Brainstem Projecting Neurons in the Rat Basal Forebrain: Neurochemical, Topographical, and Physiological Distinctions From Cortically Projecting Cholinergic Neurons

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SEMBA, K., P. B. REINER, E. G. McGEER AND H. C. FIBIGER. Brainstem projecting neurons in the rat basal forebrain: Neurochemical, topographical, and physiological distinctions from cortically projecting cholinergic neurons. BRAIN RES BULL 22(3) 501–509, 1989. — Magnocellular regions of the basal forebrain contain cholinergic neurons that project to the cerebral cortex. Neurons in the same basal forebrain regions innervate the brainstem. The present study investigated whether these brainstem projecting neurons are cholinergic, project also to the cortex, and share similar physiological properties as cortically projecting neurons. Data with retrograde tracing from various regions of the pons, medulla, and cortex combined with choline acetyltransferase immunofluorescence indicated that: 1) brainstem projecting neurons are usually segregated from cortically projecting and/or cholinergic neurons in the basal forebrain, 2) virtually no brainstem projecting neurons in the basal forebrain are cholinergic, and 3) only rarely do basal forebrain neurons have axon collaterals that project to both cortex and brainstem. Extracellular recordings from basal forebrain neurons confirmed the paucity of axonal collateralization and the topographic segregation between cortically and brainstem projecting basal forebrain neurons, and, in addition, showed that brainstem projecting neurons have a slower mean conduction velocity than cortically projecting neurons. These observations suggest that basal forebrain neurons projecting to the brainstem (pons, medulla) and the cortex represent separate cell populations in terms of projections, neurotransmitter content, distribution, and physiological properties. Preliminary results have been reported (38).

METHOD

Animals

For anatomical experiments, 69 male Wistar rats, 150–300 g in body weight, were used. An additional 28 male Wistar rats, 250–300 g, were used for physiological experiments.

Retrograde WGA-HRP Tracing

Under chloral hydrate (400 mg/kg, IP) or pentobarbital (50
mg/kg, IP) anesthesia, pressure injections (0.05–0.2 μl) of wheatgerm agglutinin-conjugated horsedarshis peroxidase (WGA-HRP; 1.5% in 0.1 M phosphate buffer) were made in the pedunculopontine tegmental nucleus [PPT; AP = 0.35, ML = 1.5, D = -1.9 from interaural zero according to König and Klippel (21)] or the dorsal raphe nucleus [DRN; AP = -7.4, ML = 0, from bregma, D = -6.0 from the surface of the brain, according to Paxinos and Watson (31)]. One to 2 days later, rats were overdosed with anesthetic, and perfused transcardially with: 1) 50 ml of 0.01 M phosphate buffered saline, 2) 500 ml of ice-cold 2% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M phosphate buffer, and 3) 500 ml of ice-cold 10% sucrose in 0.1 M phosphate buffer. Brains were removed from the skull and placed in the same sucrose solution overnight at 4°C.

Brains were cut coronally at 30 μm on a freezing microtome, and every fifth section through the forebrain and brainstem injection site was histochemically processed to visualize WGA-HRP, according to Mesulam’s (25) method using tetramethylbenzidine as a chromagen. Following reaction, sections were mounted on chrome alum coated slides, air dried, counterstained with neutral red, and coverslipped with permount.

Retrograde Labelling Combined With Immunofluorescence

Two double labelling experiments were performed: True Blue (TB) injections into various parts of the brainstem combined with either 1) additional multiple propidium iodide (PI) injections into the cortex, or 2) choline acetyltransferase (ChAT) immunofluorescence. Double labelling was examined in the basal forebrain. Some sections containing neurons double labelled from the brainstem and cortex were further processed for ChAT immunofluorescence.

Under chloral hydrate or pentobarbital anesthesia, two pressure injections of TB (Iling, 5% in distilled water, 0.1–0.2 μl each) were stereotaxically aimed at 2 of the 5 brainstem sites described below in each rat. Some rats received only one brainstem injection. Each injection was made over 10 min, and the injection needle was left in place for an additional 10 min. To avoid spillage into the cerebral cortex, the injection needle was lowered through the cerebellum for the following injection sites: the dorsal raphe nucleus/central gray [DRN/CG; AP = -11.1 from bregma, ML = 0.3, D = -8.1 from the surface of the brain at 25° directed caudorosstrally, according to Paxinos and Watson (31)]. The PPT (AP = -12.7, ML = 1.5, D = -9.5 at 35° directed caudorosstrally), the reticulo-tegmental nucleus of the pons (AP = -11.1, ML = 0.4, D = -8.7 at 20° directed caudorosstrally), and the locus ceruleus-parabrachial regions (AP = -9.8, ML = 1.3 to 1.5, D = -5.6 to -7.1 at a right angle). Control TB injections into the cerebellum resulted in no retrograde labelling in the basal forebrain. Injections into the nucleus of the solitary tract were made under visual guidance after making an incision through the cisterna magna and exposing the obex. During the initial phase of the experiments, injections were made into the DRN at 20° to midline through the cortex, rather than the cerebellum [AP = -7.8, ML = 2.0 from bregma, D = -6.1 from brain surface, according to Paxinos and Watson (31)]; subsequent PI injections (see below) were made into the cortex contralateral to this needle track. In experiments involving cortical PI injections, rats were reanesthetized two days after TB injections, and PI (Sigma, 10% aqueous solution, 0.1 μl each) was injected into at least 3 and up to 6 of the following cortical sites ipsilateral to the TB injections: medial prefrontal cortex: AP = 2.7 from bregma, ML = 0.7, D = -3.8 from the brain surface; frontal: AP = 1.5, ML = 2.5, D = -1.3; rostral cingulate: AP = 0, ML = 0.5, D = -1.5; caudal cingulate: AP = -4.0, ML = 0.5, D = -1.3; parietal: AP = -4.0, ML = 2.5, D = -0.8; hippocampus: AP = -3.3, ML = 1.7, D = -2.5 to -2.9.

Following a survival period of 4–7 days after the TB injections, rats were overdosed with anesthetic, and perfused with a buffered paraformaldehyde fixative as described in detail elsewhere (39). Following postfixation and cryoprotection, the brains were cut at 30 μm through the basal forebrain and brainstem injection sites. For basal forebrain sections to be stained also for ChAT immunofluorescence, a monoclonal antibody to ChAT (11) was used with IgG conjugated with tetramethylrhodamine isothio cyanate (TRITC). Texas Red (for sections containing TB labelling alone) or fluorescein isothiocyanate (FITC) (for sections containing both TB and PI labelling) as secondary antibody [for details of the immunohistochemical procedures, see (39)].

Microscopic Examination

Retrograde WGA-HRP labelling was examined in both bright and dark field at magnifications of 100× to 400×. Sections containing fluorescent tracers and immunofluorescence were examined in a Leitz fluorescence microscope; filter blocks A (ultraviolet and violet), N2 (green), and L2 (blue) were used to examine TB. Texas Red/TRITC/PI, and FITC labelling, respectively.

Recordings

Details of the recording procedures have been described elsewhere (33,39). Briefly, in urethane anesthetized rats extracellular recordings were made from neurons in the basal forebrain at the level of the optic chiasm by using a transphyaryngeal approach. Recording electrodes consisted of micropipettes filled with 2 M NaCl saturated with fast blue. As search stimuli, the ipsilateral PPT and DRN/CG were alternately stimulated using 0.2 msec, 2 mA pulses at 1.5 Hz. Neurons driven antidromically from these brainstem sites were characterized for spontaneous firing rate, antidromic threshold, latency, and refractory period. The antidromic criteria used included: constant latency, high frequency following (>100 Hz); and, for spontaneously firing neurons, collision. In addition to the brainstem electrodes, 4 pairs of bipolar stimulating electrodes were implanted into the ipsilateral cerebral cortex. The end of each electrode track was routinely marked by passing current to deposit fast green.

As previously described in detail (39), following recording, rats were perfused with a paraformaldehyde fixative, and the brains were cut to confirm stimulation sites in the brainstem. Sections through the PPT were routinely reacted for NADPH-diaphorase, a specific marker for cholinergic neurons in the brainstem tegmentum (48), to examine the location of electrode tips in relation to the cholinergic neurons in the PPT. In addition, alternate sections through the basal forebrain in some animals were processed for ChAT and substance P immunohistochemistry. Reconstruction of the electrode tracts against these two markers allowed localization of recorded neurons with respect to cholinergic and ventral pallidal (16,19) regions of the basal forebrain, respectively.

RESULTS

Retrograde Labelling in the Basal Forebrain Following WGA-HRP Injections Into the Brainstem

The distributions of neurons retrogradely labelled following large WGA-HRP injections into the PPT or DRN were examined within the basal forebrain, and Fig. 1 shows the results at 3 different rostrocaudal levels. From either brainstem site, occa-
FIG. 1. The distribution of retrogradely labelled neurons in the basal forebrain following large WGA-HRP injections into the PPT (left) or DRN (right). The stippling indicates the core of injection site where all neuronal elements appeared darkly stained, and the dotted line around it indicates the halo-like zone where some somata and processes were stained against an unstained background. The numbers near section drawings indicate approximate distances from the bregma. Each dot represents one retrogradely labelled neuron. Abbreviations: a, nucleus accumbens; ac, anterior commissure; bst, bed nucleus of the stria terminalis; ci, inferior colliculus; cp, caudate-putamen; ep, endopiriform nucleus; f, fornix; gp, globus pallidus; hdb, horizontal limb of the diagonal band; ic, internal capsule; lh, lateral hypothalamus; mgpa, magnocellular preoptic area; mlf, medial longitudinal fasciculus; ms, medial septum; nbm, nucleus basalis magnocellularis; poa, preoptic area; sf, septofimbrial nucleus; sm, stria medullaris; so, supraoptic nucleus; vp, ventral pallidum.

Neurons were retrogradely labelled in the medial septum, horizontal limb of the diagonal band, magnocellular preoptic area, and the nucleus basalis magnocellularis, i.e., those basal forebrain regions with high concentrations of cholinergic neurons. In addition, many neurons were retrogradely labelled in the bed nucleus of the stria terminalis, preoptic areas, and lateral hypothalamus as well as the endopiriform and cingulate cortices. More neurons were labelled ipsilaterally than contralaterally following PPT injections, and generally more neurons were labelled following PPT than DRN injections.

Double/Triple Labelling With Retrograde Tracers and ChAT Immunofluorescence

TB injections in 41 rats were located in the following brainstem...
dorsal tegmental nucleus, locus ceruleus, parabrachial regions, cerebellum, motor and sensory trigeminal nuclei, vestibular nuclei, and nucleus of the solitary tract. The injections also invaded parts of the following brainstem regions: the superior colliculus, substantia nigra pars reticulata, median raphe nucleus, nuclei of the lateral lemniscus, dorsal tegmental nucleus of Gudden, and gracile and cuneate nuclei.

Because two brainstem injections were made in most rats, only the results of combined injections could usually be ascertained. However, in some cases in which one of the two injections failed or was extremely small, it could be deduced that the injections into the sensory or motor nuclei or the cerebellum resulted in virtually no retrograde labelling in the basal forebrain. In addition, neurons retrogradely labelled from the DRN/CG and PPT were distributed in patterns similar to those seen following WGA-HRP injections; however, the number of labelled neurons was generally greater after TB than WGA-HRP injections.

Topographic relationships were examined between TB-labelled neurons, and neurons retrogradely labelled following PI injections into the cortex. Consistent with previous studies (4, 13, 26, 34, 49, 53), PI-positive neurons were present in the magnocellular basal forebrain including the medial septum, nuclei of the diagonal band, magnocellular preoptic area, and nucleus basalis magnocellularis. TB-labelled neurons were also seen in these regions, as well as other areas; in particular, those located rostrally often formed loose clusters of 6–15 neurons in the 30 μm coronal sections. These clusters were often segregated from clusters of neurons retrogradely labelled with PI from the cortex (Fig. 2).

In selective cases where many TB-labelled neurons were labelled in the basal forebrain, the distribution of TB-labelled neurons was also examined in relation to that of ChAT-immunoreactive neurons. TB injections in these cases involved the laterodorsal tegmental nucleus, PPT, DRN, CG, and/or locus ceruleus. Again, as seen in relation to PI-labelled neurons following cortical injections, TB-labelled neurons were usually segregated from ChAT-immunoreactive neurons (Fig. 3). Such segregation was seen both ipsilateral (Fig. 3A, B) and contralateral (Fig. 3C, D) to brainstem injections.

Rare TB-labelled, brainstem-projecting basal forebrain neurons were, in addition, either retrogradely labelled also with PI from the cortex (Fig. 4A, B) or ChAT-immunoreactive (Fig. 4C, D). No obvious relationship was seen between basal forebrain regions and the occurrence of these double labelled neurons. In triple labelling experiments, no neuron could be definitively demonstrated to be retrogradely labelled from both brainstem and cortex and also ChAT-immunoreactive. Most of the PI-positive neurons were ChAT-immunoreactive.

Physiological Properties of Brainstem Projecting Neurons in the Basal Forebrain

A total of 202 basal forebrain neurons met the antidromic criteria: 63 were driven from the PPT, 39 from the DRN/CG (Fig. 5), and 100 from the cortex. The neurons driven from the PPT and DRN/CG were not significantly different in spontaneous firing rate, antidromic latency, conduction velocity, refractory period, or threshold (Table I). The estimated distance between recording sites and stimulation sites were 8 mm for both PPT and DRN/CG, as well as for the cortex. The brainstem projecting basal forebrain neurons had heterogeneous antidromic latencies, ranging from 2.0–29.0 msec, and their estimated conduction velocities (1.0 ± 0.8 m/sec, mean ± S.D., n = 102) were significantly slower than those of cortically projecting neurons (1.8 ± 2.6 m/sec, n = 99) [Welch's approximate F(115) = 2.91, p<0.01]. The physiological properties of these cortically projecting basal forebrain neurons were similar...
to those reported in our previous study (33).

Each of the 102 basal forebrain neurons antidromically driven from the brainstem tegmentum was tested for additional antidromicity from the cortex, and those driven from the cortex were also tested for antidromicity from the brainstem. No neuron was encountered that could be activated antidromically from both brainstem and cerebral cortex.

All the brainstem and cortically projecting neurons described above were recorded while the electrode was advanced from the ventral surface of the brain up to 2500–3000 μm dorsally. Microdrive readings of the locations of recorded neurons indicated that the brainstem projecting neurons were located, in general, more dorsally than cortically projecting neurons; 75% of the cortically projecting neurons were recorded within 1750 μm of the ventral surface of the brain, whereas only 35% of brainstem projecting neurons were recorded within the same region (Fig. 6). The mean microdrive readings for brainstem (1967 ± 539 μm, mean ± S.D., n = 101) and cortically projecting neurons (1488 ± 569 μm, n = 100) were significantly different, t(199) = 6.1, p < 0.001. No difference in distribution was seen between neurons driven from the PPT and the DRN/CG. An example of an electrode track reconstructed against the background of superimposed ChAT and substance P immunostaining is shown in Fig. 5A. Although tissue shrinkage and distortion must be taken into account, the locations of all the brainstem projecting neurons recorded in this track appeared to be within the ventral pallidum, as defined by sub-
FIG. 4. A, B: An example of a rare neuron (arrowheads) in the nucleus basalis magnocellularis which was double labelled with TB (A) from the brainstem and PI (B) from the cortex. C, D: An example of a rare neuron (arrowheads) which was double labelled with TB (C) from the brainstem and ChAT immunofluorescence (D). Scale bar: 50 μm.

DISCUSSION

Both anatomical and physiological data in the present study are consistent with the suggestion that basal forebrain neurons projecting to the pons and medulla and those projecting to the cortex represent two separate populations in terms of neurotransmitter content, topographic distribution, and conduction velocity. Topographically, the two populations of neurons often formed small clusters which were segregated from each other, and only rarely did a neuron project to both cortex and brainstem. Almost all brainstem projecting basal forebrain neurons investigated in the present study were noncholinergic whereas the majority of cortically projecting neurons have been shown to be cholinergic [(4, 34, 49, 53), present study]. Physiologically, brainstem projecting neurons, on average, had conduction velocities about half of those of cortically projecting neurons. The above conclusion, however, should be taken with the caveat that neither the entire cortex nor the entire brainstem were injected with tracers in the present study, and also negative results such as paucity of double labelling are always open to technical questions. It should also be noted that in the present study each injection was usually large, and resulting mechanical damage at the injection site, in conjunction with type of tracer used, would permit considerable uptake of tracers by fibers of passage. This is particularly relevant in the case of brainstem injections, where the tracers might have labelled axons which descended further to terminate at levels caudal to the injection sites.

Although only a few anatomical studies have specifically investigated the brainstem projection neurons in the magnocellular basal forebrain [(27, 43, 44), present study], their presence has been noted in a number of anatomical studies investigating afferents to various pontine and medullary regions. For example, in a study on the afferents to the raphe nuclei using HRP in the rat, Aghajanian and Wang (2) observed retrogradely labelled neurons in the magnocellular preoptic area and diagonal band. Similarly, the presence of retrogradely labelled neurons in the magnocellular basal forebrain regions has been documented following tracer injections into the laterodorsal tegmental nucleus (36), nucleus reticularis tegmenti pontis (45), locus ceruleus (9), parabrachial regions (14), and nucleus of the solitary tract (37,41). The descending projections from the diagonal band and rostral sub-
FIG. 5. A, C: Example of tests of antidromicity demonstrated in basal forebrain neurons to electrical stimulation of the PPT (A) and DRN/CG (C). Upper traces demonstrate that high frequency double pulse stimulation (arrowheads) produces two action potentials with constant latency and identical waveforms. In lower traces, the same double pulse stimulation was triggered by a spontaneous action potential, which resulted in the absence of the first evoked spike (asterisks), but not the second. The second action potential triggered the double pulse stimulation again, which obscured parts of subsequent recordings. The absence of the first spike is presumptive evidence for collision between the spontaneous, orthodromic spike and the antidromic spike. Calibrations: 5 msec, 50 μV. B, D: Frequency histograms of antidromic latencies following PPT (B, n = 63) and DRN/CG (D, n = 39) stimulation. Arrows indicate mean latencies.

### TABLE 1

<table>
<thead>
<tr>
<th>Site</th>
<th>Number of Cells</th>
<th>Antidromic % (msec)</th>
<th>Antidromic Conduction Velocity (m/sec)</th>
<th>Refractory Antidromic Period (msec)</th>
<th>Antidromic Threshold (mA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPT</td>
<td>63</td>
<td>65% 13.1 ± 7.8</td>
<td>1.1 ± 0.9</td>
<td>2.1 ± 1.3</td>
<td>1.0 ± 1.3</td>
</tr>
<tr>
<td>DRN/CG</td>
<td>39</td>
<td>56% 13.2 ± 7.5</td>
<td>0.9 ± 0.5</td>
<td>1.7 ± 0.8</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>Combined</td>
<td>102</td>
<td>62% 13.2 ± 7.7</td>
<td>1.0 ± 0.8</td>
<td>2.0 ± 1.1</td>
<td>1.0 ± 1.0</td>
</tr>
<tr>
<td>Brainstem</td>
<td>44</td>
<td>66% 9.9 ± 6.4</td>
<td>1.8 ± 2.6</td>
<td>2.1 ± 1.3</td>
<td>0.7 ± 0.5</td>
</tr>
<tr>
<td>Cortex</td>
<td>100</td>
<td>52% 9.9 ± 6.4</td>
<td>1.8 ± 2.6</td>
<td>2.1 ± 1.3</td>
<td>0.7 ± 0.5</td>
</tr>
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*Where applicable, the number indicates mean ± S.D.
†The estimated distances from antidromic stimulation sites to basal forebrain recording sites were all 8.0 mm.
‡Significantly different from cortically projecting neurons [F(199) = 3.28, p < 0.01; n for cortically projecting neurons was 99].
§Significantly different from cortically projecting neurons [Welch’s t was used because of significantly different S.D.; n for cortically projecting neurons was 90].
‖Significantly different from cortically projecting neurons [F(197) = 2.64, p < 0.01; n for cortically projecting neurons was 97].

Abbreviations: PPT, pedunculopontine tegmental nucleus; DRN/CG, dorsal raphe nucleus/central gray.

The neurotransmitter content of brainstem projecting neurons...
preliminary findings indicate that these neurons do not project to perikarya are present in the magnocellular basal forebrain (47), our primarily cholinergic (3). Although somatostatin-containing projecting to the interpeduncular nucleus, which appear to be in the magnocellular basal forebrain is not known, except for those 5.

6.

FIG. 6: A: Reconstruction of an electrode track against the background of ChAT-immunoreactive neurons (large dots), and substance P-immunoreactive neuropil (stippling) to define the ventral pallidum (16,19). The immunostaining was obtained by processing adjacent sections containing the electrode track for the respective antigens, and superimposing the two patterns using the electrode track, dye spot, and histological landmarks as guides. The locations of 9 neurons antidromically driven from the cortex (n = 4) and the PPT (n = 5) are indicated from microdrive readings. Note that cortically projecting neurons are located in or near the cluster of ChAT-positive neurons whereas PPT projecting neurons are located more dorsally, in the ventral pallidum. B: Distributions, on the basis of microdrive readings, of cortically projecting (B, n = 100) and brainstem projecting (C, n = 101) neurons in the basal forebrain approximately at the level of the decussation of the anterior commissure (A). Arrows indicate means of the microdrive readings. Abbreviations: AC, anterior commissure; BS, brainstem; CTX, cortex; PPT, pedunculopontine tegmental nucleus.

in the magnocellular basal forebrain is not known, except for those projecting to the interpeduncular nucleus, which appear to be primarily cholinergic (3,52). Although somatostatin-containing perikarya are present in the magnocellular basal forebrain (47), our preliminary findings indicate that these neurons do not project to the brainstem, when basal forebrain sections containing neurons retrogradely labelled following brainstem injections in one rat were immunohistochemically processed to visualize somatostatin, no double labelled neurons were found except for one large neuron in the lateral hypothalamus (K. Semba and P. B. Reiner, unpublished observations).

To date, axonal collateralization of basal forebrain neurons has been reported to be rare, and the present findings are no exception in this regard. Very few double labelled neurons were seen in the basal forebrain following tracer injections into either midbrain central gray or interpeduncular nucleus, and the cortex (K. Semba, A. Jourdain and P. B. Reiner, unpublished observations). The axons of basal forebrain, presumably cholinergic, neurons do not appear to collateralize to innervate different regions of the neocortex in the rat [(6, 32, 35), but see (23)] and primate (50), although the presence of such collateralization has been documented in the cat (1,7). Rare dual projection has been demonstrated from basal forebrain neurons to the neo- or allocortex and the amygdala (8), or to the hippocampus and the olfactory bulb (30). Only a minority of basal forebrain neurons have been shown to project to both the cortex and the thalamic reticular nucleus (20). These findings suggest that basal forebrain neurons represent hodologically selective, but topographically more or less intermingled cell populations.

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REFERENCES


11. Eckenstein, F.; Thoenen. H. Production of specific antisera and monoclonal antibodies to choline acetyl-transferase: characterization


