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Inhibitory and excitatory amino acid neurotransmitters are utilized by the projection from the dorsal deep mesencephalic nucleus to the sublateralodorsal nucleus REM sleep induction zone

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Abstract

The sublateralodorsal nucleus (SLD) in the pons of the rat is a locus supporting short-latency induction of a REM sleep-like state following local application of a GABA_A receptor antagonist or kainate, glutamate receptor agonist. One putatively relevant source of these neurotransmitters is from the region of the deep mesencephalic nucleus (DpMe) just ventrolateral to the periaqueductal gray, termed the dorsal DpMe (dDpMe). Here, the amino acid neurotransmitter innervation of SLD from dDpMe was studied utilizing anterograde tract-tracing with biotinylated dextranamine (BDA) and fluorescence immunohistochemistry visualized with laser scanning confocal microscopy. Both markers for inhibitory and excitatory amino acid neurotransmitters were found in varicose axon fibers in SLD originating from dDpMe. Vesicular glutamate transporter2 (VGLUT2) represented the largest number of anterogradely labeled varicosities followed by vesicular GABA transporter (VGAT). Numerous VGAT and VGLUT2 labeled varicosities were observed apposed to dDpMe-labeled axon fibers indicating both excitatory and inhibitory presynaptic, local modulation within the SLD. Some double-labeled BDA/VGAT varicosities were seen apposed to small somata labeled for glutamate consistent with being presynaptic to the phenotype of REM sleep-active SLD neurons. Results found support the current theoretical framework of the interaction of dDpMe and SLD in control of REM sleep, while also indicating operation of mechanisms with a greater level of complexity.

Keywords

REM sleep; GABA; Glutamate; Glycine; Biotinylated dextran amine; Vesicular transporter

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1. Introduction

The neural mechanisms subserving rapid eye movement (REM) sleep have proven remarkably resistant to elucidation despite application of a wide variety of neuroscience techniques. Historically, a critical role for the cholinergic system was proposed based mainly on the result in cat of direct, local intracerebral administration of muscarinic agonists to sites in the pons able to induce a short latency onset and prolonged duration of REM sleep episodes (Baghdoyan et al., 1987; Vanni-Mercier et al., 1989). Sites of high sensitivity were identified in the pontine reticular formation as well as a site ventral to the locus coeruleus (LC) termed the peri-LC α (Baghdoyan et al., 1987; Vanni-Mercier et al., 1989). Questions of species generalizability of these mechanisms arose due to the failure to replicate these findings in rodent models. Sites in the caudal aspects of the rostral pontine reticular formation of rat were found to induce a long-lasting increase in REM sleep with cholinergic agonists and other neurotransmitter systems' ligands, but no evidence for a short latency "triggering" of the state had been reported in this species (Bourgin et al., 1995; Marks and Birabil, 1998).

More recently it has been found that GABA_A receptor (GABA_AR) antagonists injected into the area of the peri-LC α of cat induced REM sleep similar to muscarinic agonists, but with shorter duration of effect (<2 hr) (Xi et al., 1999). This finding was followed by the first demonstration of short latency induction of a REM sleep-like state in head-restrained rat with iontophoretic application of GABA_AR antagonists, gabazine and bicuculline, into the site homologous to peri-LC α , termed the rostral sublaterodorsal nucleus (SLD) (Boissard et al., 2002). Application of cholinergic agonists induced wakefulness and failed to increase REM sleep indicating limits to the cross-species homology. Short latency onset of a REM sleep-like state has been replicated with pressure injection of bicuculline in the SLD of the freely moving rat (Pollock and Mistlberger, 2003).

The SLD contains neurons that fire selectively in REM sleep (termed, REM-on) (Boissard et al., 2002). Iontophoretic application of the excitatory, glutamate receptor agonist, kainate, in head-restrained rat, also triggered a REM sleep-like state within minutes of the start of its ejection (Boissard et al., 2002). Ibotenic acid lesions of the SLD destroying >90% of neurons resulted in about a 60% reduction in REM sleep (Lu et al., 2006). These findings are consistent with the concept that excitation of REM-on neurons in SLD is sufficient to initiate REM sleep (Boissard et al., 2002; Fort et al., 2009; Lu et al., 2006). This is further supported by the widespread projections of SLD neurons, both rostrally and caudally, that could be responsible for recruiting various neuronal populations subserving many processes of REM sleep (Boissard et al., 2002; Luppi et al., 2007; Lu et al., 2006; Vetrivelan et al., 2009) including the two major physiological indicators of REM sleep, muscle atonia and cortical activation.

The REM sleep-inducing effect of GABA_AR antagonism in SLD was blocked by local application of kynurenic acid, a non-selective ionotropic glutamate receptor antagonist (Boissard et al., 2002). This is consistent with disinhibition of glutamate excitation being the action of GABA_AR antagonists to induce REM sleep. A current model proposed by Luppi

(Luppi et al., 2012) posits the presence of a tonic glutamatergic excitation of SLD REM-on neurons. The state-selective, REM-on pattern of discharge by these neurons is posited to be produced by a potent GABAergic inhibition that is removed only during REM sleep. Application of sufficient kainate can overcome this inhibition and GABA_AR antagonists disinhibit permitting the REM-on neurons to become active and induce REM sleep. This negative control by GABA in the SLD is viewed as one of the critical mechanisms in the distributed system of REM sleep control (Boissard et al., 2002; Fort et al., 2009; Luppi et al., 2007; Luppi et al., 2012; Lu et al., 2006; Sapin et al., 2009).

The source of this GABA disinhibition in SLD is uncertain. The SLD receives GABAergic innervation from many sources including GABAergic neurons intrinsic to the SLD (Boissard et al., 2003; Lu et al., 2006; Sapin et al., 2009). Found prominently were neurons in regions of the midbrain, pontine and medullary reticular formation retrogradely labeled from SLD and also coexpressing a marker for GABA. Less numerous, additional retrogradely labeled GABAergic neurons were found in: the area of the lateral hypothalamus; substantia nigra reticulata; pedunculo pontine tegmental nucleus; and raphe magnus.

Drawing on homology with the cat, it has long been known that bilateral electrolytic lesions in the area of the midbrain reticular formation ventral to and including the ventrolateral periaqueductal grey (vlPAG) at the level of the ponto-mesencephalic isthmus resulted in an increase of REM sleep up to 400 percent lasting several days (Petitjean et al., 1975). This strongly implicates the presence of a mechanism inhibitory to REM sleep consistent with a GABAergic innervation. Inhibiting neuronal activity with bilateral injections of the potent inhibitory GABA_AR agonist muscimol into this area also increased REM sleep, though more modestly (Sastre et al., 1996). More recently, studies utilizing ibotenic acid lesions and iontophoretically applied unilateral muscimol ejections have replicated REM sleep increases in the rat (Lu et al., 2006; Sapin et al., 2009). Bilateral injections of muscimol also resulted in large REM sleep increases in guinea pig (Vanini et al., 2007). In rat, the midbrain reticular formation, termed the deep mesencephalic nucleus (DpMe) expressed a high number of GABA neurons projecting to SLD (Boissard et al., 2003) and an indirect measure of activation, c-Fos expression, indicated that a higher number of GABAergic neurons adjacent to the vlPAG, termed the dorsal DpMe (dDpMe), were activated when REM sleep was deprived compared to a condition of increased REM sleep during recovery from REM sleep deprivation (Sapin et al., 2009). Taken together, these findings are consistent with the dDpMe as one area providing GABAergic innervation to the SLD in modulation of the REM-on neurons important to REM sleep generation.

To demonstrate this role for the dDpMe in control of REM sleep, additional data are required, such as the specific activity patterns of GABAergic neurons projecting to SLD and whether they directly innervate REM-on neurons. Preliminary to addressing these questions, we sought here to examine the anatomy of the amino-acid neurotransmitter innervation of SLD from the dDpMe. Immunohistological markers for inhibitory and excitatory amino acid vesicular transporters were coupled with anterograde axonal tracing and imaged with fluorescence confocal laser scanning microscopy. Results support some of the current

theoretical framework, while also indicating operation of mechanisms with a greater level of complexity.

2. Results

Of the 13 BDA iontophoretic ejection sites aimed at the dDpMe (see Fig. 1), all ranged between 0.6 anterior and 0.5 mm posterior to the level of the IVth cranial nerve nucleus, Bregma -7.3 mm (Paxinos and Watson, 1997). Fig. 2 is a representative example of a BDA iontophoretic ejection into the dDpMe. Data for the 13 cases are presented in Table 1. Five of the 13 sites were excluded from further analysis (in black on Fig. 1 schematic representation). Two were excluded based on the extent of the ejection's restriction to the periaqueductal gray (PAG). The remaining three were among the most lateral, but were excluded based on the low number of VGAT double-labeled varicosities on the two sections counted (total < 25). The exclusion-criteria was lying below the 95% confidence interval for the mean of the distribution of the remaining 11 sites. The two ejection-sites in central gray also fell into this low range of the distribution.

Iontophoretic ejections of BDA into the dDpMe resulted in a moderate to low density of anterogradely labeled, multi branching, varicose, axon fibers in SLD. Many of the BDA labeled varicosities were observed apposed to somata and many were not (see Fig. 3). SLD was not the sole destination of these axon projections and labeled fibers could be observed in other brain areas (not shown) particularly midbrain, pontine and bulbar reticular formation. A detailed description of the distribution of dDpMe projection sites is currently in progress.

2.1 VGAT and VGLUT2 anterogradely labeled varicosities in SLD

Two series of independently double-labeled pairs of sections for 8 BDA, dDpMe ejections were analyzed for the presence of co-labeled BDA varicosities with vesicular transporters VGAT or VGLUT2. A total of 1663 double-labeled varicosities were identified in SLD by fluorescence laser scanning confocal microscopy (see Methods Section for details). Of these, 713 were co-labeled for VGAT and 950 co-labeled for VGLUT2 (Table 1). Fig. 4 shows representative examples of double-labeled images in SLD. Fig. 4, A1–3 and B1–3, show varicose axon fibers double-labeled for VGAT and VGLUT2, respectively. There did not appear to be discernable morphological differences between the two types of fibers. Fig. 4, C and D, show dense presynaptic, vesicular transporter, single-labeled varicosities apposed to somata and proximal dendrites. It was typical for double-labeled VGAT or VGLUT2 axo-somatic appositions to comprise a minor proportion of the total, single-labeled vesicular transporter surrounding the exterior of a soma in SLD.

For the group of 8 BDA ejections, mean number of VGLUT2 double-labeled varicosities was significantly greater than mean VGAT (118.8 ± 27.1 vs 89.1 ± 22.8). A significant correlation ($r = .91$) was obtained across ejection sites between the number of double-labeled BDA/VGAT and BDA/VGLUT2 varicosities counted in SLD. Despite this correlation in number of double-labeled varicosities between series, the ratio of double-labeled VGAT to VGLUT2 exhibited variation among different ejection sites ranging from 0.447 to 1.257

with a mean of 0.758 ± 0.093 (Table 1). A ratio less than one reflects the greater number of VGLUT2 labeled varicosities.

2.2 Varicosities apposed to BDA-labeled axon fibers

Label for both VGAT and VGLUT2 varicosities appeared at high density in the neuropil of the SLD. One interesting characteristic of the BDA-labeled varicose axon fibers in SLD originating from dDpMe was the appearance of a high density of non-BDA-labeled VGAT and VGLUT2 labeled varicosities apposed to the fiber's plasma membrane. We have not observed BDA-labeled varicosities apposed to BDA-labeled fibers in SLD. Representative examples of these apposed single-labeled varicosities are shown in the confocal images of Fig. 5 for VGAT (A) and VGLUT2 (B). This finding is consistent with both excitatory and inhibitory functional modulation, within SLD, of axon fibers projecting from dDpMe.

2.3 VGAT, GLYT2 and GAD67

VGAT is a vesicular transporter utilized by both GABAergic and glycinergic neurons (Chauhdry et al., 1998). Therefore, identification of VGAT immuno-positive varicosities alone cannot distinguish between the individual amino acids or their co-localization. To gain additional insight, SLD was double-labeled for VGAT and a specific marker of glycinergic neurons, glycine transporter-2 (GLYT2) (Fig. 6A). We estimate 60% of the VGAT labeled varicosities co-expressed GLYT2 indicating the remaining 40% may be solely GABAergic. There also appeared to be specificity to the innervation in which some somatic profiles had clearly different proportions of apposed VGAT single- and VGAT/GLYT2 double-labeled varicosities (not shown).

In adjacent sections, SLD also was double-labeled for VGAT and a specific marker of GABAergic neurons, glutamic acid decarboxylase-67 (GAD67) (Fig. 6B). We estimate 80% of VGAT positive varicosities co-expressed GAD67 indicating the remaining 20% may be solely glycinergic. Assuming solely glycinergic varicosities comprise 20% and solely GABAergic varicosities comprise 40% of total VGAT, we estimate 40% of VGAT varicosities co-expressed both amino acid neurotransmitters. Double label for GLYT2 and GAD67 supports the high incidence of co-expression in SLD (Fig. 6C). In addition to a rigorous quantification, an anterograde tracer from dDpMe is needed to resolve the relative contributions of GABA and glycine from this source. These preliminary data, however, indicate the inhibitory amino acid neurotransmitter innervation of SLD to be predominantly GABA in combination with co-expressing glycine. Solely glycinergic varicosities appear to constitute a minority.

2.4 VGAT and VGLUT2 colocalized in axon fibers

Utilizing triple-labeled material, we occasionally observed BDA-labeled axon fibers in SLD containing labels for both VGAT and VGLUT2 suggesting the ability of a single fiber to release both inhibitory and excitatory amino acid neurotransmitters. Label for the two vesicular transporters could appear singly in different varicosities of the same fiber or together in the same varicosity. An example of colocalization in a single varicosity appears in the fluorescence confocal images of Fig. 7.

2.5 Glutamate labeled neurons in SLD

Preliminary to a detailed investigation examining the specific cell-types in SLD receiving input from dDpMe, several sections of SLD from BDA ejected rats were immuno-labeled for BDA, VGAT and an antibody directed against glutamate. Fig. 8 shows representative examples of double-labeled BDA/VGAT varicosities apposed to the plasma membrane of somata in the SLD. Such appositions were observed on cells both expressing the glutamate immuno-label and not. Included in this population were small glutamate-immunoreactive neurons (Fig. 8, A2), whose phenotype using the cFos-method, has been identified as belonging to the class of REM sleep-on SLD neurons (Clément et al., 2011).

3. Discussion

3.1 dDpMe projections to SLD contain VGAT and VGLUT2

The present study utilizing anterograde tracing with BDA confirms the neuronal projection from the dDpMe to the SLD previously shown by Boissard et al. (2003) utilizing anterograde transport of phaseolus vulgaris leucoagglutinin and retrograde transport of cholera toxin b-subunit (CTb). They also found the presence of GAD immunoreactivity in a portion of CTb co-labeled somata in dDpMe consistent with the current finding of VGAT in anterogradely labeled varicosities in SLD. The majority of retrogradely labeled neurons in dDpMe were not labeled for GAD67 (Boissard et al., 2003). We show for the first time VGLUT2 in anterogradely labeled varicosities in SLD, indicating presence of glutamate as at least one other neurotransmitter-phenotype projecting from dDpMe to SLD. To the extent the number of varicosities counted reflects the potential influence of the innervation, VGLUT2 varicosities outnumbered VGAT by about a mean of 45 percent from the dDpMe sites analyzed. Identification of these vesicular transporters within anterogradely labeled varicosities further supports these axon fibers as active sites of neurotransmitter release and a functional relationship among neurons in dDpMe exerting inhibitory and excitatory actions upon neurons in SLD.

It has been hypothesized that inhibition and reduction of inhibition from dDpMe shapes the activity of identified REM-on neurons in SLD responsible for generating REM sleep and this is supported by REM sleep increases following muscimol injection into dDpMe (Sapin et al., 2009). The role of the glutamatergic input to SLD from dDpMe, shown here, is not clear. To the extent ejection of kainate in SLD induces a REM sleep-like state through excitation of REM-on neurons, glutamatergic innervation of SLD from dDpMe is unlikely to directly input REM-on neurons. Muscimol applied to dDpMe also would presumably inhibit glutamatergic neurons and result in disfacilitation of REM-on neurons in SLD. Kainate applied to the homolog of the dDpMe in cat would presumably produce excitation and was reported to result in reduced REM sleep and increased wake (Crochet et al., 2006). A role for glutamatergic input to SLD is indicated by the finding of kynurenic acid, an ionotropic glutamate receptor antagonist, to block GABA_AR antagonist-induction of REM sleep when applied to the SLD (Boissard et al., 2002). Thus, facilitation of REM-on neurons appears to be required for removal of GABAergic inhibition to result in REM sleep induction. The glutamatergic input from dDpMe appears not to satisfy this need in that its inhibition by muscimol did not block induction of REM sleep. An alternative mechanism for this input is

one targeting local inhibitory neurons in SLD whose excitation could produce inhibition of REM-on neurons. It is clear from this speculation that more needs to be known of the local neuronal circuitry in SLD and its interaction with afferent inputs.

With respect to hodology and neurotransmitter utilization, the anatomical limits of the dDpMe are imperfectly defined. Here, the defining region was based on the functional results of inducing REM sleep with muscimol ejections in the rat (Sapin et al., 2009). The current targets were centered at the anterior-posterior level of the trochlear nucleus (IV n., β -7.3 mm) and ranged rostral and caudal approximately 0.5 mm. Ejections of BDA of variable size were aimed at the boarder of the vIPAG extending into the DpMe. All 13 ejection sites resulted in the appearance of anterogradely labeled, varicose axon fibers in the SLD. This result is consistent with the widespread appearance of retrogradely labeled neurons from SLD in the entire region of the PAG and DpMe (Boissard et al., 2003). To further increase the likelihood of sampling fibers for analysis originating from functionally defined dDpMe, a criteria based on the hypothesized role of GABA eliminated the sites with the lowest VGAT/BDA number of varicosities, leaving 8 sites in the group. Two sites eliminated were located in the lateral PAG and the other three located slightly lateral to the border of the vIPAG (see Fig. 1). This may indicate a delimited, circumscribed border to the lateral extent of the functional dDpMe. Our finding of a significant correlation between double-labeled VGAT and VGLUT2 varicosities between ejection sites might indicate a common dependence upon the number of anterogradely labeled axon fibers found in SLD. Even though the two most rostral sites yielded the greatest number of VGAT varicosities, further considerations of the size of BDA ejections and location did not specify additional anatomical limits of the dDpMe. In accord with our expectations, the quantity of double labeled BDA/VGAT and VGLUT2 varicosities, and the resolution of the confocal images indicates high feasibility of using these techniques to determine local anatomical relationships in SLD of the afferent inputs from dDpMe and elsewhere.

3.2 Combined labeling for BDA, VGAT and VGLUT2

To explore the dDpMe innervation of SLD further, we studied sections from the group of eight utilizing immunostaining with triple-labeling. Several interesting observations were found having implications for the interaction of dDpMe with SLD and control of REM sleep. Consistent with GABA and glutamate, respectively, being the major inhibitory and excitatory neurotransmitters in brain, the very high density of VGAT and VGLUT2 labeled varicosities in SLD indicates the importance of amino acid neurotransmission in influencing the activity of neurons in SLD. The low to moderate, density of anterogradely labeled axon fibers from even the largest ejections of BDA into dDpMe is consistent with projections from dDpMe accounting for a low proportion of total VGAT and VGLUT2 varicosities in SLD. A special role for the dDpMe amino acid neurotransmitter innervation of SLD in control of REM sleep most likely would depend upon a neuron-type-selective innervation. Identification of those neural elements in SLD receiving input from dDpMe will be critical to understanding this relationship.

3.2.1 dDpMe axon fibers in SLD appear to be subject to local modulation—In addition to VGAT and VGLUT2 punctate immunolabel found inside BDA labeled

varicosities in SLD, a very high number of these single-labeled presynaptic markers were observed apposed to the membrane of the BDA labeled fibers. This is consistent with the dDpMe projection subject to both excitatory and inhibitory local, presynaptic modulation within the SLD. We have not seen BDA labeled varicosities apposed to BDA labeled fibers, so the source of this innervation is unknown. This local modulation has several potential consequences. Among them is modulation resulting in neurotransmitter release from axon fibers having a degree of independence of action potential propagation and thus activity of somata in dDpMe. A neuron in DpMe may not necessarily express a REM sleep-related pattern of activity in order to have a REM sleep-related pattern of neurotransmitter release in SLD. Unit recordings made in the cat homolog of the dDpMe found only neurons active in wake and REM sleep compared to non-REM sleep (Crochet et al., 2006). Electrophysiologically determined activity of dDpMe neurons in rat has not been reported. Presumably, dDpMe axon fibers in SLD apposed by vesicular transporter label indicate synaptic contact with appropriate postsynaptic receptors. These receptors would be targets for drugs applied to SLD. It should not be assumed drugs applied to SLD are only directly acting upon local neurons. A similar situation may exist with the innervation of SLD in which afferent axons may not contact elements of local neurons directly. Such may be the case for the axons giving rise to varicosities apposed to dDpMe axons in SLD. These consequences discussed present an increased challenge to determining the neural network subserved by dDpMe and SLD. Awareness of the possibilities will be helpful in future investigations.

3.2.2 VGAT and VGLUT2 can be colocated in the same dDpMe axon fiber—An interesting finding observed in the material triple-labeled for BDA, VGAT and VGLUT2 was the occurrence in SLD of VGAT and VGLUT2 within the same BDA labeled axon fibers. This was seen only occasionally, but at least one axon-segment per tissue section was found. We could find no report investigating the colocalization of these neurotransmitters in soma of dDpMe, which would aid in determining its prevalence. Or do we know what role, if any, this mechanism may play in control of REM sleep. Several reports, using a variety of methods, have presented evidence in support of co-released GABA and glutamate in adult brain (Zander et al., 2010), but some controversy is still present in the current literature (Caiati, 2013). One concept expressed for colocalization of GABA and glutamate is that transmitter release can be differentially selective and dynamic (Somogyi, 2006). Based on the plasticity of REM sleep expression, such a mechanism might be involved in the interaction of dDpMe and SLD. Determination of a selective input to REM sleep-on neurons in SLD for VGAT/VGLUT2 co-labeled varicosities would add support for this idea.

3.2.3 VGAT labeled axons may innervate REM-on SLD glutamatergic neurons—Because of the critical importance of knowing the specific neuronal types targeted by afferents to SLD, we undertook a demonstration of proof of principle for a method to determine these targets. An antibody to glutamate was used to label glutamatergic neurons in SLD, VGLUT2 protein is poorly expressed at the soma. A great number of co-labeled BDA/VGAT varicosities were found in SLD apposed to somatic plasma membranes. It appears that VGAT labeled axon varicosities originating from dDpMe may directly synapse upon both non-glutamatergic and glutamatergic neurons in SLD. This included a population

of small glutamate-immunoreactive neurons, whose phenotype, using the cFos-method, has been putatively identified as that of REM sleep-on SLD neurons (Clement et al., 2011). This latter finding is consistent with dDpMe neurons exerting a potent somatic inhibition upon REM sleep-on neurons in SLD in control of their state-related activity. Encouraged by these results, we have initiated quantitative studies to identify the postsynaptic targets of VGAT and VGLUT2 varicosities with respect to several indices including neurotransmitters, postsynaptic receptor subtypes, efferent projections and cFos expression under different behavioral conditions.

3.3 VGAT identifies both GABAergic and glycinergic varicosities

VGAT is a vesicular transporter utilized by both GABAergic and glycinergic neurons (Chaudry et al., 1998). Neurons with markers for these amino acid neurotransmitters were found in the PAG, and DpMe adjacent to the vlPAG (Rampon et al., 1996; Tanaka and Ezure, 2004). Here, we conducted a preliminary investigation using specific markers for GABAergic and glycinergic neurons, GAD67 and GLYT2, respectively, in combination with VGAT. Pending a more rigorous quantification, we estimate 40% of the VGAT varicosities in SLD were solely GABAergic, 20% solely glycinergic, and 40% of varicosities expressed markers for both neurotransmitters. About 80% of VGAT positive varicosities in SLD appear to release GABA. This calculation also is subject to error owing to differences in the threshold of detection by the antibodies respectively binding GAD67 and GLYT2. Nonetheless, the result of the calculation is roughly consistent with the greater number of GABAergic neurons compared to glycinergic neurons found in the dDpMe (Boissard et al., 2003; Rampon et al., 1996).

Inasmuch as the vlPAG has a sizable projection to SLD with few being GABAergic (Boissard et al., 2003), vlPAG may be one source of glycine to SLD. Many neurons in the midbrain, pons and medulla project to SLD (Boissard et al., 2003) and these areas were shown by in situ hybridization for GAD67 and GLYT2 to possess neurons with mRNA for each, and both, markers of GABA and glycine (Tanaka and Ezure, 2004). Visual inspection of the double-labeled VGAT/GLYT2 material revealed an apparent specificity to the somatic appositions of single VGAT and double VGAT/GLYT2 varicosities in SLD (not shown). Quantification is required, but this observation is consistent with functionally different interactions of GABA and glycine in SLD.

Evidence from the cat injecting the glycine receptor antagonist, strychnine, into the homolog of SLD was without effect at sites supporting induction of REM sleep with GABA_A receptor antagonists (Xi et al., 1999). We are not aware of a report delivering strychnine to rat SLD. Even if strychnine is without effect in rat, it does not preclude a role for glycine. The presence of glycine varicosities and apparent high degree of colocalization with GABA indicates this possibility. Perhaps a more exotic mechanism, independent of glycine receptors, may be operating such as control of GABA content in synaptic vesicles through a competition for VGAT transport, or glycine modulation of N-methyl-D-aspartate glutamate receptors.

3.4 Question of dDpMe as relevant source of GABA to SLD

Recently, Lu and colleagues reported utilizing a procedure to conditionally knock-down the *vgat* gene in cells in the region of the dDpMe in mouse (Krenzer et al., 2011). Following a three-week recovery from surgery, mice failed to show any significant change in REM sleep amounts or architecture compared to controls. The authors concluded that GABA release from neurons in the manipulated area is not critical to REM sleep control. Would that the intended effect of the procedure have resulted, these data might indicate dDpMe not to be a necessary REM sleep-relevant source of GABA to SLD. Uncertainty is raised in this study by a lack of demonstration of *vgat* knockdown and, specifically, lack of VGAT protein expression in GABAergic varicosities in SLD from dDpMe at the time of the sleep study. Another issue central to the nature of the neural networks subserving REM sleep are the differences resulting from acute versus chronic manipulations. In cat, lesions of the homolog of dDpMe produced a large increase in REM sleep that recovered to baseline levels in several days (Petitjean et al., 1975). Inasmuch as recovery occurs without this source of GABA, recovery also could occur after chronic knock-down of *vgat* in the three-weeks following surgery. For that matter, destruction of the SLD does not eliminate REM sleep and the deficit in muscle atonia of REM sleep produced by these lesions also recovers in time (Sanford et al., 2001). This capacity for recovery makes it difficult to determine the role of specific cell populations or mechanisms controlling REM sleep in the unmanipulated animal. It is consistent with a distributed and redundant system having the property to recover in time when disrupted.

Conclusion

The concept emerging is that the SLD and dDpMe are two components of a more widespread and interacting system generating and controlling REM sleep. It is a system in which manipulating individual components is sufficient to alter expression of REM sleep, but no component appears necessary to its expression. The nature of such a system is consistent with its historical resistance to elucidation. Application of modern neuroscience methodologies is developing a more complete description of the neural mechanisms subserving REM sleep behavior. Here we show the feasibility of exploring the anatomical relationships among components and plan to utilize these imaging techniques toward further elucidation in the future.

4. Experimental Procedure

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 Department of Veterans Affairs, North Texas Health Care System. All efforts were made to minimize the number of animals used and their suffering.

2.1 Anterograde tracing and tissue collection

Long-Evans Hooded rats (N=13, Harlan Sprague-Dawley, Indianapolis, IN, USA) underwent aseptic, stereotaxic surgery under isoflurane anesthesia. Drawn, glass capillary tubes (tip, 15 μ m O.D.) were filled with a 5% solution of the anterograde neuronal tracer biotinylated

dextran amine (BDA, 10k MW, Molecular Probes, Grand Island, NY, USA) and aimed unilaterally at sites in the dDpMe (A-P, Bregma -6.8 to -7.8; ML, 1 to 1.5; V, 5.5 to 6.5 mm; Paxinos and Watson, 1997) using a lateral to medial angle of 14. Sites were chosen based on Luppi's studies in rat utilizing retrograde transport from SLD to identified GABAergic somata and sites supporting REM sleep induction with muscimol in the dDpMe (Boissard et al., 2003; Sapin et al., 2009). BDA was iontophoretically ejected from the pipette utilizing a positive current (3 μ A, pulsed 10sec on/10 sec off) for 20 min. After a four min. wait, the pipette was removed, wound closed and the rat allowed to recover for six days in a home cage environment under a 12/12hr light/dark cycle, 22.5 $\pm$ 1 $^{\circ}$ C, with food and water available ad libitum.

On the sixth day at five hours into the light cycle, rats were deeply anaesthetized with pentobarbital (60 mg/kg, ip) 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 until tissue sank in the solution.

Serial, coronal sections, 20 μ m-thick were cut on a freezing microtome (SM2000R, Leica, Nussloch, Germany) through the caudal midbrain and pons, and collected in 0.1M PB, with 0.005% sodium azide. The regions of interest for microscopical analyses were the area of the dDpMe, to localize the ejection site, and the SLD, ipsilateral to the BDA ejection. Sections containing SLD were identified based on previously published sites supporting induction of a REM sleep-like state in the rat (Boissard et al., 2002; Pollock and Mistlberger, 2003). The specific area is schematically represented in coronal section in Fig. 9 (after Paxinos and Watson, 1997). A more detailed definition of SLD appears below.

2.2 Immunohistochemistry and image acquisition

2.2.1 Antibodies—The main objective of this study was to use the anterograde transport of BDA to visualize varicose axon fibers in the SLD whose source is the dDpMe to determine, in these fibers, the preponderance of varicosities containing markers for the amino acid neurotransmitters GABA, glycine and glutamate. Well characterized primary antibodies were commercially obtained for this purpose and are listed, with their sources, in Table 2. Antibody raised against the vesicular GABA transporter (VGAT) was used to immuno-label varicosities utilizing GABA and/or glycine in that these two neurotransmitters share the same vesicular transporter (Chaudhry et al., 1998). In order to differentiate the two amino acids, we respectively paired immuno-labeling of VGAT with specific markers for these two transmitters. These were glycine transporter 2 (GLYT2), the isotype found in adult glycine neurons (Zafra et al., 1995) and glutamic acid decarboxylase 67 (GAD67), a GABA synthetic enzyme found in GABAergic neurons (Erlander et al., 1991). Glutamate utilizes three known vesicular transporter-isotypes (Fremeau et al., 2004). The most abundant neuronal isotype in the brainstem is type 2 and antibody raised against the vesicular glutamate transporter 2 (VGLUT2) was used to label these varicosities. To determine feasibility, for future study, of identifying the postsynaptic targets of specific dDpMe inputs to SLD, we sought to label glutamatergic neurons in SLD. Inasmuch as VGLUT2 weakly

expresses at the soma, we utilized an antibody raised against glutamate, which has been successfully utilized to identify glutamatergic neurons (Fredrich et al., 2009; Lin et al., 2000).

2.2.2 Immunohistochemistry—Tissue processed for visualization of BDA with bright field microscopy utilized the avidin-biotin complex procedure (Elite Vectastain ABC kit, Vector Labs, Burlingame, CA, USA) with nickel-diaminobenzidine and counter-stained with neutral red. For fluorescence microscopy, free-floating sections were primarily blocked with 3% normal donkey serum and 0.2% triton X-100 in PBS (PBSTDS) for 30 min. and then incubated in a mixture of two or three primary antibodies in 1% normal donkey serum PBSTDS for 18–20 hours at 4 °C (see Table 2 for dilutions). After washing with PBS, sections were incubated for 3-hours with fluorescent-tagged secondary antibodies (Cy2, Cy3 and/or Cy5 (Jackson Immuno Research Labs, West Grove, PA, USA) appropriate for the host species of their respective primary antibody. Following a 30 min. wash with PBS, sections were mounted and cover-slipped with mounting medium containing 50% glycerin and pheylenediamine. Negative controls for antibody labeling indicated a lack of specific staining when primary or secondary antibodies were omitted.

2.2.3 Image acquisition—Fluorescence confocal laser scanning microscopy was conducted on a Zeiss LSM 510 (Gottingen, Germany) equipped with argon 488 nm, helium-neon 543 nm, and helium-neon 633 nm lasers for excitation. Images were obtained using a 63x objective (water, N.A. 1.2) with a pinhole of 1 Airy unit (all channels). Optical sectioning in the Z plane was performed using multitrack-scanning at 0.33 μ m intervals.

Identification of the SLD, in this study, utilized a functional criteria based on sites reported to induce a REM-like state in rats with application of GABA_A receptor antagonists (Boisard et al., 2002; Pollock and Mistlberger, 2003). We conservatively identify a region ventral to the locus caeruleus extending caudally from the level bregma –8.9 to –9.3 mm illustrated schematically in Fig. 9. This yielded 20 sections for each rat. The region of interest in these sections was a rectangle (426 $\times$ 568 μ m) placed, using widefield fluorescence, with its dorsomedial corner just adjacent to the Barrington nucleus on the side ipsilateral to the BDA ejection. This closely approximated the rostral SLD defined by Swanson (1998). The rectangle was further divided into 12 (3 $\times$ 4), 142 μ m square fields, each the dimension of the microscope's field at 63x, zoom factor 1. Two sets of two sections (240 μ m apart) were randomly chosen from the pool of 20 for each rat and each set assigned to either the VGAT or VGLUT2 labeled series. Starting 2 μ m below the surface of a section, a z-stack of at least 10 images (0.33 μ m steps) was scanned for each field. Quantification of the data was based on these images without knowledge of their source BDA-ejection characteristics.

2.3 Image analysis

In order to minimize bias in quantification, a method of systematic random sampling was used. Counts, were performed by navigating through images of optical slices on each individual z-stack and identifying the structures of interest. Left and bottom edges were considered exclusion borders, right and top inclusion borders in the x–y planes. The uppermost optical slice was considered an exclusion plane and the 10th optical slice an

inclusion plane. Applying these criteria, BDA labeled axon fibers in SLD were identified and double-labeled varicosities in the respective VGAT and VGLUT2 series of sections were counted. Counts were totaled for the 12 fields on each section. The number of counts in the rostral compared to the caudal section of the pair of sections across ejection sites were not significantly different in either series with respect to the number of double-immunolabeled varicosities. The sum of counts for each pair of sections was used for further statistical analysis.

Because of the shared dependence on characteristics of individual BDA ejections (e.g. location and size), comparisons of counts between the VGAT and VGLUT2 series utilized a repeated measures design, t-test for dependent means, two-tail, $P < 0.05$. Measures of correlation utilized the Pearson product-moment coefficient tested to differ from zero, $P < 0.05$. Means are reported with $\pm$ standard error of the mean (SEM).

Estimates of the percentages of VGAT/GLYT2 and VGAT/GAD67 varicosities in SLD were based on counts from two randomly chosen fields in SLD for each combination of labeling. Double-labeled GLYT2/GAD67 material was not subject to counting do to the lack of a definitive marker of presynaptic varicosities, VGAT.

Images generated by the LSM 510 (TIFF format) were imported into Volocity image analysis software (v. 5.2, Improvision, Waltham, MA, USA) for creation of extended-focus and 3D-images. Photoshop software (CS4, Adobe, San Jose, CA, USA) was used for composition of figures, including cropping and global adjustments of brightness and contrast.

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Abbreviations

BDA	biotinylated dextran amine
CTb	cholera toxin b-subunit
DpMe	deep mesencephalic nucleus
dDpMe	dorsal DpMe
GABA	γ -aminobutyric acid
GABA_AR	GABA _A receptor
GAD67	glutamic acid decarboxylase-67
GLYT2	glycine transporter-2
LC	locus caeruleus
NREM	Non-REM
PAG	periaquiductal grey

REM	rapid eye movement
S.E.M	standard error of the mean
SLD	sublaterodorsal nucleus
VGAT	vesicular GABA transporter
VGLUT2	vesicular glutamate transporter-2
vlPAG	ventrolateral PAG

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Highlights

- Region ventrolateral to central grey projects to REM sleep induction zone
- Both excitatory and inhibitory amino acid neurotransmitters are utilized
- Labeled varicose axon fibers appear subject to high degree of local modulation
- Inhibitory inputs directly appose neurons of the type participating in REM sleep control

Figure 1.

Schematic coronal sections representing the 13 BDA ejections. A 1–8 (gray) denote ejections used in the grouped quantitative analysis. B 1–5 (black) were excluded from the analysis. Code-labels refer to subjects listed in Table 1. Numbers in lower right of each section indicate distance (mm) caudal to the bregma suture. III, third cranial nerve nucleus; IV, fourth cranial nerve nucleus; DpMe, deep mesencephalic nucleus; PAG, periaquiductal gray; PnO, nucleus pontis oralis; PPT, pedunculo pontine tegmental nucleus; RD, dorsal raphe; RRF, retrorubral field; xscp, decussation of the superior cerebellar peduncle.

Figure 2. Brightfield photomicrograph of a coronal section showing a representative example (A8 in Fig. 1 and Table 1) of an iontophoretic ejection of BDA into the dDpMe (arrow). Tissue counter-stained with neutral red.

Figure 3. Immunoperoxidase, brightfield image of anterogradely labeled (dark) BDA in varicose axon fibers in SLD following BDA ejection in dDpMe. Cell somata are labeled with neutral red. Scale bar in lower left.

Figure 4.

Fluorescence confocal images of single optical sections representative of colabeled varicosities in SLD. A1–3, varicosities coexpressing BDA (red) and VGAT (green) immunoreactivity. B1–3, varicosities coexpressing BDA (red) and VGLUT2 (green) immunoreactivity. Individual color channels are shown in 1 and 2, merged in 3. scale bars in A1 and B1. C, BDA and VGAT; D, BDA and VGLUT2. C and D show perisomatic locations of double labeled varicosities. Colabeling appears yellow.

Figure 5. Fluorescence confocal images of single optical sections showing BDA labeled (red) axon fibers in SLD with numerous VGAT (A) and VGLUT2 (B) labeled (green) varicosities apposed to the axon plasma membrane. Scale bar in A.

Figure 6.

Fluorescence confocal images of single optical sections in SLD of VGAT labeled varicosities (red) colabeled for GLYT2 and GAD67 in A and B (green), respectively. In C, label is for GAD67 (red) and GLYT2 (green). Approximately 60% of VGAT label colocalizes GLYT2 and 80% colocalizes GAD67. Scale bar in C.

Figure 7.

Fluorescence confocal images of a single BDA labeled varicosity (red) in SLD showing coexpression of both VGAT (green) and VGLUT2 (blue). 1–4, consecutive images in the z-plane 0.33 μm apart. A–C, paired combinations of color channels; D, merge of all three color channels. Thick arrow points to the punctate VGAT/VGLUT2 colabel. Thin arrow points to the punctate VGLUT2 label. Scale bar in A1.

Figure 8.

Fluorescence confocal images of single optical sections showing perisomatic BDA (red) and VGAT (green) colabeled varicosities in SLD. A1–3, double labeled varicosities apposed to somata labeled for glutamate (blue). B, double labeled varicosities apposed to unlabeled soma. Arrows point to an example in each image (yellow). Scale bar in A1.

Figure 9.

Schematic representation of the counting-box used on each section to tally the double labeled BDA/VGAT and BDA/VGLUT2 varicosities in SLD. The box ($426 \times 568 \mu\text{m}$), in gray, consists of 12 contiguous fields. Bar, Barrington nucleus; LC, locus caeruleus; LDT, laterodorsal tegmental nucleus; Mo5, trigeminal motor nucleus; PnC, nucleus pontis caudalis; scp, superior cerebellar peduncle.

Table 1

Double-labeled varicosities in SLD

Subject	Inj. Site (A-P)	BDA/VGAT	BDA/VGLUT2	Ratio
A1	β -6.7	228	279	0.817
A2	β -6.9	128	148	0.865
A3	β -7.3	37	59	0.627
A4	β -7.5	83	66	1.258
A5	β -7.8	89	98	0.908
A6	β -7.8	28	44	0.636
A7	β -7.8	72	161	0.447
A8	β -7.8	48	95	0.505
B1	β -7.3	25	44	0.568
B2	β -7.1	8	21	0.381
B3	β -7.8	11	21	0.524
B4	β -7.3 PAG	12	28	0.429
B5	β -7.3 PAG	22	85	0.259

Subject, refers to figure 1 schematically representing individual BDA ejection sites. A1–8, data used in the quantitative analysis; B1–5, data excluded from group analysis (see text). Inj. Site (A-P), estimate of the distance from the bregma suture (mm) to the center of each ejection site; PAG, indicates location in the periaquiductal gray. BDA/VGAT, total counts of double-labeled BDA/VGAT varicosities on two tissue sections for each ejection site; BDA/VGLUT2, total counts of double-labeled BDA/VGLUT2 varicosities on two sections for each ejection site; Ratio, ratio of double-labeled VGAT to VGLUT2.

Table 2

Primary Antibodies

Antigen	Host	Source	Cat.#	Dilution
VGAT	G.P.	SYSY	131004	1:1000
VGLUT2	G.P.	SYSY	135404	1:3000
GlyT2	G.P.	Chemicon	AB1773	1:1000
VGAT	Mouse	SYSY	131011	1:2000
GAD-67	Mouse	Sigma	G5419	1:500
VGLUT2	Mouse	Chemicon	MAB5504	1:1000
GAD-67	Rabbit	AnaSpec	53501	1:500
Glutamate	Rabbit	Sigma	G6642	1:5000
VGAT	Rabbit	SYSY	131003	1:1000
BDA	Goat	List	703	1:2000

G.P., guinea pig; SYSY, SynapticSystems; Sigma, Sigma-Aldrich; List, List Biological Labs.