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Characterization of Neurochemically Specific Projections From the Locus Coeruleus With Respect to Somatosensory-Related Barrels

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Abstract

Tactile information from the rodent mystacial vibrissae is relayed through the ascending trigeminal somatosensory system. At each level of this pathway, the whiskers are represented by a unique pattern of dense cell aggregates, which in layer IV of cortex are known as “barrels.” Afferent inputs from the dorsal thalamus have been demonstrated repeatedly to correspond rather precisely with this modular organization. However, axonal innervation patterns from other brain regions such as the noradrenergic locus coeruleus are less clear. A previous report has suggested that norepinephrine-containing fibers are concentrated in the center/hollow of the barrel, while other studies have emphasized a more random distribution of monoaminergic projections. To address this issue more directly, individual tissue sections were histochemically processed for cytochrome oxidase in combination with dopamine- β -hydroxylase, the synthesizing enzyme for norepinephrine, or the neuropeptide galanin. These two neuroactive agents were of particular interest because they colocalize in a majority of locus coeruleus neurons and terminals. Our data indicate that discrete concentrations or local arrays of dopamine- β -hydroxylase- or galanin-immunoreactive fibers are not apparent within the cores of individual barrels. As such, the data suggest that cortical inputs from the locus coeruleus are not patterned according to cytoarchitectural landmarks or the neurochemical identity of coeruleocortical efferents. While transmitter-specific actions of norepinephrine and/or galanin may not be derived from the laminar/spatial connections of locus coeruleus axons, the possibility remains that the release of these substances may mediate distinctive events through the localization of different receptor subclasses, or the contact of their terminals onto cells with certain morphological characteristics or ultrastructural components.

Keywords

galanin; norepinephrine; locus coeruleus; trigeminal; barrel; cytochrome oxidase

The face region of the rodent somatosensory cortex is recognized for the patterned arrangement of granule cells in layer IV. These neurons are organized into discrete clusters, which are separated by cell-sparse zones known as “septa” (Woosley and Van der Loos, 1970; Welker and Woosley, 1974). Due to their characteristic shape, the term “barrel” has been applied to describe and identify each neuronal aggregation. Collectively, these

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cytoarchitectonic specializations form a centralized map of the whisker pad. The topography of this cortical representation has been shown to preserve the layout of the trigeminal receptive surface and can be directly attributed to the close correspondence between a single vibrissae and the synaptic activation of an individual barrel unit.

Several histochemical and enzymatic stains including cytochrome oxidase (CO), succinate dehydrogenase, ATPase, and NADPH-diaphorase are capable of revealing the center or “hollow” of individual barrels (Wong-Riley and Welt, 1980; Land and Simons, 1985; Riddle et al., 1993; Franca and Volchan, 1995; Simpson et al., 2003). Immunoreactivity to glutamic acid decarboxylase (GAD) has also been used to demonstrate the GABAergic profiles of these structured neuronal subpopulations in barrel field cortex (Lin et al., 1985; Chmielowska et al., 1986). An additional means of discriminating different cortical sub-zones has been demonstrated through anterograde tracing studies. Previous investigations have examined labeled inputs from thalamic afferents of the ventroposterior medial nucleus (VPM) and found that they terminate primarily in the barrel center (Chmielowska et al., 1989). Whereas axon terminals from the posterior medial nucleus (POM) preferentially target the septal subregion (Koralek et al., 1988; Lu and Lin, 1993).

Using the glyoxylic acid histofluorescence technique, Lidov et al. (1978) reported that catecholamine (CA)-containing axons in mouse somatosensory cortex also exhibit a patterned innervation relative to the barrel design. Noradrenergic fibers were described as highly ramified and localized in a sunburst orientation within the center of each barrel. Such organization is supported by other studies, which have shown selective connectivity between the rodent locus coeruleus (LC) and its efferent target sites (Mason and Fibiger, 1979; Cedarbaum and Saper, 1981; Waterhouse et al., 1983; Loughlin et al., 1986a, 1986b; Morrison and Foote, 1986; Simpson et al., 1997). In general, these findings contradict other early investigations, which emphasized the diffuse projection pattern and global actions of the LC/norepinephrine (NE) system (Anden et al., 1966; Ungerstedt, 1971; Pickel et al., 1974; Nakamura and Iwama, 1975; Aston-Jones and Bloom, 1981a, 1981b; Aston-Jones et al., 1986). In fact, two experiments, one employing immunohistochemical methods (Morrison et al., 1978) and the other the glyoxylic acid histofluorescence technique (Levitt and Moore, 1978), were unable to reveal any unique geometry embedded within the monoaminergic circuitry of barrel field cortex, layer IV. Overall, dopamine- β -hydroxylase (DBH)-containing fibers tended to course obliquely through layer IV and tangentially within layers I and VI of the rat sensorimotor neocortex.

Related to the issue of barrel cortex innervation by the LC is the distribution of coeruleocortical projections that colocalize DBH and neuroactive peptides. Neurons in the LC have been found to express a variety of putative peptide transmitters. Vasopressin, somatostatin, neuropeptide Y, enkephalin, neurotensin, and galanin (Gal) are among those reported to date (Olpe and Steinmann, 1991). Gal is of particular interest since 80% of LC neurons contain both Gal and NE (Melandar et al., 1986); 50% of these neurons have been found to project to barrel cortex; and Gal-positive axons have recently been detected in somatosensory cortex (Simpson et al., 1999).

The goal of the current investigation was to evaluate the spatial relationship between LC noradrenergic efferents and functionally distinct subdomains of the somatosensory barrel field cortex. An additional objective was to determine whether the organizational properties of the LC efferent projection within different cortical subregions are contingent on the specific peptidergic composition of selective LC inputs. To elucidate any geometric overlap, individual brain sections were processed for CO and then double-stained with DBH or Gal immunohistochemical reagents. This approach permitted a direct assessment of neurochemically identified LC axon terminals with respect to the barrel design.

MATERIALS AND METHODS

Six female Long-Evans hooded rats (250 – 400 g) and three C57BL wild-type male mice were used in the present study. All animals were maintained according to guidelines approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center, and protocols conformed to the National Institutes of Health specifications for the proper treatment of animals in research. Subjects were anesthetized with Nembutal (50 mg/kg i.p.) and perfused through the heart with saline followed by 3.5% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at pH 7.2. Brains were removed and placed in the same fixative containing 20% sucrose for 12–24 hr. Fixed material was cut either in the coronal or tangential plane on a freezing microtome at 40 – 80 μm . To correlate DBH and Gal immunoreactivity with the barrel structure, sections were first stained for CO, then processed further for DBH or Gal using the ABC method with heavy metal augmentation (Adams, 1981). The visualization of immunolabeled fibers was also enhanced by silver intensification (Quinn and Graybiel, 1996) or cryoprotection with serial incubations in 5%, 10%, and 20% dimethyl sulfoxide (Lu and Lin, 1993). Both monoclonal (DBH; Chemicon) and polyclonal (DBH; Eugene Tech.; Gal, Peninsula Labs.) antibodies were utilized. Primary dilutions of antisera ranged from 1:300 to 500 and 1:3,000 to 8,000 for DBH and Gal, respectively. Labeled profiles were examined using a Nikon light microscope.

In order to analyze potential differences semiquantitatively in the noradrenergic innervation of core/hollow and septal subregions of the barrel cortex, a modified method of assessing fiber density was conducted (Horvath et al., 1999). In brief, a 50 μm $\times$ 50 μm test grid was constructed, which consisted of five vertical and five horizontal lines separated by 10 μm . The goal of the analysis was to count the number of instances a sample array of immunoreactive fibers intersected a point on the grid. A camera lucida drawing tube was used to record the distribution of labeled fibers across a territory of six barrel/septa units in two animals. Findings were plotted on a piece of paper, and the test grid was placed randomly over core and septal subregions of cortex. At least three barrel/septa units were analyzed per case, with each unit being assayed at 10 different sites within the barrel and septal subareas. In practice, a single case could yield a minimum of 60 data points, 30 from evaluations of septal subzones and 30 from inspections of core/hollow subfields. Data entries from each subarea were averaged and pooled with findings from the other case. Results were then expressed as the mean number of intersecting points/subfield (hollow or septa)/grid area. Galaninergetic fibers were not quantified due to the fact that they are relatively less abundant than NE fibers and often required several focal adjustments to follow individually labeled processes. Together, these factors made it unreasonable to survey fiber density using intersection point analysis with the 50 $\times$ 50 μm test grid.

RESULTS

The LC is regarded as the primary source of noradrenergic innervation to somatosensory divisions of the medulla, pons, and forebrain (Fuxe et al., 1968; Lindvall et al., 1974; Foote et al., 1975; Levitt and Moore, 1978, 1979) as well as Gal-containing fibers to barrel field cortex, VPM, and the principal nucleus of the trigeminal complex (PrV) (Simpson et al., 1999). By combining CO histochemistry with methods for detecting DBH and Gal immunoreactivities in the same section, the distribution of monoaminergic and galaninergetic inputs from the LC was assessed in reference to the cortical barrel organization. This staining strategy was also applied to tissue from other aspects of the trigeminal neuraxis in order to determine whether any projection patterns displayed by LC axons persist within functionally associated relay nuclei of the thalamus and brainstem. A major finding of the present study is that both DBH- and Gal-containing fibers are observed within the barrel

hollows, as well as within the septal regions between barrels. In many instances, a single DBH- or Gal-positive fiber could be traced crossing from one barrel to another. Often branches from an individual axon diverged from the main portion of the fiber and headed in a direction that was either perpendicular or parallel to the parent segment. Moreover, these fibers did not appear to be preferentially concentrated within the center of individual barrels. A representative line drawing that compares the orientation of DBH immunoreactive fibers to the position of a cortical barrel is shown in Figure 1. The apparent random trajectory of these labeled fibers was not only evident in coronal sections (Fig. 2C), but routinely observed in tangential slices (Fig. 2D). Dense tangles of monoaminergic terminals were also not detected in the barrel hollows of mouse somatosensory cortex (Fig. 3). Semiquantitative analysis further revealed that noradrenergic fibers do not preferentially target the center of an individual barrel, but rather display a more uniform distribution across layer 4. In fact, an average of 12.1 and 12.2 points of intersection were noted in core and septal subregions, respectively.

Further examination revealed that Gal-immunostained fibers in somatosensory cortex were less abundant than DBH-positive axons. In addition, the overall proportion of Gal-labeled axon profiles was slightly greater in the mouse than in the rat barrel cortex. As in the rat, Gal immunoreactive fibers in the mouse tended to avoid a strict anatomical association within the cortical barrel boundaries. Both coronal (Fig. 2A) and tangential (Fig. 2B) sections demonstrated the lack of a patterned peptidergic innervation in layer IV. The schematic diagram in Figure 4 illustrates most of these features. It is worth noting that nests of monoaminergic fibers were also not aligned with the barreloids in the VPM thalamus, nor the barrelettes in the trigeminal nuclear complex (data not shown).

DISCUSSION

By using the glyoxylic acid histofluorescence technique and the morphological landmarks of lamina IV to characterize noradrenergic projections to somatosensory cortex, Lidov et al. (1978) found that labeled tufts of axonal processes preferentially radiate within the barrel hollows. In addition to suggesting that discrete arrays of LC terminals are superimposed upon thalamocortical afferents, these findings also supported the notion that these dual inputs regulate the same local cortical circuits. The current study was conducted in an attempt to extend previous results with more conventional approaches and reveal a laminar specificity that is based on the neurochemical composition of LC efferents. However, the present data are in stark contrast with the earlier description (Lidov et al., 1978). CO staining procedures were combined with DBH or Gal immunohistochemistry in the same tissue section in order to directly evaluate the affiliation between norepinephrine/peptide-containing fibers and the barrel organization. Evidence from these recent experiments indicates that neither DBH- nor Gal-containing fibers conform to any specific dimensions relative to the barrel architecture in the rat or mouse. This information is important, because it corresponds well with other (conflicting) reports that maintain the distribution of monoaminergic fibers in rodent neocortex is uniform within individual lamina (Levitt and Moore, 1978; Morrison and Foote, 1986). These differing conclusions can most likely be attributed to technical limitations associated with using fluorescence to identify both the barrels and catecholaminergic fibers. Although the rationale for utilizing an incident illuminator to visualize barrel structures in relation to coeruleocortical afferent inputs was sound, we suspect that coupling the diffuse green background fluorescence prevalent in barrel hollows with the blue-green color typical of the catecholamine-derived fluorophore somehow distorted the spectral characteristics of the emission. The combination may have artifactually amplified the signal and lead to the conclusion that CA fibers preferentially target the barrel hollows. As demonstrated in representative photomicrographs (Lidov et al., 1978), the altered appearance of CA-containing processes inside vs. outside the barrel

hollow speaks to this possibility. Fibers situated toward the center were thick and varicose, while axonal profiles along the periphery demonstrated a fine beaded morphology. The current study, which also evaluated projection patterns with respect to the barrel architecture in an individual tissue preparation, circumvented such confounding variables by utilizing two different chromagens. In practice, this resulted in brown CO-stained barrels and black-purple Ni⁺-intensified fibers, which could easily be distinguished from one another without optical interference. Axons could often be traced within the proximity of multiple barrels. By adjusting the plane of focus, individual fibers could be followed from the septal area into neural territory associated with the barrel column. Sometimes these processes coursed diagonally through a barrel before disappearing within the supra-granular or infragranular layers.

Despite the presumed biasing influence of barrel illuminance on the labeling produced by the glyoxylic acid histofluorescence technique, material stained according to this procedure was still capable of revealing fiber trajectories similar to those documented in the present and previous reports. For example, the authors frequently encountered tangentially oriented fibers, which traversed more than one barrel and gave rise to collaterals en route. An alternative explanation for the manifestation of CA-containing fiber plexuses within the barrel hollows may be that the glyoxylic acid histofluorescence technique identifies only a selected subpopulation of monoaminergic inputs, whereas DBH immunomethodology more completely distinguishes the majority of LC efferents. However, this is unlikely, since another previous study, which utilized glyoxylic acid histochemistry (Levitt and Moore, 1978), found no evidence of dense segregated monoaminergic fiber arrays in layer IV. Species differences should also not be viewed as a contributing factor, because in the current study both rat and mouse tissue demonstrated an even distribution of noradrenergic and peptidergic projections from the LC in barrel cortex.

Although DBH- and Gal-positive coeruleocortical fibers do not appear to be organized according to any strict geometric principles (laminar or cytoarchitectonic), the possibility still remains for these transmitter substances to exert a selective influence on specific cortical components. Other investigations have mapped the distribution of receptor subtypes as a potential substrate for promoting or rendering functional specificity. Goldman-Rakic et al. (1990) found that dopaminergic, adrenergic, and serotonergic receptors are concentrated in a lamina-specific pattern within primate frontal cortex. Adrenergic subtypes have further been shown to be preferentially colocalized with cortical barrels in rat somatosensory cortex (Vos et al., 1990) and to be arranged in a complementary fashion in the thalamus and spinal cord (Rainbow et al., 1984; Stone et al., 1988; Pieribone et al., 1994). Similarly, an organized distribution of dopamine receptors has been demonstrated by the D₃ subtype, which is selectively and transiently expressed in the barrel cortex of the developing rat (Gurevich and Joyce, 2000).

Trends have also been observed with respect to the location of Gal receptors, whereby layers IV, V, and VI demonstrate a higher propensity for radio-ligand binding in feline and primate cortex (Kohler et al., 1989; Rosier et al., 1991). The recent cloning and characterization of multiple Gal subtypes (GalR1, GalR2, and GalR3) by solution hybridization/RNase protection assays, the reverse transcription-polymerase chain reaction, and in situ hybridization will likely increase the potential for detecting additional patterns of Gal receptor-related specificity in neuronal tissue (O'Donnell et al., 1999; Branchek et al., 2000; Waters and Krause, 2000). Although there appears to be some dispute over which subtypes are more prevalent in cortex, recent data indicate that GalR2 mRNA is more concentrated within layers II–III and VI.

Alternatively, target-specific actions of NE and Gal may be conferred on neurons that express certain morphological, neurochemical, or electrophysiological traits. Evidence supporting this possibility has been recently reported (Krimer et al., 1997; Kawaguchi and Shindou, 1998; Dougherty and Milner, 1999). For example, Kawaguchi and Shindou (1998) demonstrated that NE differentially regulates the excitability of specific GABAergic cell types in rat frontal cortex. Likewise, Krimer et al. (1997) reported that tyrosine hydroxylase (TH)-immunolabeled fibers selectively innervate pyramidal vs. nonpyramidal neurons. These discriminative qualities have also been observed in the hippocampus. Dougherty and Milner (1999) found that cholinergic septal afferents in the dentate gyrus preferentially contact neuropeptide-Y-containing interneurons in comparison to parvalbumin-containing interneurons.

Specific responses to NE or Gal may also be dictated by the region of the cell that receives innervation. Anderson et al. (1980) and Scharfman and Sarvey (1985) have reported that when GABA is applied near the soma, it induces a hyperpolarization associated with an increased membrane conductance. By contrast, when GABA is applied to dendrites, it causes depolarization. While data of this nature, ultrastructural or otherwise, is currently lacking with respect to either Gal- or NE-positive efferents from the LC, transmitter release and electrophysiological studies suggest that both neuroactive agents are nevertheless capable of producing a wide range of effects. Gal has been shown to inhibit the release of NE in the medulla oblongata (Tsuda et al., 1992) while enhancing the liberation of NE in the hypothalamic paraventricular nucleus (Kyrkouli et al., 1992). Gal has also been shown to exert a predominantly inhibitory influence on neuronal activity via both pre- and postsynaptic mechanisms (Sevcik et al., 1993; Sakurai et al., 1996). Likewise, diverse neuromodulatory actions have also been recorded for NE (Foote et al., 1975; Waterhouse et al., 1980, 1981, 1982, 1988, 1990; McLean and Waterhouse, 1994). For example, microiontophoretically applied NE was found to augment both the excitatory and inhibitory responses of somatosensory cortical neurons following activation of afferent input pathways (Waterhouse and Woodward, 1980).

Taken together, the present results indicate that neither the noradrenergic nor peptide-specific efferent projections of the LC innervate cortical layer IV in a unique geometric fashion consistent with the cytoarchitectonic boundaries of trigeminal somatosensory-related barrels. These findings suggest that upon activation, the LC may modulate cortical activity across several barrels as opposed to impacting barrel-related neuronal processing on an individual basis. However, it does not preclude the possibility that target-specific actions may be conferred through certain receptor subclasses or morphological/ultrastructural characteristics. Furthermore, the uniform distribution of DBH- and Gal-positive projections in both rat and mouse point to few species differences regarding the way in which the noradrenergic system influences cortical function in each animal. The current data further support the usage of different chromagens for evaluating anatomical relationships within circumscribed neural territories and suggest that the cumulative effect of combining fluorescence methodologies with similar spectral emissions may produce misleading visual distortions upon analysis.

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Fig. 1. Camera lucida line drawing illustrating the geometric relationship of DBH immunoreactive fibers with respect to the barrel structure in a tangential section through rat somatosensory cortex. Dashed lines delineate the borders of individual barrels as determined from CO staining. Note the random but uniform distribution of labeled fibers in the center/hollow of the barrel and the surrounding septal region.

Fig. 2. Distribution of Gal and DBH immunoreactive fibers with respect to rat cortical barrels. Photomicrographs of Gal-positive (**A** and **B**) and DBH-containing (**C** and **D**) axonal profiles in coronal (**A** and **C**) and tangential (**B** and **D**) sections. Dashed lines define the borders of individual barrels as revealed by CO histochemistry. Arrows indicate fibers that were identified using Ni-intensification procedures. Note the orientation of DBH- and Gal-labeled segments is not patterned relative to the cytoarchitectonic boundaries of the barrel. Scale bars = 50 μ m (**A**); 50 μ m (**B**, applies to **C** and **D**).

Fig. 3.
Camera lucida line drawing demonstrating the density and trajectory of DBH immunoreactive fibers within a coronal section through the mouse barrel field cortex. Dashed lines demarcate the borders of individual barrels. Note the unbiased arrangement of immunostained fibers that prevails within and outside the perimeter of a single barrel.

Fig. 4.

Schematic diagram depicts the overall proportion and orientation of DBH- and Gal-labeled axons in layers I–VI from representative cases in the mouse and rat. Fibers in layers I and VI tended to course parallel to the pial surface and white matter, respectively. In contrast, the axonal profiles observed in layers II–V appeared to run in many directions and to lack a lamina-specific orderliness, especially in layer IV.