Topographic Relations of Cholinergic and Noradrenergic Neurons in the Feline Pontomesencephalic Tegmentum: An Immunohistochemical Study

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REINER, P. B. AND S. R. VINCENT. Topographic relations of cholinergic and noradrenergic neurons in the feline pontomesencephalic tegmentum: An immunohistochemical study. BRAIN RES BULL 19(6) 705-714, 1987.—The immunohistochemical localization of the neurotransmitter synthesizing enzymes choline acetyltransferase, tyrosine hydroxylase and dopamine-β-hydroxylase was examined in the feline pontomesencephalic tegmentum. Examination of adjacent sections stained for either choline acetyltransferase, tyrosine hydroxylase or dopamine-β-hydroxylase immunoreactivity, as well as individual sections doubly stained for both choline acetyltransferase and tyrosine hydroxylase immunoreactivity, unequivocally demonstrated that noradrenergic and cholinergic neurons were extensively intermingled in the brainstem tegmentum of the cat. This contrasts with the situation in various other species, where neurons utilizing these two neurotransmitters are discretely localized in distinct nuclei. Furthermore, the present studies demonstrate the existence of two types of choline acetyltransferase immunoreactive neurons in the feline tegmentum: the magnocellular neurons of the pedunculopontine and laterodorsal tegmental nuclei which stain histochemically for NADPH diaphorase, plus a population of small spindle-shaped neurons in the medial and lateral parabrachial nuclei which do not stain positively for NADPH diaphorase. The data are discussed with respect to several influential hypotheses of sleep cycle control.

Locus ceruleus Lateral geniculate nucleus Acetylcholine
Pedunculopontine nucleus Laterodorsal tegmental nucleus
Noradrenaline NADPH diaphorase Sleep

The presence of noradrenergic and acetylcholine containing neurons in the pontomesencephalic reticular formation was originally suggested by histochemical studies which demonstrated that selected neurons in this region exhibit catecholamine fluorescence [10] or acetylcholinesterase staining [60]. These data provided the structural basis for several influential hypotheses regarding the functional role of aminergic and cholinergic neurons, particularly with respect to the neural control of behavioral state [21, 28].

With the development of immunohistochemical techniques for the localization of neurotransmitter synthetic enzymes, it has become possible to define the distribution of aminergic and cholinergic neurons in the pontomesencephalic reticular formation with ever increasing precision. Two complications of previous histochemical studies are alleviated by the use of immunohistochemistry. First, the specific catecholamine contained by neurons can be inferred from the presence or absence of various synthetic enzymes. For example, noradrenergic neurons, by definition, should contain both tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH), but not the adrenaline synthesizing enzyme phenylethanolamine-N-methyltransferase. Second, acetylcholinesterase staining is not a specific marker for the cholinergic neurons of the pontomesencephalic tegmentum. While most cholinergic neurons, as defined by choline acetyltransferase (ChAT) immunohistochemistry, in fact contain acetylcholinesterase [12, 34, 37, 53], noradrenergic neurons also display intense acetylcholinesterase staining, even using pharmacohistochemical procedures [1, 4, 55].

Although immunohistochemical probes have been extensively used to study the anatomy of noradrenergic [9, 17, 67, 68] and cholinergic [2, 53, 56, 65, 71-73] neurons in the brainstem of the rat, remarkably few studies have used immunohistochemistry to examine this issue in the cat [7, 31, 39, 47, 70]. In most species, pontomesencephalic noradrenergic and cholinergic neurons are localized in distinct nuclei, with noradrenergic neurons concentrated within the nucleus locus ceruleus [14] and cholinergic neurons within

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the laterodorsal tegmental and pedunculopontine nuclei [38]. The feline appears to be unique with respect to the biochemical anatomy of the pontomesencephalic tegmentum. Rather than forming a distinct nucleus locus ceruleus, noradrenergic neurons are topographically dispersed over several nuclei in the feline brainstem, and are commonly referred to as forming a locus ceruleus complex [8, 24, 26, 35, 42, 74]. The heterogeneous distribution of noradrenergic neurons in the feline locus ceruleus complex suggests that these neurons are intermingled with non-noradrenergic neurons.

One possible transmitter candidate for the non-noradrenergic neurons of the feline locus ceruleus complex is acetylcholine. Kimura and Maeda [30] examined this issue using simultaneous catecholamine histofluorescence and acetylcholinesterase techniques. However, their study was hampered by the fact that noradrenergic neurons, as mentioned previously, display intense acetylcholinesterase staining, thereby preventing simultaneous visualization of noradrenergic and cholinergic neurons. Thus, the present study has utilized the immunohistochemical localization of TH, DBH and ChAT in adjacent serial sections, as well as simultaneous immunohistochemical localization of TH and ChAT within the same section in order to examine directly the topographic relations of noradrenergic and cholinergic neurons in the feline pontomesencephalic tegmentum. Some of these data have been presented in preliminary form [69].

METHOD

The data are derived from experiments performed on four pigmented adult male cats weighing between three and four kg. The animals were deeply anesthetized with sodium pentobarbital (50 mg/kg, IP), heparinized (10,000 units, intracardiac), and perfused transcardially with 200 ml of phosphate buffered saline (0.1 M, pH 7.4) at room temperature, followed by 1500 ml of either 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) containing 1 mM MgCl₂ (N=3), or 2% paraformaldehyde and 15% saturated picric acid in 0.1 M phosphate buffer (pH 7.4, N=1) [75]. The brains were removed, postfixed for two hours in the same fixative, blocked and placed in cryoprotectant (25% sucrose, 0.1 M phosphate buffer (pH 7.4) containing 1 mM MgCl₂, 0.6% nickel ammonium sulfate, and 0.0015% H₂O₂), and then incubated in TBS containing 1% avidin-biotinylated horseradish peroxidase complex (Vector Labs), 2% normal serum and 0.3% Triton X-100, 2% normal serum and 0.01% BSA for 1 hour at room temperature. Sections were then thoroughly washed in TBS and incubated with rabbit antiserum to TH, and processed in a fashion similar to that described above until step 4 which was modified for heavy metal intensification of the second DAB reaction. In this case the final incubation of the sections was performed in 50 mM Tris buffer, pH 7.4, containing 0.02% DAB, 0.6% nickel ammonium sulfate, and 0.0015% H₂O₂. The reaction was again terminated by washing in TBS, the sections mounted and examined under the light microscope. Double immunohistochemistry for ChAT and TH in the same tissue section was accomplished by first incubating sections with the rat monoclonal antibody to ChAT as described above, followed by the standard DAB protocol, which resulted in a brown reaction product. The sections were then thoroughly washed in TBS and incubated with rabbit antiserum to TH, and processed in a fashion similar to that described above until step 4 which was modified for heavy metal intensification of the second DAB reaction. In this case the final incubation of the sections was performed in 50 mM Tris buffer, pH 7.4, containing 0.02% DAB, 0.6% nickel ammonium sulfate, and 0.0015% H₂O₂. The reaction was again terminated by washing in TBS, the sections mounted and examined under the light microscope. This combined protocol resulted in tissue sections with ChAT positive neurons displaying brown reaction product, while TH positive neurons appeared blue black. Sections stained individually for CHAT, TH, and DBH as well as those doubly labelled for TH and ChAT were used to map the distribution of cholinergic and catecholaminergic neurons in the feline midbrain and pons.

RESULTS

TH and DBH positive neurons were found dispersed throughout the dorsolateral pontine tegmentum within the nuclei locus ceruleus, parabrachialis mediais and lateralis and Kölliker-Fuse (collectively termed the locus ceruleus complex). Although it was impossible to determine if every TH positive neuron in the locus ceruleus complex also stained positively with DBH antiserum, many neurons could be shown to contain both antigens in adjacent sections stained for each (Fig. 1A, B). On the other hand, isolated neurons within the dorsal raphe nuclei (Figs. 3F, 4C), as well as the neurons of the ventral tegmental area and substantia nigra (Fig. 4A, B) stained positively for TH only. The overall distribution of ChAT immunoreactive neurons in the feline brain is described elsewhere [70]. The present report focuses upon the ChAT positive neurons of the pontomesencephalic reticular formation. A detailed description of the ChAT positive neurons of the parabigeminal nucleus has been published [70]. Two other readily distinguishable types of ChAT immunoreactive neurons were noted. Large, intensely staining ChAT positive neurons were found in the laterodorsal tegmental and pedunculopontine nuclei, including its caudal extension into the rostral end of the medial parabrachial nucleus. Isolated magnocellular ChAT positive neurons were also found in the lateral para-
FIG. 1. Distributions of DBH (A), TH (B), ChAT (C) and NADPH diaphorase (D) positive neurons in the dorsolateral pontine tegmentum. Sections A–C are adjacent 30 µm sections, and section D is separated from C by a 30 µm gap. A large blood vessel is visible in all four sections (large arrowhead). Portions of the same neurons can be seen stained for both DBH (A) and TH (B) and two examples are marked by small arrows. A and B: TH and DBH positive neurons can be seen within the locus ceruleus (lc), extending in a band across the ventral aspect of the laterodorsal tegmental nucleus (td) and into the medial parabrachial nucleus (pbm). Dendrites of TH positive neurons can be seen in the td in B. C and D: ChAT and NADPH diaphorase positive neurons can be seen in the td and pbm, but not in the lc. In D, the large neurons of the mesencephalic trigeminal nucleus also show NADPH diaphorase staining. Calibration bar: 250 µm applies to A–D.

brachial nucleus and capping the dorsal nucleus of the lateral lemniscus (Fig. 5A). In contrast to these magnocellular ChAT positive neurons, small spindle-shaped neurons which stained lightly with ChAT immunoperoxidase reaction product were found in caudal portions of the medial and especially the lateral parabrachial nuclei (Fig. 5A,B).

These two types of ChAT positive neurons could be further distinguished on the basis of NADPH diaphorase histochemistry. The distribution and morphology of neurons staining positively for NADPH diaphorase were identical to that of the magnocellular ChAT immunoreactive neurons of the pontomesencephalic tegmentum (Fig. 1C,D), while the small spindle-shaped ChAT positive neurons of the medial and lateral parabrachial nuclei did not stain positively for NADPH diaphorase. Although distinguishable on the basis of both morphology and NADPH diaphorase histochemistry, the distribution of these two types of ChAT positive neurons within the medial and lateral parabrachial nuclei overlapped somewhat; in the medial parabrachial nucleus the magnocellular neurons predominated while in the lateral parabrachial nucleus the small spindle-shaped neurons were in the majority.

The morphology of the ChAT positive somata of the laterodorsal tegmental and pedunculopontine nuclei was very similar to that of the TH/DBH immunoreactive neurons of the locus ceruleus complex (Fig. 3A,B,D,E). Both types of neurons were large and multipolar; indeed, it was impossible to distinguish the two cell types in cresyl violet stained sections. On the other hand, the morphology of the small, lightly staining, spindle-shaped ChAT positive neurons seen in the medial and lateral parabrachial nuclei was clearly distinct from the TH/DBH immunoreactive neurons of the locus ceruleus complex (Fig. 3C).

The present report focuses upon topographic relations between the ChAT positive neurons of the laterodorsal tegmental and pedunculopontine nuclei and the TH/DBH positive neurons of the locus ceruleus complex. These were studied by examination of sets of adjacent sections (Fig. 1) and sections doubly stained for ChAT and TH (Fig. 2). Within the midbrain, the ChAT positive neurons of the pedunculopontine nucleus were largely segregated from the TH positive neurons of the substantia nigra (Fig. 4B). No ChAT positive neurons were found within the substantia nigra.

At the isthmus, the ChAT positive neurons of the pedunculopontine nucleus spread dorsomedially across the tegmentum into the laterodorsal tegmental nucleus (Fig. 4C). These ChAT positive neurons intermingled somewhat with the most rostral TH/DBH positive neurons of the locus...
FIG. 2. Simultaneous immunohistochemistry for ChAT and TH in the dorsolateral pontine tegmentum. This section was stained for ChAT immunoreactive neurons using the standard diaminobenzidine protocol which results in brown immunoperoxidase reaction product in the cholinergic neurons of the laterodorsal tegmental nucleus (tld) and medial parabrachial nucleus (pbm). Subsequently, the section was stained for TH immunoreactive neurons using a nickel ammonium sulfate intensification technique (see the Method section for details) which results in blue-black immunoperoxidase reaction product in the catecholaminergic neurons of the locus ceruleus (lc) and pbm. The dendrites of TH positive neurons in the pbm can be seen extending up into the tld. 4v—fourth ventricle. Calibration: 100 μm.

FIG. 3. Morphology of pontine ChAT, TH and DBH immunoreactive neurons. (A) Large multipolar ChAT positive neurons in the laterodorsal tegmental nucleus. (B) DBH positive neurons in the locus ceruleus in a section adjacent to that shown in A. (C) Small, lightly stained ChAT positive neurons in the caudal medial parabrachial nucleus. (D) DBH positive neurons in the medial parabrachial nucleus in a section adjacent to that shown in C. (E) Large multipolar ChAT positive neurons in the rostral medial parabrachial nucleus. (F) TH positive neurons in the dorsal raphe nucleus. Fourth ventricle is at the top. Calibration bar: 25 μm for A–E; 125 μm for F.
FIGS. 4–5. Atlas of cholinergic and catecholaminergic neurons in the ratine midbrain and pons. In each figure, open circles represent ChAT positive neurons, black dots TH positive neurons and black asterisks DBH positive neurons. The atlas was constructed from adjacent 30 μm sections stained for the appropriate synthetic enzyme. Abbreviations: 3—oculomotor nucleus; 4—trochlear nucleus; 5—motor trigeminal nucleus; 7g—genu of the facial nerve; 7n—facial nerve; BC—brachium conjunctivum; CP—cerebral peduncle; cu—cuneiform nucleus; ftg—gigantocellular tegmental field; ic—inferior colliculus; ip—interpeduncular nucleus; kf—Kölliker-Fuse nucleus; lc—nucleus locus coeruleus; lld—dorsal nucleus of the lateral lemniscus; llv—ventral nucleus of the lateral lemniscus; me5—mesencephalic nucleus of the trigeminal nerve; pag—periaqueductal gray; pg—parabigeminal nucleus; pb—lateral parabrachial nucleus; pgm—medial parabrachial nucleus; pg—pontine gray; ppn—pedunculopontine nucleus; rd—dorsal raphe nucleus; sc—superior colliculus; snr—substantia nigra pars reticulata; so—superior olivary complex; TB—trapezoid body; tld—liaudororal tegmental nucleus.
The present results also raise the possibility of a noradrenergic input to the cholinergic neurons of the laterodorsal tegmental nucleus. Furthermore, both Golgi [67] and DBH immunohistochemical [17] studies in the rat have demonstrated extensive dendritic spread of noradrenergic neurons outside of the locus cereuleus, including medially in the central gray. It is of considerable interest that these dendrites are TH and DBH immunoreactive. Based upon similar observations, Björklund and Lindvall [6] hypothesized that the dendrites of substantia nigra pars compacta neurons, which extend ventrally for a considerable distance into pars reticulata, might release dopamine and this has been demonstrated both in vitro [15] and in vivo [14]. It is possible that the dendrites of locus cereuleus neurons release noradrenaline; this is supported by ultrastructural evidence of presynaptic profiles on the dendrites of rodent locus cereuleus neurons making dendro-dendritic contacts [16,59]. The spread of these dendrites into regions containing cholinergic neurons raises the possibility of multiple mechanisms for reciprocal innervation between these cholinergic and noradrenergic neurons.

Implications for Theories of Behavioral State Control

Pontomesencephalic tegmental neurons have long been thought to be involved in the phenomenology of behavioral state control. An early and influential finding was Moruzzi and Magoun's [40] demonstration that desynchronization of the cortical electroencephalogram could be evoked by electrical stimulation of the feline brainstem reticular formation. Although widespread areas of the brainstem were implicated, the most effective region was centered in the midbrain, essentially coincident with the pedunculopontine nucleus. Shute and Lewis [60], using acetylcholinesterase histochemistry in the rat, were the first to suggest that this so-called ascending reticular activating system might originate with cholinergic neurons. The present data demonstrate the existence of such a population of cholinergic neurons in the feline brainstem reticular formation. Furthermore, anatomical and immunohistochemical studies have confirmed that at least in the rat, pontomesencephalic cholinergic neurons have ascending projections consistent with this purported physiological role. In particular, brainstem cholinergic neurons project directly to limited portions of the cerebral cortex [52,72], to midline thalamic nuclei [56,62], as well as to the cholinergic basal forebrain [58]. An important experimental question is to determine the extent to which thalamic [63] and basal forebrain [64] mechanisms contribute to the phenomenon of EEG desynchronization produced by brainstem reticular stimulation.

Electrolytic lesion of the dorsolateral pontine tegmentum selectively abolishes the muscular atonia characteristic of paradoxical sleep [20,29]. Based upon these and other data, Jouvet [27,28] hypothesized that the noradrenergic neurons of the caudal locus cereuleus complex were "executive" elements in the generation of atonia during paradoxical sleep. In partial support of this hypothesis, Jones et al. [12] found that large locus cereuleus complex lesions which significantly reduced cerebral noradrenaline content eliminated the atonia of paradoxical sleep. However, they concluded that the effect was not dependent upon noradrenergic neurons per se since 6-hydroxydopamine lesions did not affect the atonia of paradoxical sleep to any appreciable extent [33]. The present finding of cholinergic neurons within the locus cereuleus complex suggests another cell group which might be implicated in the atonia of paradoxical sleep. Indeed, Hendricks et al. [19] have demonstrated that much smaller electrolytic lesions of the pedunculopontine tegmentum result in decreases in cerebral noradrenaline which can be reversed by cholinergic agents. Thus, it is possible that the atonia of paradoxical sleep is mediated by cholinergic neurons in the pontomesencephalic region and that both cholinergic and noradrenergic input to the caudal pontomesencephalic region are necessary for the generation of paradoxical sleep.

DISCUSSION

The major finding of the present study is the unequivocal demonstration that noradrenergic and cholinergic neurons are extensively intermixed in the feline pontomesencephalic tegmentum. Previous studies using simultaneous acetylcholinesterase histochemistry and catecholamine histochemistry [30] had suggested that this might be the case in the cat brain. The present data clearly demonstrate that this is so and provide details regarding the topographic relationships of these cell groups.

The present data also provide evidence for the existence of two types of cholinergic neurons in the feline dorsolateral tegmentum. In addition to the magnocellular neurons of the pedunculopontine and laterodorsal tegmental nuclei, which appear to comprise the feline homologue of the caudal cholinergic column in the rat and primate [53-55, 71-73], a distinct population of small neurons displaying ChAT immunoreactivity was seen in the medial and lateral parabrachial nuclei. Similar neurons have not been reported in other species. That these small spindle-shaped ChAT positive neurons comprise a separate population is suggested by both their distinct morphology and the absence of NADPH diaphorase staining of their somata. These ChAT positive cells were also found to be extensively intermixed with TH/DBH positive cells in the parabrachial nuclei.

Several lines of evidence suggest that the noradrenergic neurons of the locus cereuleus may receive a cholinergic input. Thus, these neurons contain acetylcholinesterase [1,4,32,55], possess muscarinic receptors [48], and are excited by acetylcholine [5,13,18,66]. The proximity of the caudal cholinergic column to the locus cereuleus in the rat [30] and primate [55] suggests that the presumptive cholinergic innervation of noradrenergic neurons might arise from these ChAT immunoreactive neurons. In the cat, this possibility is amplified by the fact that these neurochemically distinct neurons are intermingled within the dorsolateral pontine tegmentum.

The present results also raise the possibility of a noradrenergic input to the cholinergic neurons of the laterodorsal tegmental nucleus. Dendrites of TH positive neurons in the medial parabrachial nucleus were observed extending up into the laterodorsal tegmental nucleus. The present results also raise the possibility of a noradrenergic input to the cholinergic neurons of the laterodorsal tegmental nucleus. Dendrites of TH positive neurons in the medial parabrachial nucleus were observed extending up...
lesions of the region just medial to the locus ceruleus complex was most efficacious in producing the phenomenon. These lesions essentially coincide with the distribution of ChAT positive neurons in the laterodorsal tegmental and (caudal) pedunculopontine nuclei. Using retrograde tracing techniques [51] it has been shown that neurochemically unidentified neurons in this region project to the lower brainstem, including the so-called medullary inhibitory area of Magoun and Rhines [36]. Taken together, these observations suggest that cholinergic neurons of the pontomesencephalic tegmentum might be involved in the active generation of muscular atonia during paradoxical sleep.

Hobson et al. [22] suggested that reciprocal connections between 'cholinergic' neurons of the gigantocellular tegmental field and noradrenergic neurons of the locus ceruleus complex might be responsible for the cyclical alteration of the sleep-wake cycle. This controversial hypothesis was supported by the findings of Kimura et al. [31], who reported the presence of cholinergic/cholinceptive neurons in the gigantocellular tegmental field. Although our anatomical evidence in fact supports the notion of reciprocal cholinergic-noradrenergic interactions (see above), we found no evidence for ChAT immunoreactive neurons in the gigantocellular tegmental field [70] using a well characterized monoclonal antibody [12]. These observations, in concert with previous physiological data which challenged fundamental aspects of the hypothesis [61], provide strong evidence that the original reciprocal interaction hypothesis was incorrect in several important respects. However, it is intriguing to note that a small number of neurons with the precise state-specific firing patterns originally predicted for cholinergic neurons by this hypothesis, namely silent during all states but paradoxical sleep, have been recorded within the medial parabrachial nucleus of the unanesthetized cat [44.51]. It would be premature to suggest that such neurons are cholinergic; this remains a challenging question for future study.

The most enduring and robust observation implicating pontomesencephalic tegmental neurons in sleep cycle control is the finding that within the locus ceruleus complex, there exists a subpopulation of neurons whose members exhibit stereotypical variation in their mean and absolute discharge rate across behavioral states when recorded in the unanesthetized cat, failing silent during paradoxical sleep [21, 22, 43, 46, 49, 50]. Although it has long been suspected that these so-called PS-off cells are noradrenergic, the diffuse distribution of noradrenergic neurons in the feline locus ceruleus complex has made definitive identification difficult. The results of the present study add to this complication by demonstrating the extensive intermingling of cholinergic neurons with noradrenergic neurons within the locus ceruleus complex. However, the observations that neurons in the homogeneously noradrenergic locus ceruleus of the rat also exhibit the PS-off phenomenon [3], and that PS-off cells in the feline locus ceruleus complex are inhibited by systemic administration of clonidine [45], provide strong evidence that locus ceruleus complex PS-off cells are in fact noradrenergic.

The physiological properties of brainstem cholinergic neurons are unknown. The present data provide the anatomical detail required to examine this issue. The evidence implicating brainstem cholinergic neurons in sleep related phenomena suggests that studies of the behavioral neurophysiology of these neurons will significantly further our understanding of the cellular and biochemical control of behavioral state.

After submission of this manuscript, a report has appeared by Jones and Beaudet (J Comp Neurol 261: 15-32, 1987) who found a similar distribution of cholinergic and catecholaminergic neurons in the feline pontomesencephalic tegmentum using slightly different techniques.

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