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Implications for REM Sleep Physiology

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ABSTRACT: Recent evidence indicates that pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal polypeptide (VIP) might play an important role in rapid eye movement sleep (REMS) generation at the pontine level in rats. We have thus examined the immunohistochemical distribution of VIP and PACAP in the pontine and mesencephalic areas known to be involved in REMS control in rats. A dense network of VIP-immunoreactive cell bodies and fibers was found in the dorsal raphe nucleus. A large number of PACAP-positive perikarya and nerve fibers was observed in the area known as the REMS induction zone within the pontine reticular formation (PRF). The present results provide an anatomical basis to our previous functional data, and suggest that PACAPergic mechanisms within the PRF play a critical role in long-term regulation of REMS.

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INTRODUCTION

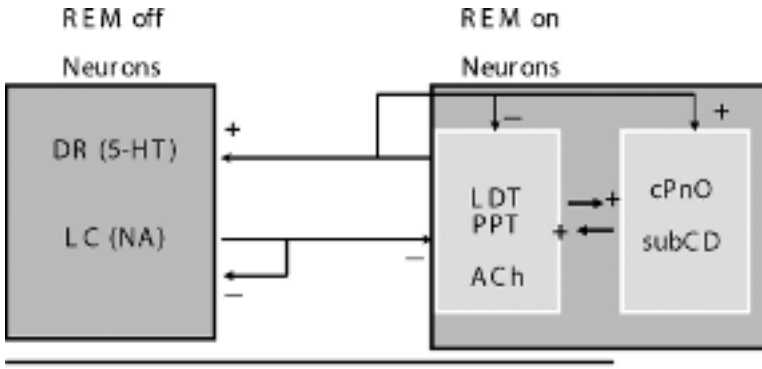
A role for Vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) in rapid eye movement sleep (REMS) regulation has been early suggested by studies reporting REMS enhancement following intracerebroventricular (i.c.v.) injection of VIP or PACAP.[1–3] Only a few studies, however, have examined the effects of direct intracerebral administration of VIP and PACAP on REMS.[4–7] These latter reports allowed the identification of brain areas involved in this sleep-promoting effect, i.e., the dorsal raphe nucleus[4] (DR) and the area within the pontine reticular formation (PRF), known as the "REMS induction zone"[5,6] in rat, and the amygdala, a structure strongly connected to the pontine generators of REMS, in cat.[7]

REMS regulation is known to be under the control of a reciprocal interaction between "REM off" and "REM on" structures located in the brain stem.[8] The "REM off" structures, i.e., the DR, that contains serotoninergic neurons, and the locus caeruleus (LC), that contains noradrenergic neurons, and the "REM on" structures, i.e., the pontine tegmentum and the PRF, are silent and active, respectively, during REMS (FIG. 1). The pontine tegmentum comprises the laterodorsal and pedunculopontine nuclei (LDT and PPT), two areas that provide a dense cholinergic innervation to the PRF[9] (FIG. 1). There is now ample evidence that cholinergic mechanisms at the pontine level are essential to the generation of REMS. In particular, direct local microinjection of carbachol (cholinergic agonist) allowed the identification of an "REMS induction zone" defined as the caudal part of the oral pontine reticular nucleus (cPnO) and the adjacent dorsal subcaeruleus nucleus (subCD) in rats.[10–12]

Interestingly, we found that a single microinjection of a low dose of VIP or PACAP into the REMS induction zone promotes sustained REMS for up to 12 days.[5,6] This long-term enhancement of REMS, which depends on VIP-PACAPergic/muscarinic receptor interactions,[13,14] is well suited for the study of both functional aspects and molecular mechanisms of this state of vigilance.

Both VIP and PACAP increase the intracellular content of cyclic AMP and act through activation of specific common receptors.[15] In order to determine the respective roles of each peptide as REMS promotor, and to establish the physiological relevance of their shared pharmacological effect, it is necessary to investigate whether endogenous VIP and/or PACAP actually exist in the areas targeted by the microinjections.

Here, we conducted an immunohistochemical study of VIP and PACAP in the rat brain stem in order to determine their respective distributions in the REMS induction zone and in "REM on" and "REM off" structures.



	VIP		PACAP	
	cell bodies	fibers	cell bodies	fibers
cPnO	-	±	++	++
subCD	-	±	++	++
LDT	±	±	-	+
PPT	-	+	-	+
DR	++++	+++	-	-
LC	-	+	+++*	++

FIGURE 1. The reciprocal model of "REM off"–"REM on" interaction[8] with the distribution of VIP- and PACAP-immunoreactive perikarya and fibers as determined in the components of the model. *Observed in colchicine-treated animals only. cPnO = caudal part of the oral pontine reticular nucleus; subCD = dorsal subcaeruleus nucleus; LDT = laterodorsal tegmentum; PPT = pedunculopontine tegmentum; DR = dorsal raphe nucleus; LC = locus caeruleus.

MATERIALS AND METHODS

Adult male Sprague Dawley rats, weighing 250–300 g, were used in the present study. The animals were handled in agreement with the ethical rules for experimentation on laboratory animals (DHEW Publication; 80–23; Office of Science and Health Reports, DRR/NIH, Bethesda, MD, USA; 1980). One day before sacrifice, half of the animals received an i.c.v. injection of colchicine. Briefly, 80 μg colchicine in 4 μL of phosphate buffered saline (PBS, pH 7.4) was injected over 10 min into the lateral ventricle under pentobarbital anesthesia (stereotaxic coordinates: P: 0.9 mm, L: 1.5 mm, V: −3.5 mm under the brain surface). Twenty-four hours later, the animals were deeply anesthetized (50 mg/kg of sodium pentobarbital) and perfused via the left

ventricle with 100 mL of PBS followed by one of the fixative solutions described below. Brains were then removed and processed for immunostaining.

Three different antibodies were used with specific fixative conditions and dilutions as determined in previous experiments. *(1)* Anti-VIP antibodies (INCSTAR Corp., Stillwater, MN); dilution, 1:8000; fixation, 4% paraformaldehyde + 0.2% picric acid; 50-μm-thick free-floating coronal sections (vibratome). *(2)* Anti-PACAP27 antibodies (Peninsula, San Carlos, CA); dilution, 1:10,000; fixation, 3% paraformaldehyde + 1% glutaraldehyde; 15 μm-thick coronal sections on slides after cryoprotection (cryostat). *(3)* Monoclonal antibody against PACAP27 and PACAP38[16]; dilution, 1:5; fixation, 4% paraformaldehyde, 15-μm-thick coronal sections on slides after cryoprotection (cryostat). The latter two antibodies yielded comparable results except for the staining of nerve fibers that was greater using the monoclonal anti-PACAP antibody.

Tissue sections were pretreated with 0.1% H_2O_2 in PBS for 30 min to inhibit endogenous peroxidase activities. The sections were subsequently incubated overnight at 4°C with the primary antiserum supplemented with 1% normal goat serum and 0.2% Triton X-100. Then, the sections were incubated with a biotinylated secondary antibody (Vector, dilution, 1:250) for 2 h at room temperature. Thereafter, immunohistochemical staining was performed using the avidin-biotin-peroxidase method and H_2O_2-diamino-benzidine for revelation.

To control the specificity of the immunostaining, the primary antibodies were preabsorbed with an excess of respective peptide, VIP or PACAP. Immunostaining was not modified when the primary antibody directed against VIP or PACAP was preabsorbed with the heterologous peptide.

RESULTS

The distribution of PACAP- and VIP-immunoreactive elements appeared to be heterogeneous within brain stem structures known to participate in REMS regulation (FIG. 1). No VIP-positive cell bodies were visualized in the PRF, subCD, LDT, PPT, or LC nuclei. In contrast, numerous VIP cell bodies were observed in the DR nucleus, mainly in its caudal part. These neurons were fusiform, mainly uni- or bipolar, with short dendritic profiles, and were oriented in the dorsoventral direction within the DR (FIG. 2 B). Scarce VIP immunostained nerve fibers were observed in the PRF, subCD, LDT, PPT, and LC nuclei. They appeared as long varicose fibers and sometimes occurred individually (FIG. 2 D). A much greater number of short thin nerve fibers immunostained for VIP were identified in the DR where they exhibited a dorsoventral orientation (FIG. 2 B).

In sharp contrast with that found with anti-VIP antibodies, anti-PACAP antibodies labeled neuronal perikarya in the PRF, notably in the cPnO and adjacent subCD corresponding to the "REMS induction zone" (FIG. 1). These PACAP-immunoreactive neurons, of various sizes, were mostly multipolar, with very long dendrites and many of them corresponded to gigantocellular

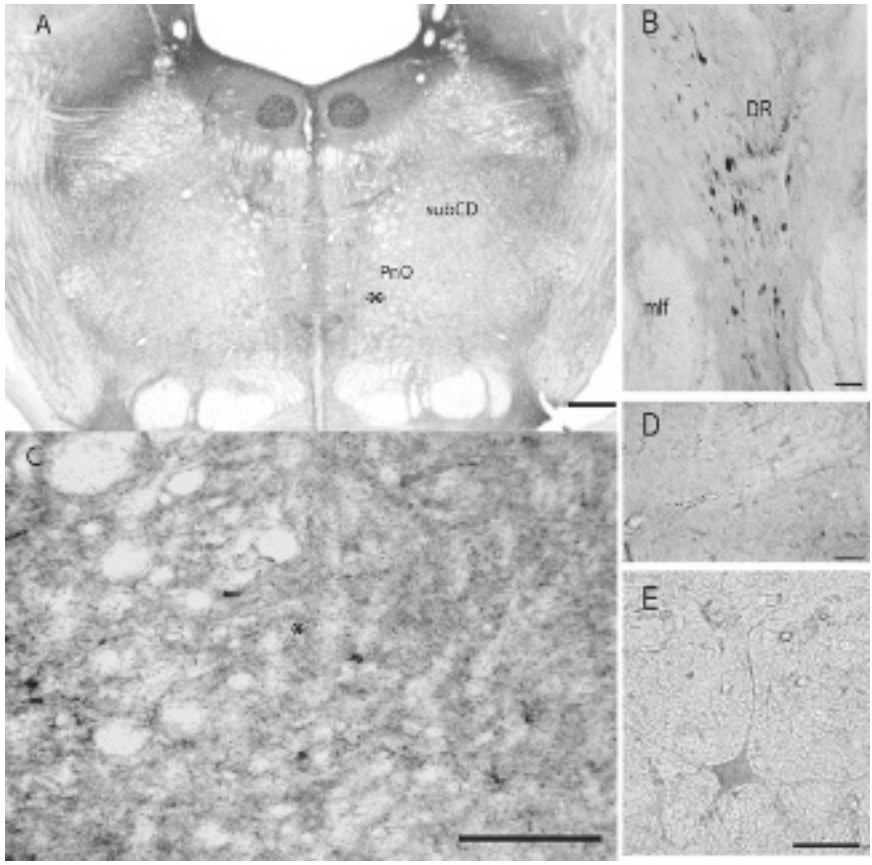


FIGURE 2. Photomicrographs of coronal sections showing VIP and PACAP immunoreactivity in REMS control areas. **(A)** PACAP immunoreactivity at level 8.8 mm posterior to bregma; **(B)** VIP immunoreactivity in the DR; **(C)** PACAP immunoreactivity in the cPnO; **(D)** scarce VIP positive nerve fibers in the cPnO; **(E)** PACAP-positive perikaryon with long dendrites in the cPnO. Scale bars: 500 μm in A, 25 μm in B, 100 μm in C, 50 μm in D, 50 μm in E. Abbreviations as in FIGURE 1.

nerve cell bodies (FIG. 2 E). A high density of PACAP-immunoreactive nerve fibers of various sizes without any particular orientation was also observed in the same area (FIG. 2 C). In rats treated with colchicine, a large number of PACAP-positive neurons were also visualized in the LC (FIG. 1).

DISCUSSION

Here, we report, for the first time, the presence of PACAP, but not VIP, in the REMS induction zone, an area defined as the caudal part of the PnO and

the adjacent subCD nucleus. This PACAPergic labeling corresponds to both *(a)* fibers, suggesting the existence of a naturally occurring release of PACAP in this area, and *(b)* cell bodies with long dendritic ramifications, raising the question of the functional role of these cells, in particular whether they belong to the "REM on" cell component. Thus, further experiments are needed in order to measure the local release of PACAP and the firing rate of PACAP-positive cells *in vivo* across the sleep wakefulness cycle. The presence of PACAP but not VIP is also functionally coherent with the existence of specific binding sites with higher affinity for PACAP than VIP in the PRF.[6] Retrograde labeling studies have to be performed in order to determine the origin of the PACAP-positive fibers present in the REMS induction zone. Anterograde labeling studies from the LC would also be needed in order to determine the projection sites of the PACAP-positive cells located in this structure. Indeed, previous reports[17,18] and our observation of PACAP-positive cells in the LC of colchicine-treated animals suggest that PACAP might modulate wakefulness, in addition to its role in REMS regulation.

A large number of VIP-positive cell bodies as well as a dense VIPergic innervation were observed in the DR as previously reported.[19] In rats, microinjection of VIP into the DR, where serotoninergic neurons that project to the forebrain are located, has been shown to produce an increase in slow wave sleep (SWS) at low doses, and to promote REMS at larger doses.[4] VIP injected i.c.v. during the light period enhances REMS whereas it increases both SWS and REMS when injected during the dark period.[1,2] Altogether, these data suggest that VIPergic mechanisms within the DR may play a critical role in sleep wakefulness regulation, possibly through an inhibition of the serotoninergic system.

Our data do not support the hypothesis of a direct modulation of the "REM on" component by the VIPergic system. At variance with a previous report,[20] we did not detect any VIP-immunoreactive neuron in the pontine tegmentum. Only a very low density of VIP- and PACAP-immunoreactive fibers could be observed in this latter structure.

In conclusion, our immunocytochemical data showing that PACAP-positive cell bodies and fibers do exist in the REM sleep induction zone provide further support to the idea that PACAPergic mechanisms within the PRF play a critical role in long-term regulation of REMS.[3,6,14]

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