

GABAergic Neuron Distribution in the Pedunculopontine Nucleus Defines Functional Subterritories

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ABSTRACT

γ -Aminobutyric acid (GABA)ergic neurons are widely distributed in brainstem structures involved in the regulation of the sleep-wake cycle, locomotion, and attention. These brainstem structures include the pedunculopontine nucleus (PPN), which is traditionally characterized by its population of cholinergic neurons that have local and wide-ranging connections. The functional heterogeneity of the PPN is partially explained by the topographic distribution of cholinergic neurons, but such heterogeneity might also arise from the organization of other neuronal populations within the PPN. To understand whether a topographical organization is also maintained by GABAergic neurons, we labeled these neurons by *in situ* hybridization for glutamic acid decarboxylase mRNA combined with immunohistochemistry for choline acetyltransferase to reveal cholinergic neurons. We an-

alyzed their distribution within the PPN by using a method to quantify regional differences based on stereological cell counts. We show that GABAergic neurons of the rat PPN have a rostrocaudal gradient that is opposite to that of cholinergic neurons. Indeed, GABAergic neurons are predominantly concentrated in the rostral PPN; in addition, they form, along with cholinergic neurons, a small, high-density cluster in the most caudal portion of the nucleus. Thus, we provide evidence of heterogeneity in the distribution of different neuronal populations in the PPN and show that GABAergic and cholinergic neurons define neurochemically distinct areas. Our data suggest that the PPN is neurochemically segregated, and such differences define functional territories. *J. Comp. Neurol.* 515:397–408, 2009.

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Indexing terms: brainstem; cholinergic neurons; *in situ* hybridization; stereology; topography; basal ganglia

GABAergic neurons are widely distributed across the brainstem (Ford et al., 1995). Although their functional roles have not been fully elucidated, they are likely to play diverse roles in the regulation of behavioral states, such as sleep homeostasis (Brown et al., 2008; Datta, 2007; Torterolo et al., 2001), locomotor control (Takakusaki et al., 2004), and attentional processes (Inglis et al., 2001; Winn, 2006). The pedunculopontine nucleus (PPN), a structure conventionally defined by the boundaries of the CH5 cholinergic neuronal cell group (Mesulam et al., 1983), has been identified as also containing GABAergic neurons (Ford et al., 1995; Jia et al., 2003), which outnumber the cholinergic neurons (Wang and Morales, 2009). Although it is known that PPN neurons have ascending and descending long-range projections (Mena-Segovia et al., 2008a; Semba and Fibiger, 1992), it remains to be determined the extent to which the GABAergic neurons contribute to the regulation of the local activity and how they influence their distant targets in, for instance, the thalamus and basal ganglia. Previous reports have shown that at least a fraction of GABAergic neurons in the PPN send their axons to regions such as hypothalamus (Ford et al., 1995), the subthalamic nucleus (Bevan and Bolam, 1995), and the midbrain dopaminergic systems (Charara et al., 1996), but the difficulty in

labeling GABAergic projection neurons by conventional immunohistochemical methods has slowed the progress in their characterization.

Because the PPN and related regions receive and give rise to dense connections with the basal ganglia (Mena-Segovia et al., 2004) and other functionally distinct systems in the brain

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(Saper et al., 2005), it is likely that some topographic organization defines functional domains within the PPN. It has been proposed that two different regions in the PPN can be distinguished on the basis of the arrangement and density of cholinergic neurons, although the boundary between these two regions is not clearly defined. Thus, a rostral part consists of sparsely distributed cholinergic neurons showing a layer-like cellular arrangement that is referred to as *pars dissipata*, whereas the caudal part has a higher density of cholinergic neurons but lacks the layer-like organization and is referred to as *pars compacta* (Honda and Semba, 1995; Rye et al., 1987). In line with this, such a division is also supported by the interconnections of the PPN with the basal ganglia and its efferents to the midbrain dopaminergic systems: the rostral part is preferentially innervated by the basal ganglia output nuclei and projects mainly to the substantia nigra *pars compacta*, whereas the caudal portion does not receive such inputs and innervates preferentially neurons in the ventral tegmental area (Grofova and Zhou, 1998; Oakman et al., 1995; Shink et al., 1997; for review see Mena-Segovia et al., 2008b). Indeed, recent studies have shown that there are also functional differences between the two portions of the PPN, which have been evaluated in terms of behavioral responses following excitotoxic lesions or electrical stimulation (Alderson et al., 2006, 2008; Andero et al., 2007). Taken together, these anatomical and behavioral studies suggest that the rostral PPN is specialized in motor control and associative learning, whereas the functions of the caudal PPN remain to be fully elucidated.

To determine the distribution of different neuronal types in the PPN, as an initial step toward understanding their contribution to functionally distinct areas, we labeled cholinergic neurons by using conventional immunohistochemical techniques for choline acetyltransferase (ChAT) and GABAergic neurons by using in situ hybridization for glutamic acid decarboxylase (GAD) and designed a method to identify topographic differences based on stereological cell counts. We identified a neuronal distribution that supports the idea of a functional segregation and propose a standardized method to divide the rostral from the caudal PPN.

MATERIALS AND METHODS

Animals and tissue preparation

Nine adult male Sprague-Dawley rats (250–310 g; Charles River, Margate, United Kingdom) were used in accordance with the Animals (Scientific Procedures) Act, 1986 (UK). Animals were deeply anesthetized using a mixture of ketamine (30 mg/kg⁻¹, IP; Ketaset, Willow Francis, Crawley, United Kingdom) and xylazine (3 mg/kg⁻¹, IP; Rompun, Bayer, Germany) and intracardially perfused with 0.05 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were removed and post-fixed in the same solution for about 12 hours. Sagittal sections (35 μ m) of the brainstem were obtained with a vibratome (Leica Microsystems, United Kingdom), collected in six series, and stored in PBS. Solutions used for the preparation of brains and subsequent in situ hybridization steps were treated with an RNase inhibitor (0.1% diethyl pyrocarbonate [DEPC]).

In situ hybridization

One of the six series of sagittal sections was hybridized for GAD mRNA (both isoforms, 65 and 67; n = 6), with labeled

riboprobes that have been described previously (for primer sequences see Table 1 in Nair-Roberts et al., 2008). Eight to ten free-floating sections, encompassing an entire 1:6 series, were combined in a single well for processing. The sections were washed several times in PBS containing 0.1% Tween 20 (PBS-T) before transfer to hybridization buffer (HB; 50% formamide, 5 \times SSC, 100 μ g/ml denatured salmon sperm DNA, 50 μ g/ml heparin, 0.1% Tween 20) for preincubation (1 hour at 65°C). Digoxigenin (DIG)-labeled riboprobes (diluted in HB) were added to sections, and hybridization was allowed to take place for 12 hours at 65°C. After a series of high- to low-stringency washes, the sections were transferred to PBS for detection of DIG-labeled riboprobes with a mouse monoclonal anti-DIG antibody conjugated to alkaline phosphatase (Roche Diagnostics, United Kingdom; 1:2,000 in PBS applied overnight at 4°C). Sections were washed in PBS and transferred to a high-pH buffer (HP; 100 mM NaCl, 100 mM Tris, pH 9.5, 0.1% Tween 20, 50 mM MgCl₂). A staining solution consisting of NBT/BCIP (nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate) phosphatase substrate (Roche Diagnostics) diluted 1:50 in HP buffer was applied for 2.5 hours. The phosphatase reaction was terminated by washing in PBS. Optimal concentrations and incubation times were determined in preliminary dilution series and time course experiments (results not shown). An incubation time of 2.5 hours at room temperature was found to produce specific labeling of cell bodies in known GABAergic cell regions, without significant background. In situ hybridization procedures were also carried out with sense DIG-labeled riboprobes to control for the occurrence of nonspecific signals (for details see Nair-Roberts et al., 2008). No cellular labeling was observed in sections hybridized with sense riboprobes (results not shown).

Immunohistochemistry

After in situ hybridization, sections were further processed to reveal ChAT immunoreactivity, along with the sections of three additional brains that were not exposed to any previous processing. Sections that were not incubated with the primary antibody were used as controls for our protocol; in these sections, no immunolabeling was observed. The free-floating sections were incubated overnight with a goat anti-ChAT antibody (1:500; Chemicon, Temecula, CA; AB144P, LV1450147, immunogen: human placental enzyme), followed by a secondary horse anti-goat biotinylated antibody (1:500; Vector, Burlingame, CA) for 4 hours. The ChAT antiserum recognized a single band of ~68–70 kD on Western blot of rat brain (information provided by the manufacturer). Furthermore, with the same antibody, Wang and Morales (2009) observed a 100% correspondence between ChAT immunolabelling and ChAT mRNA labelling in the PPN. Avidin-biotin peroxidase complex (ABC; Vector) was then applied to the sections and incubated for a further 2 hours. The immunoreactivity was then revealed by incubations in diaminobenzidine tetrahydrochloride (DAB; 0.025% w/v; Sigma, St. Louis, MO) and hydrogen peroxide (0.002% w/v; Sigma). Sections were mounted on gelatin-coated slides and left to dry before being dehydrated with alcohols of increasing concentration and xylene and finally covered with resin (XAM, BDH) and coverslips. The level of detection of ChAT immunoreactivity was similar in the sections processed for in situ hybridization (n = 6) and the sec-

tions that were only exposed to the immunohistochemical procedure ($n = 3$).

Image processing

Images of single ChAT labeling or combined with GAD mRNA in situ hybridization were captured with a DVC camera (DigitalVideoCamera, TX) attached to an Eclipse 80i microscope (Nikon) with Neurolucida software (MicroBrightField Inc., Colchester, VT). The brightness and contrast were subsequently adjusted in Photoshop (Adobe Systems Inc., Mountain View, CA). The montage shown in Figure 3A was formed from nine images taken with a $\times 10$ objective, overlapping $\sim 10\%$, and then merged automatically using built-in functions in Photoshop.

Estimation of area and division into segments

The PPN has an irregular, wedge-like shape that extends dorsocaudally from the caudal border of the substantia nigra *pars reticulata* (SNR) toward the superior cerebellar peduncle (scp), at an angle of approximately 60° from a vertical line. To define subregions of the PPN, it was necessary to divide it into segments of equal width that were large enough to obtain a significant estimation of the number of neurons in each segment but small enough to identify gradients in the distribution of neurons along the entire nucleus. Because the anatomical relationship between SNR and PPN is constant across the different mediolateral levels (see Results; see Fig. 2D), the SNR made a good anatomical reference for the PPN. To assess how constant the spatial relationship between SNR and the width of the brainstem is, we arbitrarily defined a second reference point given by the center of an imaginary line crossing the cerebellar peduncle (Fig. 1A). The distance between the center of the SNR (see below) and the middle point of this line (scp centrum) was constant across animals ($4,125 \pm 43 \mu\text{m}$, $n = 6$; mediolateral level with respect to Bregma: 1.9 mm; measured using Neurolucida software; Paxinos and Watson, 1986), further supporting the use of the SNR to reference the PPN subdivisions. Some shrinkage was observed in the X and Y planes as a result of the fixation, processing and mounting protocols but was not taken into account as all sections were treated in a similar manner. The perimeter of the SNR was defined by the dense population of GAD-positive cells surrounded by the fibers of the medial lemniscus and the compacta region. Lines measuring the longest diameter in the horizontal and vertical planes of the SNR were aligned to each other's midpoints to define the center of the SNR. Around this central point, circles were centered with radii increasing in steps of $300 \mu\text{m}$ (including those within the SNR), which divided the PPN in up to 10 segments (Fig. 1A,B). The perimeter of the PPN was determined by cholinergic (ChAT-positive) neurons. The criteria to define the perimeter were as follows: a line was drawn passing by approximately $50 \mu\text{m}$ of the ChAT-positive neurons that delimit the border of the PPN and was relatively smoothed to fit with previous descriptions of the nucleus (Paxinos and Watson, 1986). If a ChAT-positive neuron was more than $150 \mu\text{m}$ from its nearest neighbor and did not fall within a smooth line around the other ChAT-positive neurons, it was excluded as an outlier. There was never more than one outlier per section.

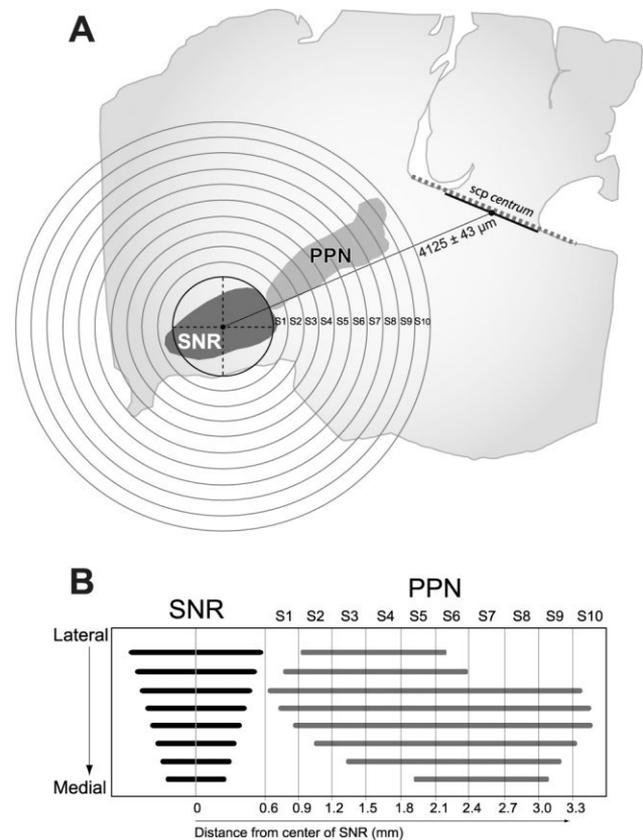


Figure 1. Neurons in the PPN were counted following a radial segmentation. **A:** Schematic of a sagittal section showing the position of the SNR in the midbrain and the PPN in the brainstem. The center of the SNR in the rostrocaudal plane was used as the central reference for the circles that delimit each of the $300\text{-}\mu\text{m}$ segments (S1–S10). The relationship between the SNR and the brainstem was evaluated by the constant distance between its center and the scp centrum (see Materials and Methods). **B:** On average, eight sagittal sections (from a 1:6 series) containing the PPN were obtained from one hemisphere of each brain (the most lateral sections containing only SNR were not included). In each section, segments were nominated by their distance from the center of the SNR (point 0). Segment 1 represents the closest distance of the PPN to the center, which was in all cases $\sim 600 \mu\text{m}$. No more than 10 segments were obtained in any single brain.

Cell counting

Stereological cell counts were carried out using Stereo Investigator software (version 8; MicroBrightField Inc.), an Eclipse 80i microscope (Nikon) with a computer-controlled motorized stage (LUDL Electronic Products) and a Lucivid module projecting the computer's display into the drawing tube of the microscope. The sections (1:6 series, spaced $175 \mu\text{m}$ apart) were examined and aligned, and boundaries of the PPN were drawn while viewing with a $\times 4$ objective. Each segment was outlined as a closed contour. The optical fractionator probe was used to move automatically between counting frames. The counting frame was $75 \mu\text{m}$ square, and the sampling grid was the same size (every positive neuron for each marker was counted). The sections were cut at $35 \mu\text{m}$ on the vibratome but were typically less than $6 \mu\text{m}$ thick once processed and mounted. A $\times 100$ 1.4 NA oil-immersion objec-

tive was used for all cell counts for low depth of field, providing greater accuracy of measurement in the Z plane. A cell was included if the top surface of its nucleus was below the surface of the section. Because ChAT-positive neurons have strongly labeled, large-diameter proximal dendrites that sometimes make it difficult to define the soma, we opted to count cell nuclei instead of "the top of the cell below the surface." The same criterion was used for GAD-positive neurons. Cells that lay outside the contour were not counted. It was judged that, if the nucleus of a cell was more than 50% outside the boundary, then it was outside the contour. The total number of positive neurons for each marker was obtained for each segment at each mediolateral level. To obtain the total number of positive neurons in the PPN of each animal, we summed all the segments at all mediolateral levels and multiplied by 6. The density of positive neurons per segment was obtained by dividing the number of neurons by the original volume of the segment in which they were contained, comprising the boundaries set by the cholinergic neurons and the segment boundaries. Finally, the densities were averaged across animals after summing the corresponding segments across mediolateral levels.

Statistical analysis

The density (number of positive cells per cubic millimeter) of ChAT-positive and GAD-positive neurons in each of the 300- μm segments into which the PPN had been divided was calculated. To eliminate volume as a variable and allow comparisons between segments, we used density instead of total cell counts. For the statistical analysis, the volumes of each segment at all mediolateral levels were summed and the densities of cells averaged between animals. An ANOVA was used to determine significance across the different segments for both markers. Student's *t*-test was used to define differences in the total cell counts for both markers of the whole PPN. The level of significance was set at $P < 0.05$.

RESULTS

Distribution of cholinergic neurons in the PPN

Neurons showing immunoreactivity for ChAT, identified by the typical brown DAB precipitate, were defined as cholinergic neurons. Sagittal brain sections were analyzed following a lateral-to-medial direction. The first cholinergic neurons in the lateral portions of the PPN were identified at ~ 2.3 mm lateral to Bregma. The distribution was consistent with previous work both in PPN and in other regions identified as containing cholinergic neurons (Mesulam et al., 1983). Cholinergic neurons consisted of irregularly shaped neurons with staining in the cytoplasm, dendrites, and axon, and the cell bodies extended caudally and dorsally in the more medial sections, as has been extensively reported in the literature (Rye et al., 1987). Cholinergic processes in the PPN were frequently observed to form varicosities, and some of the processes were traced back to their cell bodies within the PPN (not shown). The cholinergic neurons that were closest to the boundary of the SNR, as delimited by the medial lemniscus, clearly extended neural processes in a rostral direction (Fig. 2A). Some cholinergic neurons were identified within the boundaries of the SNR (Fig. 3F,G), but the number was minimal and not representative of the total or segmental PPN cholinergic population. Neurons in this rostral area were more sparsely dis-

tributed than in more caudal segments (compare Fig. 2A and B). Most of the cholinergic neurons in the most rostral segments were aligned following the fibers of the scp, whereas caudal cholinergic neurons did not show such an arrangement. The total cell counts revealed that cholinergic neurons became more numerous as the distance from SNR increased, but this was directly related to the area that cholinergic neurons occupied (Fig. 2C). This is partially the consequence of the fact that the actual borders of the PPN are defined by the cholinergic neurons. Therefore, rather than an increased density, we observed an increased area of the PPN as it is extended caudally (Fig. 2D), in agreement with previous descriptions of the nucleus (Paxinos and Watson, 1986).

GAD mRNA-positive neurons in the PPN

Neurons showing positive labeling for GAD mRNA, which was identified by the nitroblue tetrazolium reaction product formed by the alkaline phosphatase reaction, were defined as GABAergic neurons. They were also identified in other brain areas known to contain GABAergic neurons, such as the SNR and the zona incerta. They were found throughout the rostro-caudal extent of PPN and were easily differentiated from cholinergic neurons that contained the brown peroxidase reaction product (Fig. 3). GABAergic neurons were intermingled among the cholinergic neurons but did not show the layer-like arrangement characteristic of cholinergic neurons in the rostral PPN (Fig. 3B,C). No GAD-positive processes were observed, because the labeling was confined to the cell body. In the caudal PPN, where the higher density of cholinergic processes produced a dense brown staining, the GAD signal was still clearly detectable (Fig. 3D,E). The distribution of GABAergic neurons did not follow the same boundaries set by cholinergic neurons. It was still possible to observe GAD-positive neurons beyond the limits set by the cholinergic neurons that did not follow a particular pattern. However, in the most rostral part of the PPN, cholinergic neurons were observed to cross the boundary set by the medial lemniscus into the SNR (Fig. 3F). It was thus apparent that in this transition zone there was a mixing of cholinergic and possibly GABAergic neurons from the PPN. The processes of cholinergic neurons gave rise to varicosities that formed appositions with GABAergic somata with no apparent rostrocaudal proclivity, although they were more easily identifiable in the rostral PPN, where the background tended to be lighter (Fig. 3G'-G''').

GAD mRNA-positive neurons are differentially distributed within the PPN

The density of GABAergic neurons was greater than the density of cholinergic neurons across all mediolateral levels of the PPN, and they were heterogeneously distributed (Fig. 4A,B). From the rostralmost appearance of ChAT-positive neurons in the first lateral section (corresponding to approximately 2.35 mm from Bregma), GAD-positive neurons were already present at a higher density than ChAT-positive neurons. In all the rostral segments (S1-S5) of the PPN, GABAergic neurons have a higher density than cholinergic neurons (Fig. 4C). This trend is inverted in the caudal segments of the lateral sections, where cholinergic neurons have a higher density and surpass the GABAergic neurons by up to 3:1.

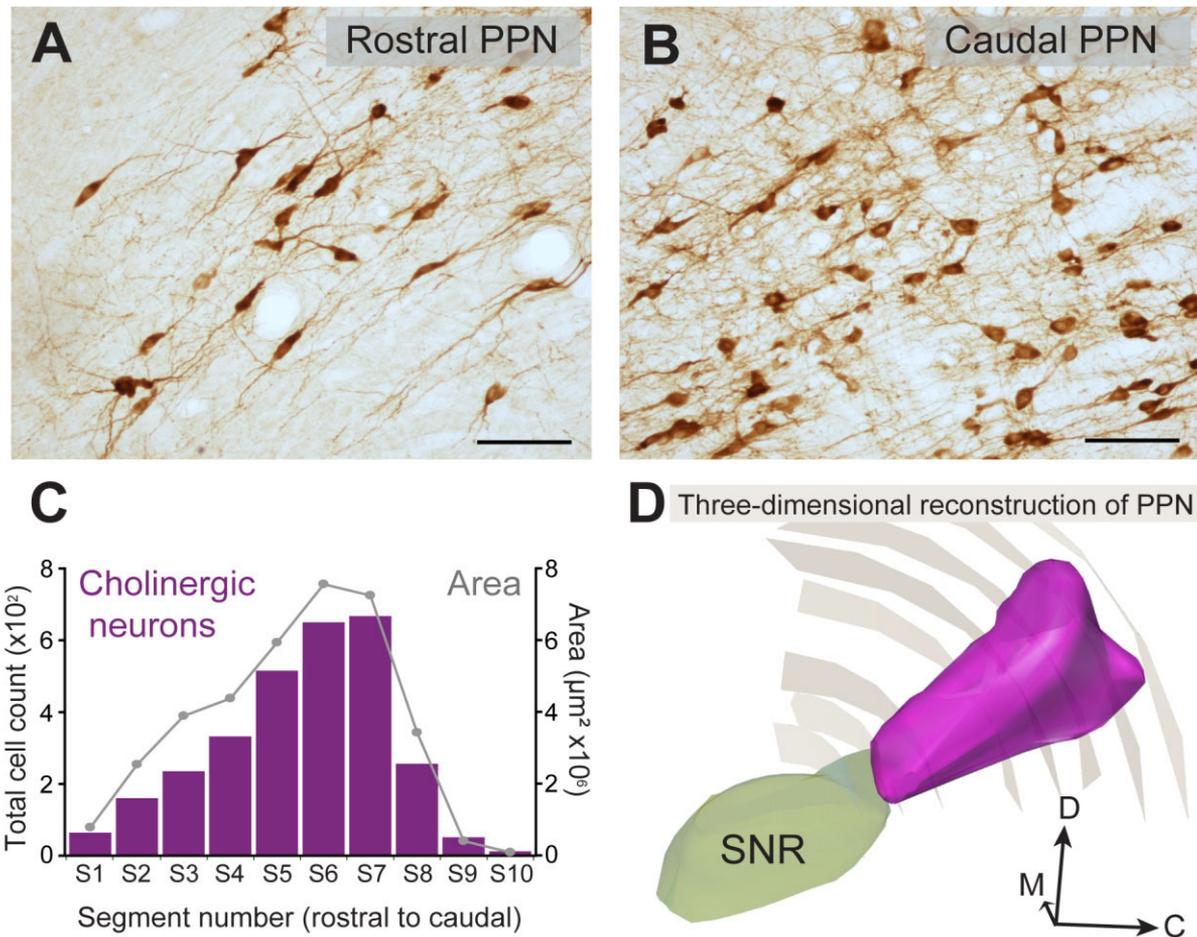


Figure 2.

The PPN as defined by the distribution of cholinergic neurons. **A,B:** Differences in the number and arrangement of cholinergic neurons are some of the characteristics that histologically differentiate the rostral from the caudal PPN, although no anatomical hallmark provides an adequate resolution for such division. **C:** The number of cholinergic neurons gradually increases toward the caudal segments of the PPN in a similar proportion as the area does. No reference other than the area delimited by the cholinergic cell bodies can determine the extent of the PPN. **D:** Three-dimensional reconstructions of the SNR and PPN show a close anatomical relationship across all mediolateral levels. This reconstruction was formed from the rendered sections after stereological counts; the semicircular lines (pale brown) represent the different segments joined together, showing that the shape of the PPN was maintained even after use of an external reference (the center of the SNR) to align the cell counts. C, caudal; D, dorsal; M, medial. Scale bars = 100 μm.

The numbers of neurons in different mediolateral segments were then summed in each animal to obtain the total cell density for a given segment and then averaged between animals to obtain the rostrocaudal distribution in the PPN (Fig. 5). We observed a significantly higher density of GAD-positive neurons in the rostral five segments (S1–S5) compared with the more caudal five segments (S6–S10), indicating an heterogeneous distribution of GABAergic neurons and a clear difference between rostral and caudal PPN (Fig. 5A; S1: $F_{1,11} = 125.28$, $P < 0.001$; S2: $F_{1,11} = 50.07$, $P < 0.001$; S3: $F_{1,11} = 91.07$, $P < 0.001$; S4: $F_{1,11} = 40.42$, $P < 0.001$; S5: $F_{1,11} = 11.13$, $P < 0.05$). On the other hand, ChAT-positive neurons showed the opposite trend, although this distribution was not similar in magnitude to that of GABAergic neurons. The total cell counts (Fig. 5B) revealed that there are twice as many GABAergic neurons as cholinergic neurons in the PPN (GAD-positive: $6,571 \pm 818$ neurons; ChAT-positive: total $2,942 \pm 122$ neurons; $n = 6$, $P < 0.05$).

The comparison of the distribution of GABAergic neurons of the first five segments vs. the last five revealed a dramatic rostral-to-caudal gradient. Because this trend was observed at all mediolateral levels, the distribution of the GABAergic neurons can be used to divide the PPN accurately into rostral and caudal parts with a spatial resolution of no more than 300 μm (the size of one segment). Thus, a semiperpendicular division (80°) to the line that crosses from the SNR center to the scp centrum, located at 2.1 mm from the SNR center (or 51% of that distance, if other protocols or fixation techniques that affect tissue shrinkage are used; this corresponds to the end of S5), would consistently divide the rostral from caudal PPN (Fig. 6). This division, based primarily on GABAergic neurons, is also consistent with the differences in number and organization of cholinergic neurons (described previously) between the so-called *pars dissipata* and *pars compacta*.

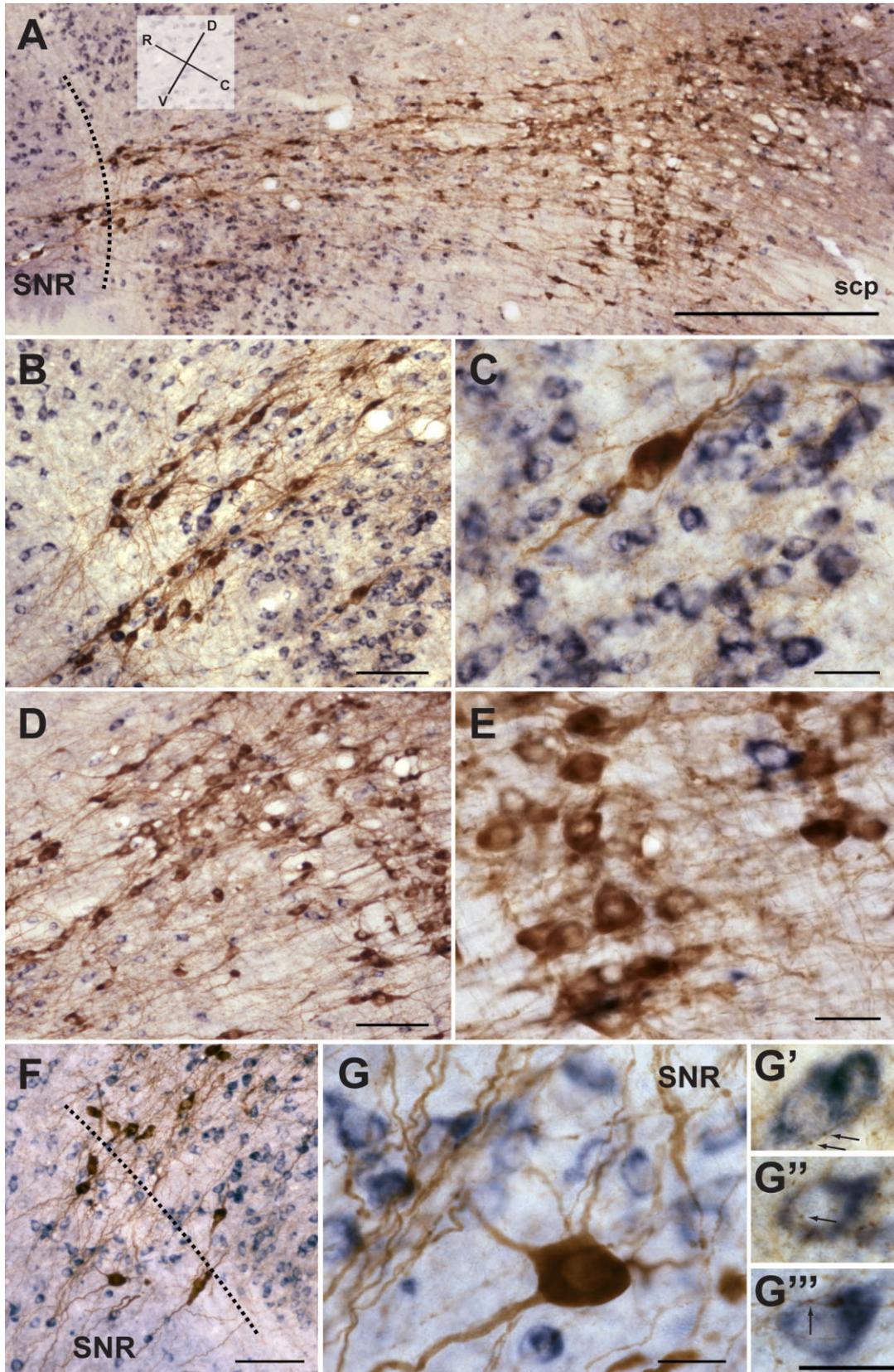


Figure 3

Dorsal cluster of GABAergic neurons with dense cholinergic processes

We observed, in the caudal and most dorsal part of PPN, a small cluster of GAD-positive neurons embedded in an area of high-density cholinergic cell bodies and processes (Fig. 7A,B). This cluster of GAD-positive neurons was present in all animals ($n = 6$) and is very specific to the mediolateral level (Bregma ~ 1.8 mm) and can be observed in only one or two sagittal sections (spaced $175 \mu\text{m}$). It measures $521 \pm 13 \mu\text{m}$ in its longest diameter and $315 \pm 22 \mu\text{m}$ in its shorter diameter ($n = 6$), and it is usually embedded between S6 and S8 (Fig. 7C). The densities of GAD-positive and ChAT-positive neurons within the cluster are clearly different from their densities in the rest of the segment in which the cluster is embedded (Fig. 7D). This cluster could represent a functional subdivision in the caudal PPN. Further experiments are needed to determine the connectivity and other neurochemical features of this area.

DISCUSSION

Our results provide the first topographically based stereological quantification of the PPN and show that two neurochemically distinct neuronal populations are heterogeneously distributed within it, the rostral PPN being predominantly GABAergic. In contrast, although the density of cholinergic neurons remains relatively constant, there are larger numbers of neurons in the caudal PPN compared with its rostral portion. Although the PPN has traditionally been considered as a cholinergic nucleus, our findings demonstrate that, in fact, GABAergic neurons are more numerous. In addition, GABAergic neurons show a very constant pattern of distribution between animals, providing a reliable marker for dividing the PPN into rostral and caudal domains. These differences in the distribution of neurochemically distinct populations of neurons are consistent with previous anatomical and functional findings, thus supporting the idea of functional subterritories within the PPN.

Neuronal heterogeneity in the PPN

The PPN is composed of at least three main neuronal populations [cholinergic, GABAergic, and glutamatergic neurons (Clements and Grant, 1990; Ford et al., 1995)] and a variety of other neuronal markers, including calcium-binding proteins and neuropeptides (Fortin and Parent, 1999; Vincent, 2000). Although colocalization of different markers has been reported, the degree of overlap is not yet clear (Bevan and Bolam, 1995; Lavoie and Parent, 1994; Sidibé et al., 2002). Recently, it has been reported that the number of glutamatergic neurons (as revealed by *in situ* hybridization for VGluT2) is greater than the number of cholinergic neurons and that there is a very low level of coexpression of the two markers (Wang and Morales, 2009). These data together with the data presented here strongly suggest that cholinergic neurons represent only a minority of the neuronal population present in the PPN. It should be noted, however, that the lower number of cholinergic neurons does not detract, in any way, from the significant influence they provide in terms of the modulation of distant systems and their own local activity (Garcia-Rill et al., 2007; Mena-Segovia et al., 2008a). What these findings indicate is that noncholinergic neurons should be considered in terms of the functional properties of the PPN. The idea of neuronal heterogeneity in the PPN is not recent and is supported by the work of several groups (Bevan and Bolam, 1995; Clements and Grant, 1990; Ford et al., 1995; Grofova and Zhou, 1998; Lavoie and Parent, 1994; Mena-Segovia et al., 2008a; Steininger et al., 1997; Takakusaki et al., 1996; Vincent, 2000). However, our findings provide the first evidence of neurochemical segregation among the neuronal populations of the PPN.

Two neurochemically distinct areas coincide with functional topography in the PPN

Behavioral studies have shown evidence of functional differences between the rostral PPN, or *pars dissipata*, and the caudal PPN, or *pars compacta*. Excitotoxic lesions in the two regions of the PPN produce different effects when the rats are exposed to systemic nicotine: lesions in the caudal PPN increase nicotine self-administration and do not depress locomotor activity following nicotine administration, whereas lesions in the rostral PPN decrease baseline locomotor activity and have no effect on nicotine self-administration (Alderson et al., 2006, 2008). On the other hand, lesions in the caudal PPN were more likely to reduce global attention than lesions in the rostral PPN (Inglis et al., 2001). In addition, it has been shown that electrical stimulation of the rostral PPN prior to training improved the outcome in an acquisition task (Andero et al., 2007). The same stimulation did not produce any effect when applied to the caudal PPN. The results for these groups strongly support a functional difference between the rostral and the caudal PPN. These differences are likely to be related to the efferent connectivity and the neurochemical subtypes present in the two portions of the PPN. Our present work contributes to the notion of a functional division in PPN by providing the anatomical substrate that might explain it and suggests a direct involvement of the GABAergic neuronal population in the functions associated with the rostral PPN, in particular, motor control and associative learning. On the other hand, the large numbers of cholinergic neurons in the caudal PPN, compared with the rostral portion, produce a

Figure 3.
GAD mRNA is widely distributed in the PPN. **A:** Montage of nine images showing the PPN in the sagittal plane between the SNR and the cerebellar peduncle. In this and the subsequent images, ChAT-positive neurons appear as brown and GAD-positive neurons as blue. This mediolateral level is approximately 1.9 mm lateral to Bregma, showing the PPN at its maximal length. The image was tilted 30° clockwise. **B:** The rostral portion of the PPN shows a high density of GAD-positive neurons compared with cholinergic neurons, which show the typical orientation toward the SNR and following the fibers of the scp. **C:** Higher magnification of the rostral PPN in an area rich in GAD-positive neurons. **D:** The caudal portion of the PPN has a higher density of cholinergic neurons, which at this level do not show a layer-like organization. **E:** Higher magnification of the caudal PPN in an area rich in ChAT-positive neurons. **F:** ChAT-positive neurons were observed to cross the boundary of the SNR, as delimited by the fibers of the medial lemniscus (dotted line). There was a similar density of the two markers on both sides of the boundary. **G:** High magnification of the caudal SNR and rostral PPN showing GAD-positive neurons apposed by ChAT-positive processes. Large numbers of GAD-positive neurons were found to have ChAT-positive varicosities in close apposition to their cell bodies (arrows; G'-G'''). SNR, substantia nigra *pars reticulata*; scp, superior cerebellar peduncle; D, dorsal; V, ventral; R, rostral; C, caudal. Scale bars = 0.5 mm in A; 100 μm in B,D,F; 50 μm in C,E; 20 μm in G; 20 μm in G''' (applies to G'-G''').

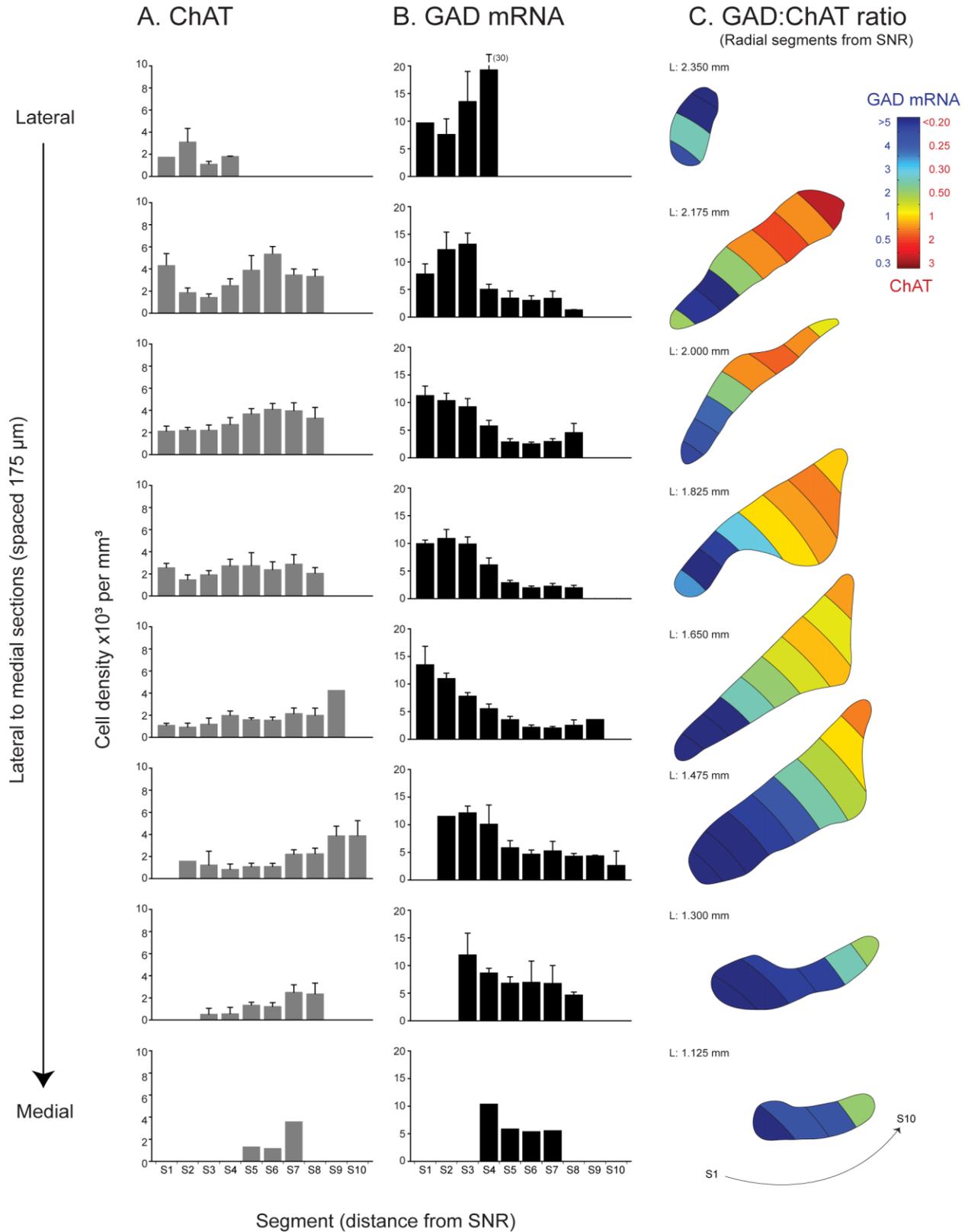


Figure 4

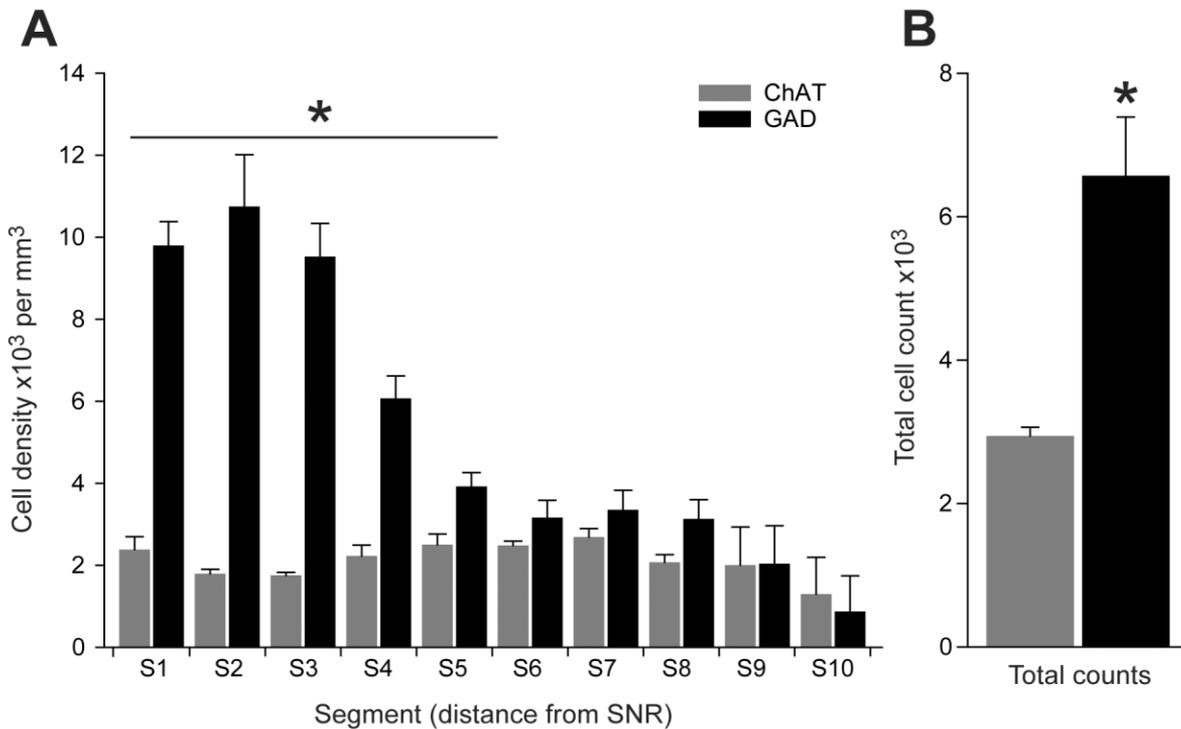


Figure 5. GABAergic neurons predominate over cholinergic neurons. **A:** Average cell density for all mediolateral segments depicted in Figure 4. **B:** Total number of each population obtained from the total cell count, showing that GAD-positive neurons are more numerous than ChAT-positive neurons. The statistical analysis of the different segments showed that segments 1–5 were significantly different from segments 6–10 (see Results for details of statistical analysis). The distribution of cholinergic neurons remained relatively constant across segments, but, in contrast to the case for GABAergic neurons, cholinergic neurons showed an increase in density in the caudal segments of the lateral sections (caudal PPN; see also Fig. 4). Error bars indicate SEM. **P* < 0.05.

larger cholinergic ascending projection that is likely to have a greater influence in the thalamus (Oakman et al., 1999), thus having an impact on arousal levels and sleep-wake regulation. Cholinergic neurons in the caudal PPN are also able to exert a modulatory role over dopamine neurons in the ventral tegmental area, maintaining a steady level of acetylcholine release but also the ability to respond to phasic signals (Floresco et al., 2003; Miller and Blaha, 2005). The putative

Figure 4. Mediolateral distribution of cholinergic and GABAergic neurons. **A,B:** Density of ChAT-positive and GAD-positive neurons, respectively, along the mediolateral axis of the PPN, showing the average of corresponding segments from all animals (*n* = 6; error bars indicate SEM). The different plots represent the different bregma levels (L) shown in C, from lateral (top) to medial (bottom). Each level is separated by 175 μm, and each bar represents a different 300 μm segment (from S1 to S10). **C:** Ratio of cholinergic and GABAergic neurons for each of the segments. The ratios of all segments were composed on average schematic representations of the PPN from L 2.350 to L 1.125 (in mm). Thus, cold colors represent a ratio favoring GAD-positive neurons over ChAT-positive neurons from 2:1 to 22:1 (only shown up to 5:1; blue scale), whereas warm colors represent a ratio favoring ChAT-positive neurons over GAD-positive neurons from 2:1 to 3:1. A constant tendency across all mediolateral levels shows the predominant presence of GABAergic neurons in the rostral segments, whereas cholinergic neurons are more numerous in the caudal segments of the lateral sections.

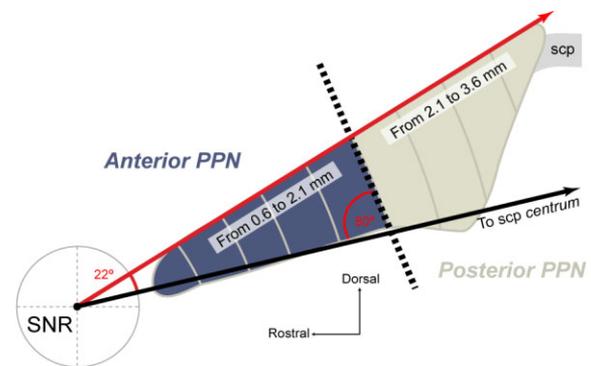


Figure 6. Subregions of the PPN based on neurochemical segregation. Schematic representation of the PPN showing the rostral segments (characterized by GABAergic prevalence, blue) and the caudal segments (characterized by a higher cholinergic tendency, yellow-green) separated by a line situated between segments 5 and 6 (equivalent to 2.1 mm from the center of the SNR), as observed from the statistical segregation shown in Figure 5. The vertex of such line has an angle of 80° in relation to the line crossing from the SNR center to the scp centrum (as shown in Fig. 1). The use of this line even in unstained brains, following diverse experimental paradigms, would accurately divide between the predominantly GABAergic (rostral) and the evenly mixed GABAergic/cholinergic (caudal) PPN.

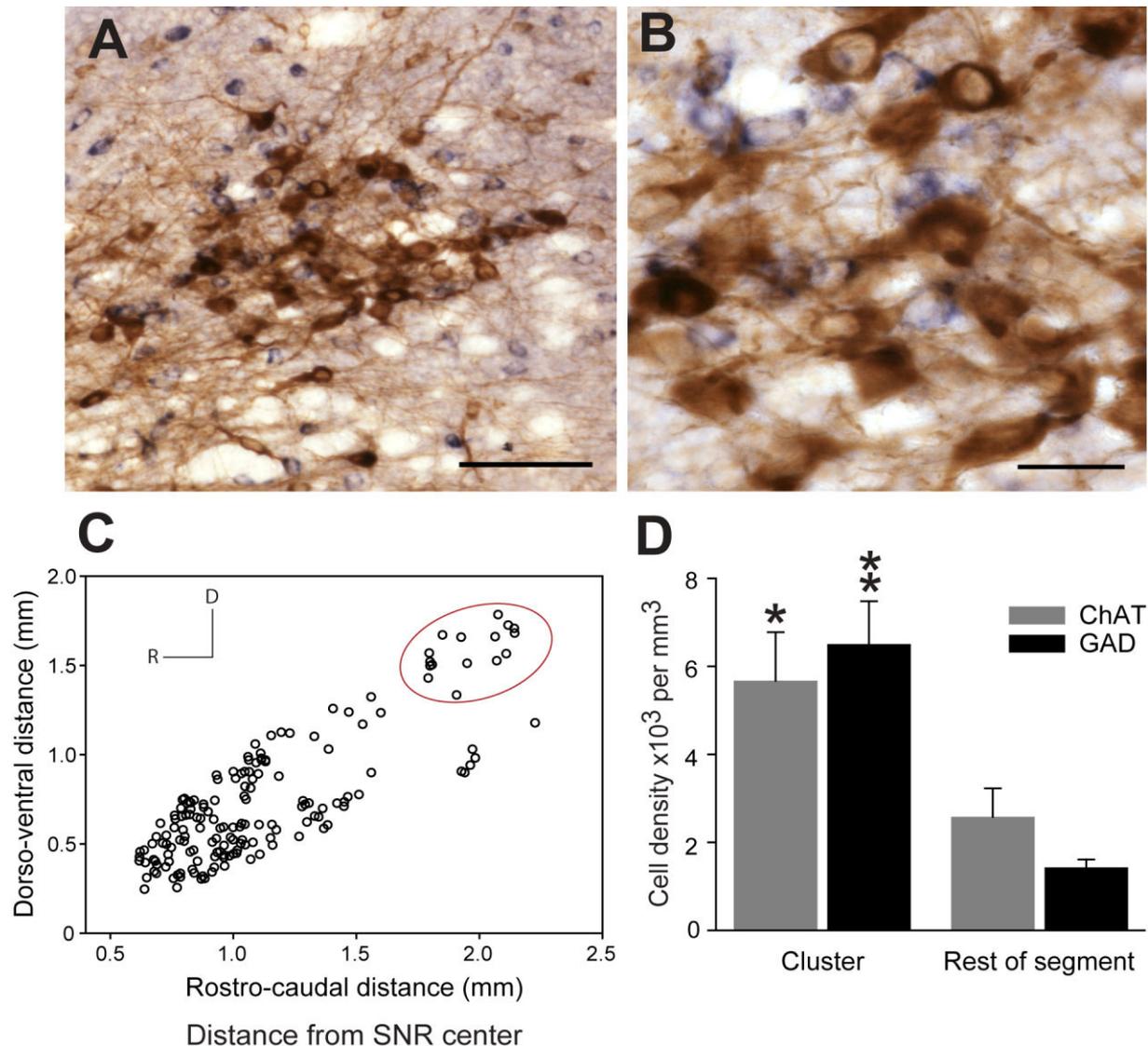


Figure 7. GABAergic neurons form a small cluster in the caudal PPN. **A,B:** ChAT-positive and GAD-positive neurons were found to form a dense cluster near the dorsocaudal border of the PPN at approximately 1.8 mm lateral to Bregma. **C:** This cluster was more clearly identified by the presence of GAD-positive neurons because of their low density in the caudal segments. **D:** The density of positive neurons for both markers was significantly higher within the cluster than the density of neurons in the rest of the segments in which the cluster was embedded (typically S6–S8). Error bars indicate SEM. * $P < 0.05$ between ChAT densities. ** $P < 0.05$ between GAD densities. Scale bars = 100 μm in A; 50 μm B.

interconnections between cholinergic and GABAergic neurons, as suggested here (Fig. 3) and from our previous work (Mena-Segovia et al., 2008a), would form the basis of a functional microcircuit able to carry out early processing of sensory events before being conveyed to forebrain structures.

GABAergic neurons as a precise marker for delimiting rostral and caudal PPN

Although the differences in the number and arrangement of cholinergic neurons have been useful to separate two distinct regions of the PPN, as shown by connectivity and behavioral studies, it is not possible to locate such a boundary precisely nor to reproduce it at all stereotaxic levels. Our analysis,

based on the division of the PPN into 300- μm segments, showed that the distribution of GAD-positive neurons makes a radical change after the fifth segment caudal to the SNR (equal to 1.5 mm from the start of the PPN and 2.1 mm from the center of the SNR). We observed this pattern at all mediolateral levels in all the animals that we examined. This change in GAD mRNA expression is correlated with the typical parameters used to separate *pars compacta* from *pars dissipata* in the PPN and with the stereotaxic coordinates used by previous groups aiming to identify differences between the two parts of the nucleus (Alderson et al., 2006, 2008; Andero et al., 2007). This evidence strongly suggests that the previously identified divisions of the PPN correspond to the neu-

rochemical divisions reported here. It is therefore reasonable to suggest the use of the segmental division in the PPN to set the boundary between rostral and caudal PPN. This method will standardize the regional differences in the PPN and will provide a division with a resolution of 300 μm , which is a significant improvement considering the lack of prominent anatomical reference points around the PPN (with the exception of the SNR and scp that were used for this method). The separation of functionally distinct regions of the PPN also has important implications for Parkinson's disease, in which PPN has been shown to be involved and is currently being used as a therapeutic target for deep brain stimulation (Androulidakis et al., 2008; Jenkinson et al., 2004; Mazzone et al., 2005; Pahapill and Lozano, 2000).

Possible role of GABA in the PPN

Because of its connections with many parts of the forebrain (e.g., thalamocortical neurons, basal ganglia output nuclei, midbrain dopaminergic systems, hypothalamic arousal systems), the PPN is able to promote global brain activation, most likely mediated through the generation of intrinsic and extrinsic neuronal oscillations (Leszkowicz et al., 2007; Lorincz et al., 2008; Mena-Segovia et al., 2008a; Steriade et al., 1991). Many types of GABAergic neurons in different regions of the central nervous system provide the pacemaker activity that determines the frequency of a wide range of oscillations (Buzsaki and Draguhn, 2004; Llinas et al., 2005; Mann and Paulsen, 2007; Somogyi and Klausberger, 2005). Thus, GABAergic neurons in the PPN could be playing a determinant role in the modulation of such intrinsic and extrinsic neuronal oscillations: locally as interneurons or as projection neurons in distant targets (as in the case of hypothalamus and subthalamic nucleus). Evidence from extracellular recordings and single-cell labeling shows important differences among the neuronal subtypes in the PPN: cholinergic neurons show particular physiological properties different from those of noncholinergic neurons (Mena-Segovia et al., 2008a). This suggests that different neurochemical types of PPN neurons projecting to the same targets may exert different, if not opposite, effects, perhaps related to specific neuronal subtypes, thus contributing to the known functions of the PPN. Any input reaching the PPN would then have to find a balance between the excitatory cholinergic (nicotinic) and glutamatergic output vs. the inhibitory cholinergic (M2-muscarinic) and GABAergic output.

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LITERATURE CITED

- Alderson HL, Latimer MP, Winn P. 2006. Intravenous self-administration of nicotine is altered by lesions of the posterior, but not anterior, pedunculopontine tegmental nucleus. *Eur J Neurosci* 23:2169–2175.
- Alderson HL, Latimer MP, Winn P. 2008. A functional dissociation of the anterior and posterior pedunculopontine tegmental nucleus: excitotoxic lesions have differential effects on locomotion and the response to nicotine. *Brain Struct Funct* 213:247–253.
- Andero R, Torras-Garcia M, Quiroz-Padilla MF, Costa-Miserachs D, Coll-Andreu M. 2007. Electrical stimulation of the pedunculopontine tegmental nucleus in freely moving awake rats: time- and site-specific effects on two-way active avoidance conditioning. *Neurobiol Learn Mem* 87:510–521.
- Androulidakis AG, Mazzone P, Litvak V, Penny W, Dileone M, Doyle Gaynor LM, Tisch S, Di Lazzaro V, Brown P. 2008. Oscillatory activity in the pedunculopontine area of patients with Parkinson's disease. *Exp Neurol* 211:59–66.
- Bevan MD, Bolam JP. 1995. Cholinergic, GABAergic, and glutamate-enriched inputs from the mesopontine tegmentum to the subthalamic nucleus in the rat. *J Neurosci* 15:7105–7120.
- Brown RE, McKenna JT, Winston S, Basheer R, Yanagawa Y, Thakkar MM, McCarley RW. 2008. Characterization of GABAergic neurons in rapid-eye-movement sleep controlling regions of the brainstem reticular formation in GAD67-green fluorescent protein knock-in mice. *Eur J Neurosci* 27:352–363.
- Buzsaki G, Draguhn A. 2004. Neuronal oscillations in cortical networks. *Science* 304:1926–1929.
- Charara A, Smith Y, Parent A. 1996. Glutamatergic inputs from the pedunculopontine nucleus to midbrain dopaminergic neurons in primates: *Phaseolus vulgaris*-leucoagglutinin anterograde labeling combined with postembedding glutamate and GABA immunohistochemistry. *J Comp Neurol* 364:254–266.
- Clements JR, Grant S. 1990. Glutamate-like immunoreactivity in neurons of the laterodorsal tegmental and pedunculopontine nuclei in the rat. *Neurosci Lett* 120:70–73.
- Datta S. 2007. Activation of pedunculopontine tegmental PKA prevents GABAB receptor activation-mediated rapid eye movement sleep suppression in the freely moving rat. *J Neurophysiol* 97:3841–3850.
- Floresco SB, West AR, Ash B, Moore H, Grace AA. 2003. Afferent modulation of dopamine neuron firing differentially regulates tonic and phasic dopamine transmission. *Nat Neurosci* 6:968–973.
- Ford B, Holmes CJ, Mainville L, Jones BE. 1995. GABAergic neurons in the rat pontomesencephalic tegmentum: codistribution with cholinergic and other tegmental neurons projecting to the posterior lateral hypothalamus. *J Comp Neurol* 363:177–196.
- Fortin M, Parent A. 1999. Calretinin-immunoreactive neurons in primate pedunculopontine and laterodorsal tegmental nuclei. *Neuroscience* 88:535–547.
- Garcia-Rill E, Heister DS, Ye M, Charlesworth A, Hayar A. 2007. Electrical coupling: novel mechanism for sleep-wake control. *Sleep* 30:1405–1414.
- Grofova I, Zhou M. 1998. Nigral innervation of cholinergic and glutamatergic cells in the rat mesopontine tegmentum: light and electron microscopic anterograde tracing and immunohistochemical studies. *J Comp Neurol* 395:359–379.
- Honda T, Semba K. 1995. An ultrastructural study of cholinergic and non-cholinergic neurons in the laterodorsal and pedunculopontine tegmental nuclei in the rat. *Neuroscience* 68:837–853.
- Inglis WL, Olmstead MC, Robbins TW. 2001. Selective deficits in attentional performance on the 5-choice serial reaction time task following pedunculopontine tegmental nucleus lesions. *Behav Brain Res* 123:117–131.
- Jenkinson N, Nandi D, Miall RC, Stein JF, Aziz TZ. 2004. Pedunculopontine nucleus stimulation improves akinesia in a Parkinsonian monkey. *Neuroreport* 15:2621–2624.
- Jia HG, Yamuy J, Sampogna S, Morales FR, Chase MH. 2003. Colocalization of gamma-aminobutyric acid and acetylcholine in neurons in the laterodorsal and pedunculopontine tegmental nuclei in the cat: a light and electron microscopic study. *Brain Res* 992:205–219.
- Lavoie B, Parent A. 1994. Pedunculopontine nucleus in the squirrel monkey: cholinergic and glutamatergic projections to the substantia nigra. *J Comp Neurol* 344:232–241.
- Leszkowicz E, Kusmierczak M, Matulewicz P, Trojniar W. 2007. Modulation of hippocampal theta rhythm by the opioid system of the pedunculopontine tegmental nucleus. *Acta Neurobiol Exp* 67:447–460.
- Llinas R, Urbano FJ, Leznik E, Ramirez RR, van Marle HJ. 2005. Rhythmic and dysrhythmic thalamocortical dynamics: GABA systems and the edge effect. *Trends Neurosci* 28:325–333.
- Lorincz ML, Crunelli V, Hughes SW. 2008. Cellular dynamics of cholinergically induced alpha (8–13 Hz) rhythms in sensory thalamic nuclei in vitro. *J Neurosci* 28:660–671.
- Mann EO, Paulsen O. 2007. Role of GABAergic inhibition in hippocampal network oscillations. *Trends Neurosci* 30:343–349.
- Mazzone P, Lozano A, Stanzione P, Galati S, Scarnati E, Peppe A, Stefani A. 2005. Implantation of human pedunculopontine nucleus: a safe and

- clinically relevant target in Parkinson's disease. *Neuroreport* 16:1877–1881.
- Mena-Segovia J, Bolam JP, Magill PJ. 2004. Pedunclopontine nucleus and basal ganglia: distant relatives or part of the same family? *Trends Neurosci* 27:585–588.
- Mena-Segovia J, Sims HM, Magill PJ, Bolam JP. 2008a. Cholinergic brainstem neurons modulate cortical gamma activity during slow oscillations. *J Physiol* 586:2947–2960.
- Mena-Segovia J, Winn P, Bolam JP. 2008b. Cholinergic modulation of midbrain dopaminergic systems. *Brain Res Rev* 58:265–271.
- Mesulam MM, Mufson EJ, Wainer BH, Levey AI. 1983. Central cholinergic pathways in the rat: an overview based on an alternative nomenclature (Ch1–Ch6). *Neuroscience* 10:1185–1201.
- Miller AD, Blaha CD. 2005. Midbrain muscarinic receptor mechanisms underlying regulation of mesoaccumbens and nigrostriatal dopaminergic transmission in the rat. *Eur J Neurosci* 21:1837–1846.
- Nair-Roberts RG, Chatelain-Badie SD, Benson E, White-Cooper H, Bolam JP, Ungless MA. 2008. Stereological estimates of dopaminergic, GABAergic and glutamatergic neurons in the ventral tegmental area, substantia nigra and retrorubral field in the rat. *Neuroscience* 152:1024–1031.
- Oakman SA, Faris PL, Kerr PE, Cozzari C, Hartman BK. 1995. Distribution of pontomesencephalic cholinergic neurons projecting to substantia nigra differs significantly from those projecting to ventral tegmental area. *J Neurosci* 15:5859–5869.
- Oakman SA, Faris PL, Cozzari C, Hartman BK. 1999. Characterization of the extent of pontomesencephalic cholinergic neurons' projections to the thalamus: comparison with projections to midbrain dopaminergic groups. *Neuroscience* 94:529–547.
- Pahapill PA, Lozano AM. 2000. The pedunclopontine nucleus and Parkinson's disease. *Brain* 123:1767–1783.
- Paxinos G, Watson C. 1986. The rat brain in stereotaxic coordinates. San Diego: Academic Press. 119 p.
- Rye DB, Saper CB, Lee HJ, Wainer BH. 1987. Pedunclopontine tegmental nucleus of the rat: cytoarchitecture, cytochemistry, and some extrapyramidal connections of the mesopontine tegmentum. *J Comp Neurol* 259:483–528.
- Saper CB, Scammell TE, Lu J. 2005. Hypothalamic regulation of sleep and circadian rhythms. *Nature* 437:1257–1263.
- Semba K, Fibiger HC. 1992. Afferent connections of the laterodorsal and the pedunclopontine tegmental nuclei in the rat: a retro- and anterograde transport and immunohistochemical study. *J Comp Neurol* 323:387–410.
- Shink E, Sidibé M, Smith Y. 1997. Efferent connections of the internal globus pallidus in the squirrel monkey: II. Topography and synaptic organization of pallidal efferents to the pedunclopontine nucleus. *J Comp Neurol* 382:348–363.
- Sidibé M, Paré JF, Raju D, Smith Y. 2002. Anatomical and functional relationships between intralaminar thalamic nuclei and basal ganglia in monkeys. In: Nicholson LFB, Faull RLM, editors. *The basal ganglia VII*. Boston: Kluwer Academic/Plenum Publishers. p 409–420.
- Somogyi P, Klausberger T. 2005. Defined types of cortical interneurone structure space and spike timing in the hippocampus. *J Physiol* 562:9–26.
- Steininger TL, Wainer BH, Rye DB. 1997. Ultrastructural study of cholinergic and noncholinergic neurons in the pars compacta of the rat pedunclopontine tegmental nucleus. *J Comp Neurol* 382:285–301.
- Steriade M, Dossi RC, Pare D, Oakson G. 1991. Fast oscillations (20–40 Hz) in thalamocortical systems and their potentiation by mesopontine cholinergic nuclei in the cat. *Proc Natl Acad Sci U S A* 88:4396–4400.
- Takakusaki K, Shiroyama T, Yamamoto T, Kitai ST. 1996. Cholinergic and noncholinergic tegmental pedunclopontine projection neurons in rats revealed by intracellular labeling. *J Comp Neurol* 371:345–361.
- Takakusaki K, Oohinata-Sugimoto J, Saitoh K, Habaguchi T. 2004. Role of basal ganglia-brainstem systems in the control of postural muscle tone and locomotion. *Prog Brain Res* 143:231–237.
- Tortorolo P, Yamuy J, Sampogna S, Morales FR, Chase MH. 2001. GABAergic neurons of the laterodorsal and pedunclopontine tegmental nuclei of the cat express c-fos during carbachol-induced active sleep. *Brain Res* 892:309–319.
- Vincent SR. 2000. The ascending reticular activating system—from aminergic neurons to nitric oxide. *J Chem Neuroanat* 18:23–30.
- Wang H, Morales M. 2009. Pedunclopontine and laterodorsal tegmental nuclei contain distinct populations of cholinergic, glutamatergic and GABAergic neurons in the rat. *Eur J Neurosci* (in press).
- Winn P. 2006. How best to consider the structure and function of the pedunclopontine tegmental nucleus: evidence from animal studies. *J Neurol Sci* 248:234–250.