Published in final edited form as:

Brain Res. 2009 November 10; 1297: 32–40. doi:10.1016/j.brainres.2009.08.045.

A Novel GABAergic Afferent Input to the Pontine Reticular Formation: the Mesopontine GABAergic Column

Chang-Lin Liang and Gerald A. Marks

Department of Veterans Affairs North Texas Health Care System and Department of Psychiatry, University of Texas Southwestern Medical Center at Dallas

Abstract

Pharmacological manipulations of γ -aminobutyric acid (GABA) neurotransmission in the nucleus pontis oralis (PnO) of the rat brainstem produce alterations in sleep/wake behavior. Local applications of GABA_A receptor antagonists and agonists increase REM sleep and wake, respectively. These findings support a role for GABAergic mechanisms of the PnO in the control of arousal state. We have been investigating sources of GABA innervation of the PnO that may interact with local GABA_A receptors in the control of state. Utilizing a retrograde tracer, cholera toxin-B subunit (CTb), injected into the PnO and dual-label immunohistochemistry with an antibody against glutamic acid decarboxylase-67 (GAD67), we report on a previously unidentified GABAergic neuronal population projecting to the contralateral PnO appearing as a column of cells, with long-axis in the sagittal plane, extending through the midbrain and pons. We refer to these neurons as the mesopontine GABAergic column (MPGC). The contiguous, columnar, anatomical distribution suggests operation as a functional neural system, which may influence expression of REM sleep, wake and other behaviors subserved by the PnO.

Keywords

REM sleep; GABA; nucleus pontis oralis; retrograde labeling; cholera toxin-B subunit

1. Introduction

The medial pontine reticular formation has been implicated in a variety of physiological and behavioral functions. One behavior influenced by this region is REM sleep. Specifically, the oral pontine reticular formation (PnO) is considered a REM sleep induction zone in the region because of the increase in REM sleep following the intracerebral injection of several different neurotransmitter-receptor ligands directly into this area (Ahnaou et al., 1999; Ahnaou et al., 2000; Baghdoyan et al., 1987; Bier and McCarley, 1994; Bourgin et al., 1999; Bourgin et al., 1995; Bourgin et al., 1997; Marks and Birabil, 1998; Marks et al., 2003; Yamuy et al., 1995). The ability of pharmacological agents interacting with these brainstem mechanisms to significantly alter the expression of state strongly implicates the PnO as a region concerned with the natural regulation of REM sleep.

Corresponding author: Gerald A. Marks Dallas VA medical Center MC# 151 4500 South Lancaster Road Dallas, Texas 75216 Tel. (214) 857-0279 Mobile (214) 240-1654 Fax (214) 462-4966 gerald.marks@utsouthwestern.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Recently, a role for GABAergic neurotransmission has been supported by the action of GABA_A receptor antagonists and GABA synthesis inhibitors, injected into PnO of rat, to induce REM sleep (Marks et al., 2008; Sanford et al., 2003; Watson et al., 2008). This implies the action of GABA in the PnO exerts negative control of REM sleep and withdrawal of its actions promotes REM sleep expression. In addition, GABA_A receptor agonists and GABA reuptake inhibitors, injected into PnO of rat result in decreased REM sleep and increased wake (Camacho-Arroyo et al., 1991; Sanford et al., 2003; Watson et al., 2008). This implies a positive action of GABA in PnO in the control of wakefulness.

GABAergic neurotransmission is a prominent mechanism in the pontine reticular formation. It is estimated, based on ultra-structural examination, that 30% of all synapses in the ventral PnO of the cat are GABAergic (De La Roza and Reinoso-Suarez, 2006). The PnO is densely innervated by GABAergic fibers (De La Roza and Reinoso-Suarez, 2006; Ford et al., 1995) whose sources have yet to be completely identified. Afferents of the PnO are from widespread regions along the neuraxis with almost half originating from other areas of the brain reticular formation, including the contralateral PnO (Shammah-Lagnado et al., 1987). Some of these afferents, in addition to collateral axons of local neurons, are the sources of GABAergic innervation of the PnO that may be involved in control of arousal state.

We have initiated a project with the long-term objective to identify the sources of GABAergic innervation of the PnO and determine whether these identified neuronal populations have a role in the control of state. Utilizing a retrograde tracer, cholera toxin-B subunit (CTb) injected into the PnO and dual-label immunohistochemistry with an antibody against glutamic acid decarboxylase-67 (GAD67), we report here on a previously unidentified GABAergic neuronal population projecting to the contralateral PnO appearing as a column of cells, with long-axis in the sagittal plane, extending through the midbrain and pons. We refer to these neurons as the mesopontine GABAergic column (MPGC).

2. Results

Observations in this report are restricted to the anatomical definition of a novel neuronal population based on hodology and neurotransmitter utilization. These neurons are identified with CTb-immunoreactive somata following microinjections of this retrograde tracer into the PnO on the contralateral side of the brain. Additionally, these neurons co-express immunoreactivity to the GABA synthetic enzyme, GAD67. The population is a collection of GABAergic neurons projecting to the PnO organized as a column extending caudally from the level of the rostral pole of the pedunculopontine tegmental nucleus (PPT) to the caudal pons.

2.1. Ctb injections and retrograde transport

Injections of 0.5% CTb were aimed at the caudal aspects of the PnO. With either 13.8 or 9.2 nL volumes, diffusion in the anterior and posterior directions tended to take an unpredictable course dorsally or ventrally. The four rats chosen for analysis were based on the injection being confined to the PnO (see figure 1 for representative example). An additional, fifth, rat was prepared with a small iontophoretic deposit of CTb well within the borders of the nucleus. The CTb deposit in this subject was the smallest of the group and, as might be expected, resulted in the fewest number of retrogradely labeled neurons.

In the region examined from the caudal midbrain through the pons, the distribution of retrogradely labeled CTb-containing, neuronal, somata was very similar to that described by Shammah-Lagnado and colleagues (Shammah-Lagnado et al., 1987) for the rat following injections of horseradish peroxidase or horseradish peroxidase conjugated with wheat germ agglutinin into the PnO. Here we find a large number of CTb-labeled somata in the periaqueductal central grey matter and midbrain reticular formation, with a predominance on

the side ipsilateral to the injection. Labeled neurons were found in the deep layers of the superior colliculus, with a small contralateral dominance. A consistent aggregate of labeled neurons was found contralateral to the CTb injection caudal to the substantia nigra reticulata (SNR) and dorsolateral to the medial lemniscus (ml). Our investigation of the neurotransmitters utilized by this latter cell population lead to uncovering the MPGC (see Discussion).

In the pons, CTb labeled cells continued to be observed in the central grey and most caudal midbrain reticular fields as well as appearing in the raphe nuclei, parabrachial area, Kolliker-Fuse nucleus, and several nuclei of the dorsal pontine tegmentum. The largest number of labeled neurons resided within the pontine reticular formation. A high density of labeled neurons was always observed in a location roughly symmetrical to the injection site on the contralateral side. Labeled neurons were found bilaterally in reticularis pontis caudalis (PnC).

The relative distribution of CTb labeled neurons did not differ among rats with PnO injections of variable size. The absolute number observed was dependent on the extent of the injection. In the 14 sections of five rats analyzed for the region of the MPGC, the mean number of CTb labeled neurons counted was 403 ± 161 (mean $\pm$ SEM). The rat with the largest injection resulted in 1024 and the smallest, deposited by iontophoresis, 87 (see table 1).

2.2. The mesopontine GABAergic column

Figure 2a is a bright-field photomicrograph of the PnO on the side contralateral to the injection. The tissue is immuno-labeled for GAD67 and CTb utilizing the immunoperoxidase method. The field is typical of the material used to count and plot labeled neuronal somata. The CTb-immuno-labeling is observed as granular, blue-black cytoplasmic staining with nickel-intensified diaminobenzidine tetrahydrochloride (DAB). The yellow-brown DAB staining for GAD67 immunoreactivity labels cell-bodies of various sizes and fibers in the neuropil, which can achieve a high density. Presence of both labels in a neuronal somata constituted the criterion for counting the cell as a GABAergic neuron projecting to the PnO on the contralateral side.

The same pattern of labeling was observed utilizing the higher resolution afforded by laser scanning confocal fluorescence microscopy. Figure 2b is an example of a double-labeled neuron in the midbrain portion of the MPGC. Viewing the individual channels and their merging clearly demonstrates the colocalization of CTb and GAD67 in the neuron shown. Every second image of a z-stack is shown in Figure 2c using a focal plane of 1 μ m. Both double and single-CTb labeled neurons are shown in this field in the caudal PnO contralateral to the CTb injection. Typical of the pontine reticular formation are GAD67 immuno-labeling of a dense fiber network in the neuropil and the presence of GAD67 immuno-positive somata with a relatively large nucleus and small cytoplasmic compartment.

The neuronal population described here in the rat consists of somata co-immunopositive for antibodies directed against CTb and GAD67, with the CTb injected into the contralateral PnO. They comprise a contiguous distribution in the form of a column extending caudally from the rostral pole of the PPT in the midbrain to the most caudal aspects of the pontine reticular formation, a distance of approximately 3.5 mm. Figure 3 is a schematic diagram from one rat representative of the distribution of double-labeled and single CTb-only labeled neurons in the region. At its most rostral extent, Bregma -6.4 to -6.7 mm, the GABAergic neurons of the column are intermingled with cholinergic neurons of the PPT as well as in close association to the serotonergic B9 cell group (Vertes and Crane, 1997). Double-labeled neurons are found ventral to the retrorubral field and dopaminergic A8 cell group (German and Manaye, 1993). We have not observed any neurons immunopositive for choline acetyltransferase (ChAT) or tyrosine hydroxylase (TH) to also label for CTb at this level (see supplemental figure 1). Proceeding in the caudal direction, cholinergic neurons of the PPT maintain a position lateral to the decussation of the superior cerebellar peduncle (xscp) in its shift dorsad, while neurons

of the column retain their ventral position dorsal to the medial lemniscus and ventral to the xscp. The column extends further caudal through the PnO into the PnC where it is reduced in number and appears to drift medially, to include the raphe interpositus nucleus. The most caudal extent is at the level of the exiting VII nerve, Bregma -10.0 mm.

The mean total percentage of double-labeled CTb⁺/GAD67⁺ cells to the total number of CTb labeled cells was quite high and similar across animals, 65.6±2.8% (mean±SEM). Variation in the means across the 14 sections analyzed also was small, SEM=±2.4. Thus, despite variation in the absolute numbers of cells across animals, the proportions of double-labeled cells remained quite constant (see table 2). No statistical differences were detected in the percentage either across sections or across subjects (Kruskal-Wallis one-way ANOVA).

Although cell-counts were not performed, we have observed that CTb injections into the PnC also give rise to GAD67, double-labeled neurons in the column. Thus, the GABAergic innervation by column-neurons appears not to be restricted to the contralateral PnO.

3. Discussion

In a preliminary survey looking for GABAergic neurons projecting to the PnO, we identified several populations of neurons labeled with CTb injected into the PnO and positively immunostained for GAD67 (Marks et al., 2006). One area of interest was a dense aggregation of these double-labeled neurons in the area of the rostral pole of the PPT on the side contralateral to the CTb injection. Another area was within the dense region of CTb somatic-labeling consistently found in the PnO contralateral to the injection. Subsequent examination of the series of closely spaced coronal sections through the entire midbrain and pons revealed that these two areas were part of a contiguous column of GABAergic neurons defined by their projections to the contralateral PnO.

Neurons in the rostral aspects of the column were described by Shammah-Lagnado and colleagues (Shammah-Lagnado et al., 1987) in their studies of afferents to the PnO in the rat where it was suggested that they may be part of the A8 catecholamine cell group. We report here that these neurons are not immunopositive for either ChAT or TH. This brain area also contains the serotonergic neurons of the B9 group (Vertes and Crane, 1997). Although we have not examined this directly, it has been reported in the rat that virtually no serotonin-immunoreactive neurons in this region also contain GABA (Stamp and Semba, 1995). This would indicate neurons of the column also do not express the serotonergic neurotransmitter phenotype of the B9 group. We can not comment on colocalization of other neurotransmitters, such as glycine (Tanaka and Ezure, 2004).

The rostral region of the GABAergic column, medial to the PPT, has been termed the midbrain extrapyramidal area based on cellular morphology and reciprocal connectivity with nuclei part of the extrapyramidal motor system (Lee et al., 1988). There also appears to be some overlap between this area and that of the mesencephalic locomotor region defined by electrically induced locomotion on a treadmill (Skinner and Garcia-Rill, 1984). From studies in cat, the region occupied by the column has been designated as a motor inhibitory system called the ventral mesopontine junction in which electrical stimulation can induce global inhibition of muscle tone in decerebrate animals (Lai and Siegel, 1990) and chronic lesions induced by neurotoxins result in selective suppression of the muscle atonia accompanying REM sleep (Lai et al., 2008).

A similar role in muscle inhibition during REM sleep has been ascribed to the pontine reticular formation based on lesion experiments (Sanford et al., 2001). In rat, unilateral lesions in the pontine reticular formation are reported to induce postural asymmetry and/or rotation towards

the side contralateral to the lesion (Costall et al., 1976) and electrical stimulation induces ipsiversive circling behavior (Miliaressis and Philippe, 1984).

Although there is an association of the anatomical location of the MPGC with these mechanisms related to motor activity, it is not clear that the GABAergic neurons of the column share in any of these roles. We have reported unilateral microinjection of GABA_A receptor antagonists, injected into PnO of rat, result in long-lasting increases in REM sleep and also can elicit short-lasting, vigorous, ipsiversive circling behavior (Marks et al., 2008). High-dose, bilateral injections of bicuculline, an antagonist, have been reported to induce circling and “escape-like” behaviors (Sanford et al., 2003). Unilateral Injection of muscimol, an agonist, can elicit circling to the contralateral side (unpublished observation). Therefore, at least one component of the role PnO plays in motor control appears to have dependence upon a GABAergic innervation.

With little doubt, the ubiquity of GABAergic synapses in the PnO (De La Rosa and Reinoso-Suarez, 2006) and the many functions ascribed to the PnO are consistent with multiple roles of GABA's action. Even though injection of GABA_A receptor antagonists into PnO of rat can have effects on both motor behavior and REM sleep, the mechanisms are independent as evidenced by the different time courses of action and that the REM sleep induction is blocked by atropine while the induction of circling behavior is not (Marks et al., 2008).

In the most caudal aspects of the MPGC, neurons migrate to a more medial position and are found in the nucleus raphe interpositus, a site of the location of omnipause neurons (OPN) (Buttner-Ennever et al., 1988). OPN are involved in the generation of fast saccades and inasmuch as their cessation of firing accompanies the rapid eye movements of wake and REM sleep they appear to be involved in the generation of both (Matsui et al., 1994). OPNs have diverse projections, one of which is distributed over parts of the PnO (Langer and Kaneko, 1983). The morphological description of OPNs as bipolar with dendrites oriented horizontally appearing in vertical columns adjacent to the midline matches that observed here (Buttner-Ennever et al., 1988). One discrepancy is that OPNs in monkey, cat and human are immunoreactive for glycine and are devoid of GABA immunostaining (Horn et al., 1994). By our definition of MPGC neurons, the neurons we describe are immunoreactive to GAD67. This may represent a species difference and OPNs in rat may express both GABA and Glycine. Such colocalization has been described for neurons in the raphe interpositus of the rat (Tanaka and Ezure, 2004).

Preliminary data indicate GABAergic neurons in the region of the column do not project exclusively to the contralateral PnO. Retrogradely labeled neurons were observed with tracer injections placed more caudal in the PnC (data not shown). GABA_A receptor agonists injected into PnC of rat reduce REM sleep (Sanford et al., 2003). In addition, Boissard and colleagues (Boissard et al., 2003) investigated GABAergic neurons projecting to the sublaterodorsal nucleus (SLD), another REM sleep induction zone in the rat, and report neurons at locations that coincide with the MPGC. These areas include the rostral pole of the PPT and the PnO. The SLD supports induction of REM sleep by GABA_A receptor antagonists in rat, though action of agonists to induce wake has not been reported (Boissard et al., 2002; Pollock and Mistlberger, 2003). Effects on both REM sleep and wake have been reported for antagonists and agonists, respectively, injected into a homologous region in the cat (Xi et al., 1999). Several species differences exist in physiological responses to intracerebral injection, and possibly neuroanatomy, between rat and cat warranting caution in making inferences drawn from one species to the other (discussed in Marks et al., 2008). Continued work will be required to determine the extent of the projections of the column, whether individual GABAergic neurons have multiple targets and whether this anatomical feature is present in species other than rat.

The neural population defined here in rat as the MPGC based on utilization of the neurotransmitter GABA, common projections to the contralateral PnO and contiguous columnar anatomical distribution suggest operation as a functional neural system. Inasmuch as this is so, determination of function will require additional investigation. One candidate function of the MPGC is as the source of GABA innervation of PnO demonstrated to exert negative control over the expression of REM sleep and positive control over wakefulness (Camacho-Arroyo et al., 1991; Marks et al., 2008; Sanford et al., 2003; Watson et al., 2008). Additional association of the column with regions involved in motor control and generation of rapid eye movements, both REM sleep and wake accompanying phenomena, reinforce the need to study this novel system as a mechanism of state control.

4. Experimental procedure

All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by local Institutional Animal Care and Use Committees at UT Southwestern Medical Center and the Dallas VA. All efforts were made to minimize the number of animals used and their suffering.

4.1. Delivery of retrograde tracer

Five, male, Long-Evans Hooded rats (Harlan) weighing between 150 and 300 g were deeply anaesthetized with isoflurane and placed in a stereotaxic apparatus. A glass micropipette filled with a 0.5% solution of CTb (List Biological Labs) was aimed unilaterally at the PnO (coordinates: bregma -8.3 to -8.8 , lateral 1.0 , 8.0 mm from skull). In four rats, delivery was achieved by pressure injection (Nanoinjector, WPI) of either 13.8 or 9.2 nL through a pipette with lumen 25 μ m (ID). In one rat, the CTb was ejected iontophoretically ($+1$ μ A, $10/10$ sec on/off, 30 min) through a pipette with lumen 5 μ m (ID).

4.2. Tissue preparation and Immunohistochemistry

At the end of a post-injection survival period of 11 days, animals were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and then perfused through the ascending aorta with 30 ml heprinized saline and 550 ml of 4% paraformaldehyde in 0.1 M 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 submerged. Sections, 20 μ m-thick, were cut on a freezing microtome (SM2000R, Leica). Serial sections, including the midbrain and pons, were collected in 0.1 M PB, with 0.005% sodium azide, and divided into 12 series of sections 240 μ m apart. A total of 14 sections spanned the area of interest and was used in the analysis for each rat.

Both immunoperoxidase and fluorescence immunohistochemical methods were used in this study. For immunoperoxidase staining, the frozen sections were washed in phosphate buffered saline (PBS, pH 7.4), treated with 1% hydrogen peroxide in PBS and blocked in 5% normal serum in PBS. Sections were then incubated with diluted goat CTb polyclonal antibody ($1:40,000$, List Biological Labs) over-night at room temperature. This was followed by incubation with 1.5 μ g/ml biotinylated secondary antibody (Vector Labs) for 30 minutes and with avidin/biotin/peroxidase reagent ($1:250$, ABC Elite, Vector Labs) for 1 hour. Sections were reacted in acetate buffer (pH 6.0) containing 0.035% diaminobenzidine tetrahydrochloride (DAB), 2.5% nickel ammonium sulfate and 0.001% hydrogen peroxide (nickel-DAB), for $7-8$ minutes, to produce a blue/black stain. After a 30 min. wash in PBS and 1.5% normal horse serum (Elite Vectastain kit) for blocking, sections were immersed in diluted mouse GAD67 monoclonal antibody ($1:8,000$, Chemicon) or mouse ChAT monoclonal antibody ($1:1000$, Chemicon) at room temperature overnight. 1.5 μ g/ml biotinylated secondary antibody and avidin/biotin/peroxidase reagent ($1:250$), from the same ABC Elite kit, were used

and a standard DAB solution (0.035% DAB in 0.1M PBS, pH 7.4 with 0.001% of hydrogen peroxide) was employed, for 8-10 min, to produce a brown/yellow stain. All incubations were done on a shaker with PBS washes, 10 min. $\times$ 3, between each major step.

For fluorescence staining, free-floating sections were primarily blocked for 1 hour with donkey normal serum in PBS with 0.3% triton $\times$ 100 and then incubated in a mixture of primary antibodies: either goat CTb polyclonal antibody(1:5,000) and mouse GAD67 polyclonal antibody(1:1,000) or goat CTb polyclonal antibody and rabbit TH polyclonal antibody (1:1000, Protos Biotech) at 4 °C for 40 hours. After three PBS washes, sections were incubated with a mixture of secondary antibodies (anti-goat IgG and anti-mouse IgG) respectively conjugated to FITC and Cy3 in a solution of PBS, triton and donkey serum. All secondary antibodies used were raised from donkey and affinity-purified (Jackson ImmunoResearch). Finally, the sections were washed with PBS, mounted, and cover-slipped with a mounting medium containing glycerin and phenylenediamine to reduce fading of immunofluorescence.

All ABC and fluorescence immunolabeling were completely abolished when primary or secondary antibodies, described above, were omitted.

4.3. Data analysis

Series of sections labeled for GAD67/CTb and ChAT/CTb, processed with the immunoperoxidase method, were examined by bright-field microscopy under a 40x objective utilizing an Olympus BX-51 microscope equipped with a motorized xyz stage and CCD camera (Microfire, Optronics). Every identified single CTb- and double-labeled somata in the area of interest were mapped and counted with the aid of computer-based image analysis software (Neurolucida, MicroBrightField). The area of interest was on the side of the brain contralateral to the CTb injection site and defined rostrally by the most rostral pole of the PPT and most caudal pole of the substantia nigra reticulata, bordered dorsally by the retrorubral field and ventrally by the medial lemniscus. This area extended caudally into the pontine reticular formation to include all of the PnO and PnC. The atlas of Paxinos and Watson was used as a reference (Paxinos and Watson, 1997). The criteria for identifying a CTb-positive neuronal somata was the presence of at least three nickel-DAB clusters of particles. CTb-positive somata were then determined to be double-labeled by the presence or absence of DAB staining. Single-CTb and double-labeled somata were marked, plotted and automatically counted to generate the data for tables and schematic maps.

Fluorescence images of CTb and GAD67- or TH-immunoreactivity were obtained with a laser scanning confocal microscope (LSM 510 META, Zeiss) using 63x or 100x objectives with two laser lines (emission wavelengths 488 and 543 nm). Pinhole under each laser set to 1 airy units. Images were restricted to the top 3-9 μ m of the section where penetration of all antibodies was confirmed. Scanning and optical sectioning in the z-dimension, through the thickness of the section, were performed using multitrack scanning with a section thickness of 0.33 μ m or 1 μ m. Typically, z-stacks of 6-15 consecutive images (512 $\times$ 512 pixels) were recorded with the photomultiplier settings adjusted for sampling over the area of interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the Department of Veterans Affairs (Merit Review) and The National Institutes of Health (R01MH57434). We thank Ginger Zhou for her technical assistance in immunohistochemistry.

Abbreviations

7n, facial nerve
 A8, A8 dopamine cells
 CLi, caudal linear raphe nucleus
 cp, cerebral peduncle
 CTb, cholera toxin-B subunit
 DAB, diaminobenzidine tetrahydrochloride
 DMTg, dorsomedial tegmental area
 GABA, γ -aminobutyric acid
 GAD67, glutamic acid decarboxylase-67
 IPc, interpeduncular nucleus caudalis
 ml, medial lemniscus
 MnR, median raphe nucleus
 Mo5, motor trigeminal nucleus
 MPGC, mesopontine GABAergic column
 OPN, omnipause neurons
 Pa6, paraabducens nucleus
 PBS, phosphate buffered saline
 PmR, paramedian raphe
 Pn, pontine nuclei
 PnC, reticularis pontis caudalis
 PnO, nucleus pontis oralis
 PnV, ventral pontine reticular nucleus
 PPT, pedunculopontine tegmental nucleus
 Pr5, principal sensory trigeminal nucleus
 py, pyramidal tract
 RPO, rostral periolivary region
 RRF, retrorubral field
 rs, rubrospinal tract
 RtTg, reticulotegmental nucleus
 SLD, sublateral rodorsal nucleus
 SNR, substantia nigra reticulata
 Sp5, spinal trigeminal nucleus
 ts, tectospinal tract
 VLL, ventral nucleus of the lateral lemniscus
 VTA, ventral tegmental area
 VTg, ventral tegmental nucleus
 xscp, decussation of the superior cerebellar peduncle

References

- Ahnaou A, Basille M, Gonzalez B, Vaudry H, Hamon M, Adrien J, Bourgin P. Long-term enhancement of REM sleep by the pituitary adenylyl cyclase-activating polypeptide (PACAP) in the pontine reticular formation of the rat. *Eur. J. Neurosci* 1999;11:4051–4058. [PubMed: 10583493]
- Ahnaou A, Laporte AM, Ballet S, Escourrou P, Hamon M, Adrien J, Bourgin P. Muscarinic and PACAP receptor interactions at pontine level in the rat: significance for REM sleep regulation. *Eur. J. Neurosci* 2000;12:4496–4504. [PubMed: 11122360]
- Baghdoyan HA, Rodrigo-Angulo ML, McCarley RW, Hobson JA. A neuroanatomical gradient in the pontine tegmentum for the cholinceptive induction of desynchronized sleep signs. *Brain Research* 1987;414:245–261. [PubMed: 3620930]

- Bier MJ, McCarley RW. REM-enhancing effects of the adrenergic antagonist idazoxan infused to the medial pontine reticular formation of the freely moving cat. *Brain Research* 1994;634:333–338. [PubMed: 7907526]
- Boissard R, Fort P, Gervasoni D, Barbagli B, Luppi P-H. Localization of the GABAergic and non-GABAergic neurons projecting to the sublaterodorsal nucleus and potentially gating paradoxical sleep onset. *Eur. J. Neurosci* 2003;18:1627–1639. [PubMed: 14511341]
- Boissard R, Gervasoni D, Schmidt MH, Barbagli B, Fort P, Luppi P-H. The rat ponto-medullary network responsible for paradoxical sleep onset and maintenance: a combined microinjection and functional neuroanatomical study. *Eur. J. Neurosci* 2002;16:1959–1973. [PubMed: 12453060]
- Bourgin P, Ahnaou A, Laporte AM, Hamon M, Adrien J. Rapid eye movement sleep induction by vasoactive intestinal peptide infused into the oral pontine tegmentum of the rat may involve muscarinic receptors. *Neuroscience* 1999;89:291–302. [PubMed: 10051236]
- Bourgin P, Escourrou P, Gaultier C, Adrien J. Induction of rapid eye movement sleep by carbachol infusion into the pontine reticular formation in the rat. *NeuroReport* 1995;6:532–536. [PubMed: 7766858]
- Bourgin P, Lebrand C, Escourrou P, Gaultier C, Franc B, Hamon M, Adrien J. Vasoactive intestinal polypeptide microinjections into the oral pontine tegmentum enhance rapid eye movement sleep in the rat. *Neuroscience* 1997;77:351–360. [PubMed: 9472395]
- Büttner-Ennever JA, Cohen B, Pause M, Fries W. Raphe nucleus of the pons containing omnipause neurons of the oculomotor system in the monkey, and its homologue in man. *J. Comp. Neurol* 1988;267:307–321. [PubMed: 2830321]
- Camacho-Arroyo I, Alvarado R, Manjarrez J, Tapia R. Microinjections of muscimol and bicuculline into the pontine reticular formation modify the sleep-waking cycle in the rat. *Neurosci. Lett* 1991;129:95–97. [PubMed: 1656343]
- Costall B, Naylor RJ, Marsden CD, Pycock C. Circling behavior produced by asymmetric median raphe nuclei lesions in rats. *J. Pharm. Pharmacol* 1976;28:248–249. [PubMed: 6702]
- De La Rosa C, Reinoso-Suarez F. GABAergic structures in the ventral part of the oral pontine reticular nucleus: an ultrastructural immunogold analysis. *Neuroscience* 2006;142:1183–1193. [PubMed: 16916586]
- Ford B, Holmes CJ, Mainville L, Jones BE. GABAergic Neurons in the rat pontomesencephalic tegmentum: codistribution with cholinergic and other tegmental neurons projecting to the posterior hypothalamus. *J. Comp. Neurol* 1995;363:177–196.
- German DC, Manaye KF. Midbrain dopaminergic neurons (nuclei A8, A9, and A10): three-dimensional reconstruction in the rat. *J. Comp. Neurol* 1993;331:297–309. [PubMed: 8514911]
- Horn AK, Büttner-Ennever JA, Wahle P, Reichenberger I. Neurotransmitter profile of saccadic omnipause neurons in nucleus raphe interpositus. *J. Neurosci* 1994;14:2032–2046. [PubMed: 7908956]
- Lai YY, Siegel JM. Muscle tone suppression and stepping produced by stimulation of the midbrain and rostral pontine reticular formation. *J. Neurosci* 1990;10:2727–2734. [PubMed: 2388085]
- Lai YY, Hsieh K-C, Nguyen D, Peever J, Siegel JM. Neurotoxic lesions at the ventral mesopontine junction change sleep time and muscle activity during sleep: An animal model of motor disorders in sleep. *Neuroscience* 2008;154:431–443. [PubMed: 18487021]
- Langer TP, Kaneko CR. Efferent projections of the cat oculomotor reticular omnipause neuron region: an autoradiographic study. *J. Comp. Neurol* 1983;217:288–306. [PubMed: 6886055]
- Lee HJ, Rye DB, Hallanger AE, Levey AI, Wainer BH. Cholinergic vs. noncholinergic efferents from the mesopontine tegmentum to the extrapyramidal motor system. *J. Comp. Neurol* 1988;22:469–492. [PubMed: 2461392]
- Marks GA, Birabil CG. Enhancement of rapid eye movement sleep in the rat by cholinergic and adenosinergic agonists infused into the pontine reticular formation. *Neuroscience* 1998;86:29–37. [PubMed: 9692741]
- Marks, GA.; Birabil, CG.; Liang, C-L. Extra-reticular sources of GABA implicated in control of REM sleep and wakefulness.. *Neuroscience Meeting Planner* 458.16.; Atlanta, GA: Society for Neuroscience. 2006. CD-ROM

- Marks GA, Sachs OW, Birabil CG. Blockade of GABA, type A, receptors in the rat pontine reticular formation induces rapid eye movement sleep that is dependent upon the cholinergic system. *Neuroscience* 2008;156:1–10. [PubMed: 18706488]
- Marks GA, Shaffery JP, Speciale SG, Birabil CG. Enhancement of rapid eye movement sleep in the rat by actions at A1 and A2a adenosine receptor subtypes with a differential sensitivity to atropine. *Neuroscience* 2003;116:913–920. [PubMed: 12573729]
- Matsui H, Sasaki Y, Tamai A. Firing patterns of the pontine omnipause neurons during sleep in the cat. *Nippon Ganka Gakkai Zasshi* 1994;98:245–250. [PubMed: 8154381]
- Miliaressis E, Philippe L. The pontine substrate of circling behavior. *Brain Research* 1984;293:143–152. [PubMed: 6704711]
- Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates: Compact Third Edition CD-ROM*. Academic Press; San Diego: 1997.
- Pollock MS, Mistlberger RE. Rapid eye movement sleep induction by microinjection of the GABA-A antagonist bicuculline into the dorsal subcoeruleus area of the rat. *Brain Research* 2003;962:68–77. [PubMed: 12543457]
- Sanford LD, Cheng C, Tang X, Silvestri A, Mann G, Ross RJ, Morrison AR. Sleep and behavior in rats with pontine lesions producing REM sleep without atonia. *Sleep Res Online* 2001;4:1–5.
- Sanford LD, Tang X, Xiao J, Ross RJ, Morrison AR. GABAergic regulation of REM sleep in reticularis pontis oralis and caudalis in rats. *J. Neurophysiol* 2003;90:938–945. [PubMed: 12672782]
- Shammah-Lagnado SJ, Negrao B, Silva A, Ricardo JA. Afferent connections of the nuclei reticularis pontis oralis and caudalis: a horseradish peroxidase study in the rat. *Neuroscience* 1987;20:961–989. [PubMed: 2439943]
- Skinner RD, Garcia-Rill E. The mesencephalic locomotor region (MLR) in the rat. *Brain Research* 1984;323:385–389. [PubMed: 6525525]
- Stamp JA, Semba K. Extent of colocalization of serotonin and GABA in the neurons of the rat raphe nuclei. *Brain Research* 1995;677:39–49. [PubMed: 7606468]
- Tanaka I, Ezure K. Overall distribution of GLYT2 mRNA-containing versus GAD67 mRNA-containing neurons and colocalization of both mRNAs in midbrain, pons, and cerebellum in rats. *Neurosci. Res* 2004;49:165–178. [PubMed: 15140559]
- Vertes RP, Crane AM. Distribution, quantification, and morphological characteristics of serotonin-immunoreactive cells of the suprallemniscal nucleus (B9) and pontomesencephalic reticular formation in the rat. *J. Comp. Neurol* 1997;378:411–24. [PubMed: 9034900]
- Watson CJ, Soto-Calderon H, Lydic R, Baghdoyan HA. Pontine reticular formation (PnO) administration of hypocretin-1 increases PnO GABA levels and wakefulness. *Sleep* 2008;31:453–464. [PubMed: 18457232]
- Yamuy J, Morales FR, Chase MH. Induction of rapid eye movement sleep by the microinjection of nerve growth factor into the pontine reticular formation of the cat. *Neuroscience* 1995;66:9–13. [PubMed: 7637879]
- Xi M-C, Morales FR, Chase MH. Evidence that wakefulness and REM sleep are controlled by a GABAergic pontine mechanism. *J. Neurophysiol* 1999;82:2015–2019.

Fig. 1. Representative example of a CTb injection into the caudal aspects of the PnO. The injection site is visualized by the large, dark spot (arrow) immuno-labeled for CTb in this coronal section counter-stained with neutral red. Bar represents 1 mm.

Fig. 2.
 (A-C) Examples of immuno-labeled neurons in the MPGC. (A) Bright-field immunoperoxidase labeled neurons in the PnO on the side contralateral to the CTb injection. CTb is labeled with nickel-intensified DAB (bluish-black) and GAD67 with DAB (yellowish-brown). Double-labeled, CTb/GAD67 somata are indicated by large arrows. Single-labeled, CTb-only somata are indicated by small arrows. Several GAD67-only somata appear and are not indicated by arrows. (B) Laser scanning fluorescent image of a double-labeled neuron in the midbrain portion of the column. Panels, B1 and B2, are the individual color channels; CTb, green and GAD67, red. Panel B3 is the merged image, colocalization is yellow. (C) Laser scanning fluorescent z-stack of a field in the PnO using a 1 μm focal plane. The four panels,

C1-4, are a series of every second image in the stack. Colors are as they appear in (B). Small arrows in C3 indicate double-labeled, CTb/GAD67 somata and large arrow, CTb-only. (A-C) Bar indicates scale.

Fig. 3. Schematic diagram of the distribution of double-labeled, CTb/GAD67 somata (filled circles) and single-labeled, CTb-only somata (open circles). (A-H) Coronal sections, anterior to posterior, of a representative animal through the region of the MPGC on the side contralateral to the CTb injection. Sections are labeled with their approximate distance (mm) posterior to the Bregma suture. Each symbol represents the count of three somata on that section. Diagrams adapted from Paxinos and Watson (1997).

Table 1

Numbers of Labeled Cells Counted

Subject	Label	Section Level Posterior to Bregma (mm)										10.0	TotalGrand Totals			
		6.4	6.7	7.0	7.2	7.5	7.8	8.1	8.3	8.6	8.9			9.2	9.4	9.7
TR-51	CTb/GAD	8	17	18	15	17	9	23	13	12	14	13	16	11	9	195
	CTb only	2	3	12	7	5	7	8	6	7	9	4	8	2	1	81
TR-52	CTb/GAD	9	24	21	23	16	18	11	15	10	9	11	12	5	7	191
	CTb only	1	13	5	12	5	12	10	30	8	14	5	3	3	4	125
TR-56	CTb/GAD	11	22	23	27	18	23	18	24	21	8	8	6	5	7	221
	CTb only	4	4	7	8	5	15	8	14	12	5	3	2	2	2	91
TR-68	CTb/GAD	8	36	74	59	67	78	75	39	33	19	20	17	18	17	560
	CTb only	4	10	45	33	42	47	56	77	69	28	24	18	6	5	464
TR-80*	CTb/GAD	0	4	7	4	7	6	1	2	8	6	3	2	2	3	55
	CTb only	2	2	9	2	4	2	3	2	2	1	1	2	0	0	32
Total	CTb/GAD	36	103	143	128	125	134	128	93	84	56	55	53	41	43	1222
	CTb only	13	32	78	62	61	83	85	129	98	57	37	33	13	12	793
Grand Totals		49	135	221	190	186	217	213	222	182	113	92	86	54	55	2015

Section levels approximately referenced to the Paxinos and Watson atlas (1997).

* denotes rat with iontophoretic ejection of CTb.

Table 2

Percentage of All CTb Labeled Neurons Double-Labeled for GAD

Subject	Section Level Posterior to Bregma (mm)														MeanSEM
	6.4	6.7	7.0	7.2	7.5	7.8	8.1	8.3	8.6	8.9	9.2	9.4	9.7	10.0	
TR-51	80.00	85.00	60.00	68.18	77.27	56.25	74.19	68.42	63.16	60.87	76.47	66.67	84.62	90.00	72.22 2.79
TR-52	90.00	64.86	80.77	65.71	76.19	60.00	52.38	33.33	55.56	39.13	68.75	80.00	62.5	63.64	63.77 4.17
TR-56	73.33	84.62	76.67	77.14	78.26	60.53	69.23	63.16	63.64	61.54	72.73	75.00	71.43	77.78	71.79 1.94
TR-68	66.67	78.26	62.18	64.13	61.47	62.40	57.25	33.62	32.35	40.43	45.45	48.57	75	77.27	57.50 4.08
TR-80*	0.00	66.67	43.75	66.67	63.64	75.00	25.00	50.00	80.00	85.71	75	50.00	100	100.00	62.96 7.40
mean	62.00	75.88	64.67	68.37	71.37	62.84	55.61	49.71	58.94	57.54	67.68	64.05	78.71	81.74	65.65 2.42
SEM	15.97	4.31	6.59	2.29	3.63	3.20	8.61	7.27	7.75	8.52	5.71	6.40	6.39	6.18	

Section levels approximately referenced to the Paxinos and Watson atlas (1997).

* denotes rat with iontophoretic ejection of CTb. SEM is the standard error of the mean.