in random order, 5 sites received double-injections of atropine/saline and atropine/DMCM 10^{-2} M, 3 of these sites received an additional atropine/GBZ. No site was included in the analysis in which a final injection did not reproduce a previous injection in the series with a significant increase in REM sleep. This was usually a DMCM 10^{-2} M dose. This procedure was used to support the sites= pharmacological responsiveness throughout the series of injections.

Polygraphic recordings were obtained for 8 hrs following each injection-procedure. Electrical potentials were digitized (256 S/s) and stored in the European Data Format. Recordings were visually scored (Rodent Sleep Stager, Grass Instruments, West Warwick, RI, USA) utilizing standard criteria without knowledge of the experimental condition. Each 15-s epoch was assigned a score of wake, NREM sleep or REM sleep. From these data, stage totals, bout frequency, and mean bout duration were determined for each hour. Bout frequency was a count of the number of times an epoch was scored a particular stage that was preceded by an epoch scored a different stage. Mean bout duration was computed by dividing the time in stage by the bout frequency. REM sleep and NREM sleep latencies were computed based on the interval from stylette reinsertion after injection to the first continuous 30-s episode (~2 epochs) of each respective sleep state.

After completion of the injection experiments, rats were killed by decapitation under deep isoflurane anaesthesia and injection sites were determined in coronal sections (30 μm) stained with cresyl violet or neutral red and mapped with the aid of the atlas of Paxinos and Watson (1997).

4.3 Immunohistochemistry and microscopy

Details of the immunohistochemistry and fluorescence laser scanning confocal microscopy have been previously described (Liang and Marks, 2009). Briefly, an additional two male

rats (Harlan Sprague-Dawley, Indianapolis, IN, USA) were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and then perfused through the ascending aorta with 30 ml heparinized saline and 500 ml of 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4) for 30 minutes. The brains were removed, blocked in the coronal plane, post-fixed in the same fixative for 1 hour and cryoprotected by immersion in 20% sucrose in PB. Serial, coronal sections, 20 μ m-thick were cut on a freezing microtome (SM2000R, Leica, Nussloch, Germany) through the pons, and collected in 0.1M PB, with 0.005% sodium azide. The region of interest was the PnO defined as the area of the pontine reticular formation approximately 7.8 to 8.8 mm posterior to the bregma suture (Paxinos and Watson, 1997).

Free-floating sections were primarily blocked with 3% normal donkey serum and 0.3% Triton X-100 in PBS for 60 min and then incubated in a mixture of primary antibodies raised against VAcHT, 2 GABA_AR subunit and, VGAT for 18–20 hours at 4°C (see Table 1). After washing with PBS, sections were incubated with fluorescent-tagged (Cy2, Cy3 and Cy5) secondary antibodies (Jackson ImmunoResearch, West grove, PA, USA) for 3 hours. After 30 min wash with PBS, sections were mounted and cover-slipped with mounting medium containing glycerin and phenylenediamine.

Confocal microscopy was performed to examine relationships among VAcHT, 2 and, VGAT positive boutons in PnO. Fluorescence (Cy2, Cy3 and Cy5) images were obtained with a laser-scanning confocal microscope (objective: 63 $\times$, water, NA 1.2) using three lasers with excitation wave lengths of 488, 543, and 633 nm, respectively (LSM510-META, Zeiss, Göttingen, Germany). Confocal images were imported into Photoshop software (CS4, Adobe, San Jose, CA, USA) for composition of merged images and global adjustments of brightness and contrast.

4.4 Statistical analyses

All statistical analyses were performed in SigmaPlot12 (Systat Software, Richmond, CA, USA) using an $\alpha = 0.05$ to determine significance. One-way repeated measures ANOVAs were used for each dependent variable in experiments based on 8-hour recordings with individuals receiving multiple drug injections. In instances where main effects were found significant, multiple comparisons using the Bonferroni t-test were conducted to determine significant differences between treatments and control. Means are reported $\pm$ standard error of the mean (SEM). Correlational analyses were computed as Pearson product-moment coefficients and tested for differences from zero with two-tailed tests, $\alpha = 0.05$. Confidence intervals were computed two-tailed at the 95% level.

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Abbreviations

ACh	acetylcholine
BZ	benzodiazepine
DMCM	methyl-6,7-dimethoxy-4-ethyl- -carboline
DMSO	dimethyl sulfoxide
ESZ	eszopiclone

GABA_AR	GABA _A receptor
GBZ	gabazine
IC	inferior colliculus
LDT	laterodorsal tegmental nucleus
Non-REM	NREM
PAG	periaquiductal grey
PBS	phosphate buffered saline
PnO	nucleus pontis oralis
PPT	pedunclopontine tegmental nucleus
S.E.M.	standard error of the mean
THIP	4,5,6,7-tetrahydroisoxazolo (5,4-c)pyridin-3(ol)
VACHT	vesicular acetylcholine transporter
VGAT	vesicular GABA transporter
VTg	ventral tegmental nucleus of Gudden

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Highlights

!BZ inverse agonist, DMCM, increased REM sleep when injected into the PnO of rat !
 REM sleep induction was completely blocked by atropine at a dose not affecting REM
 sleep ! 2 GABA_AR-subunit was observed colocalized to many cholinergic varicosities in
 PnO !Data support a GABA_AR subtype binding BZ in control of REM sleep through
 control of ACh in PnO

Individual Differences in Drug Effects REM Sleep as Percentage of Mean Control

Fig. 1.

Schematic coronal sections of the pons (after Paxinos and Watson, 1997). Injection sites are represented by oval symbols. Dark symbols (red, N=5) indicate GBZ increases in REM sleep greater than the 95% confidence interval of control mean (GBZ significant). Light symbols (yellow, N=3) indicate increases not exceeding the 95% confidence interval (GBZ not significant). All increases following DMCM were above the 95% confidence interval. Numbers to the lower left of each section indicate distance (mm) caudal to the Bregma suture. DR, dorsal raphe; LDT, laterodorsal tegmental nucleus; PnO, nucleus pontis oralis; PPT, pedunculopontine tegmental nucleus; SC, superior colliculus; VTg, ventral tegmental nucleus of Gudden; xscp, decussation of the superior cerebellar peduncle.

Mean REM Sleep Increase Grouped by Gabazine Effect

Fig. 2. Time course effects on the means of total: A, REM sleep; B, NREM sleep; and C, wake following intracerebral injection of the BZ inverse agonist DMCM at a concentration of 10^{-2} M (60 nl). The eight hours are divided into four consecutive blocks starting at the time of injection. Each block is the mean of the percentage of control value (N=8). Error bars are $\pm$ S.E.M.

Fig. 3. Effects of GBZ (10^{-4} M) and three doses of DMCM (10^{-3} , 10^{-2} , 10^{-1} M) on REM sleep parameters as a percentage of control (100%) for the eight hours following injection into PnO. The parameters are: A, total time in REM sleep; B, REM sleep bout frequency per hour. * indicates values significantly different from 100% ($P < 0.05$, $N = 8$). Error bars are $\pm$ S.E.M.

Fig. 4. Effects of DMCM (10^{-2} M) and atropine (4×10^{-3} M) on the means of REM sleep totals as a percentage of control (100%) for the eight hours following injection into PnO. The drug conditions are: DMCM, DMCM alone; Atr/sal, double-injection of atropine followed by normal saline; and Atr/DMCM, double-injection of atropine followed by DMCM. * indicates values significantly different from 100% as well as significantly different from Atr/sal and Atr/DMCM ($P < 0.05$, $N = 5$). Error bars are $\pm$ S.E.M.

Fig. 5. Effects of DMCM (10^{-2} M) and GBZ (10^{-4} M) on the means of REM sleep totals as a percentage of control (100%) for the eight hours following injection into PnO. Results of injections at the eight sites are divided into two groups: One group of sites at which GBZ increased REM sleep above the 95% confidence interval for mean control (GBZ significant, N=5); and the other group at which GBZ failed to increase REM sleep (GBZ not signif., N=3). All REM sleep increases following DMCM are above the 95% confidence interval. Error bars are $\pm$ S.E.M.

Fig. 6. Responses of the three individual sites (A–C) receiving both atropine followed by GBZ and DMCM on the means of REM sleep totals as a percentage of control for the eight hours following injection into PnO. The conditions are: GZ-4, GBZ (10^{-4} M) alone; DM-2, DMCM (10^{-2} M) alone; A/sal, atropine (4×10^{-3} M) preceding normal saline; A/GZ-4, atropine preceding GBZ (10^{-4} M); and A/DM-2, atropine preceding DMCM (10^{-2} M). Pairs of dashed lines indicate the 95% confidence interval for the mean of four, saline, vehicle-control injections at each site.

Fig. 7. Fluorescence confocal image representative of double-labeled varicosities in PnO co-expressing VACHT (blue) and the $\gamma 2$ GABA_AR-subunit (red) apposed by a varicosity labeled for VGAT (green). Arrowhead points to the apposition of VGAT- and a VACHT/ $\gamma 2$ -labeled varicosity (arrow). Scale bar: 5 μ m.

Table 1

Primary Antibodies used in the Study

Antigen	Species Raised in	Source and Code	Dilution	Specificity Reference
VACHT	Goat	Chemicon, AB1578 Temecula, CA, USA	1:2,000	(Arvidsson et al., 1997) (Henny and Jones, 2006)
2	Rabbit	PhosphoSolutions 832-GG2C Aurora, CO, USA	1:500	(Sperk et al., 1997) (Pirker et al., 2000)
VGAT	Mouse	Synaptic Systems 131 011 Göttingen, Germany	1:1,000	(Takamori et al., 2000)