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PHYSIOLOGICAL EVIDENCE FOR SUBPOPULATIONS OF CORTICALLY PROJECTING BASAL FOREBRAIN NEURONS IN THE ANESTHETIZED RAT

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Abstract—Sixty-three cortically projecting basal forebrain neurons were identified in chloral hydrate anesthetized rats by antidromic activation from the cerebral cortex. Two subpopulations were noted: type I neurons exhibited two antidromic action potentials of constant latency and identical waveform in response to double pulse cortical stimulation. In contrast, type II neurons exhibited two antidromic action potentials of constant latency but differing waveforms in response to the double pulse paradigm. The phenomenon exhibited by type II cortically projecting basal forebrain neurons is interpreted as evidence for loss of the somatodendritic portion of the antidromic action potential with high frequency stimulation. The median latency to antidromic activation of type II neurons (13.5 ms) was significantly longer than that of type I neurons (3.9 ms). Spontaneous firing rates varied over a wide range (0–49 Hz), and there was no significant difference between the rates of type I and type II neurons.

These data underscore the physiological heterogeneity of this presumptive cholinergic cortical afferent system. Anatomical studies have shown that most, but possibly not all cortically projecting basal forebrain neurons are cholinergic.[33,43,55,59,60] The relative proportions of type I (87%) and type II (13%) neurons encountered in this study suggest that type I neurons might be cholinergic and type II neurons non-cholinergic. If substantiated, this hypothesis would permit cholinergic and non-cholinergic cortically projecting basal forebrain neurons to be distinguished using a simple test of antidromicity.

The cholinergic neurons of the basal forebrain[2,21,24,45] provide a major extrinsic cholinergic input to the cerebral cortex.[17,54,56] At present, relatively little is known of the physiological characteristics of these neurons. Several groups have recorded unidentified basal forebrain neurons *in vivo* in unanesthetized rats,[34] cats,[15,47,52] and primates.[1,12–14,41] Observations on basal forebrain neurons antidromically activated from the cerebral cortex have been reported in anesthetized rats[4–6,27] and cats,[29] and unanesthetized cats.[51] More recently, several groups have studied the physiology of basal forebrain neurons *in vitro*, using brain slices[19,53] as well as cultured basal forebrain neurons[35,50] followed by histochemical demonstration of acetylcholinesterase in the recorded neurons.

As a first step towards physiological characterization of basal forebrain cholinergic neurons, the properties of cortically projecting basal forebrain (CPBF) neurons were studied in the anesthetized rat. Some of these data have been presented in preliminary form.[39]

EXPERIMENTAL PROCEDURES

The subjects were adult male Wistar rats weighing between 250 and 300 g, anesthetized with chloral hydrate (400 mg/kg, i.p.) and placed in a stereotaxic apparatus with the incisor bar set 3.3 mm below the interaural line.[36] Animals were maintained in a deep plane of anesthesia (lack of flexion response to pinch of hind paw) by supplemental doses of chloral hydrate delivered through an i.v. catheter in the left jugular vein. A rectal probe connected to a heating pad kept core temperature at 37°C.

Bipolar stainless steel stimulating electrodes with tip separations of 0.75–1.0 mm were implanted in frontal (AP +2.5 mm, ML ±2.5 mm, all coordinates are given in relation to bregma), rostral cingulate (AP +2.0 mm, ML ±0.5 mm) and caudal cingulate (AP −4.0, ML ±0.5 mm) cortices. For frontal cortex stimulation, the electrodes were lowered 1 mm below the cortical surface, while rostral and caudal cingulate electrodes were lowered 1.5 mm below the cortical surface. Electrodes were cemented into place, and connected to the output of a stimulus isolation unit driven by a stimulator to provide pulses of 0.2–4.3 mA intensity and 0.2–0.5 ms duration. Square wave pulses were delivered continuously at 1.7 Hz to one of the three stimulating electrodes while advancing the microelectrode through the basal forebrain and searching for neurons activated by cortical stimuli.

Microelectrodes made of 0.5 mm (i.d.) microfilament glass tubing were freshly pulled and filled with either: (1) 4% horseradish peroxidase in 0.05 M Tris and 0.2–0.5 M KCl, or (2) 2 M NaCl saturated with Pontamine Sky Blue. Direct current resistance of horseradish peroxidase filled electrodes ranged between 40 and 80 MΩ, while those filled with NaCl were between 2 and 10 MΩ. Horseradish peroxidase filled electrodes were connected via chlorided silver wire to an intracellular amplifier whose unfiltered output was simultaneously displayed on the oscilloscope as a low gain direct current coupled signal, and a higher gain alternating current coupled trace. NaCl filled electrodes were connected to an extracellular amplifier whose output was filtered (300–10,000 Hz) and displayed on the oscilloscope.

The basal forebrain was systematically explored by lowering the electrode from dorsal to ventral using a hydraulic microdrive and searching for cells which could be antidrom-

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Abbreviations: CPBF neuron, cortically projecting basal forebrain neuron.

ically activated from the ipsilateral cortex. Coordinates used were: AP = +0.3 to −1.8 mm; ML = ±1.0 to 2.5 mm. One to five penetrations were made per animal. CPBF neurons were operationally defined by the antidromic criteria of constant latency, high frequency following (> 100 Hz) and, for spontaneously active neurons, collision.[28]

Once a CPBF neuron was encountered, data on its threshold and latency to antidromic activation were obtained directly from the oscilloscope. Spontaneous firing rates were computed from a 60 s sample. When horseradish peroxidase filled electrodes were used, extracellular characterization was followed by penetration of the cell (using alternating current "ringing"); horseradish peroxidase was deposited intracellularly by passage of 7 nA of positive current for 60–120 s with a 50% duty cycle. When extracellular electrodes were used, the end of a track was marked by passing 6 μA negative current through the electrode for 5 min, depositing Pontamine Sky Blue into the extracellular space.

At the end of the experiment, the animals were heparinized, given an overdose of anesthetic, and perfused transcardially with phosphate-buffered saline (0.1 M, pH 7.6) followed by 3% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate-buffered saline. Brains were removed, postfixed for 2 h in the fixative, blocked, and placed in 30% sucrose overnight. Pontamine Sky Blue spots were localized in 50 μm thick frozen sections counterstained with Neutral Red, while horseradish peroxidase filled neurons were visualized according to the protocol described in the companion paper.[49] The locations of CPBF neurons were reconstructed and plotted on standardized coronal sections of the rat brain.[36]

RESULTS

The data were derived from recordings of 975 basal forebrain neurons in 58 rats. Of these, 63 neurons satisfied the criteria of constant latency and high frequency followed to cortical stimulation and were operationally defined as CPBF neurons: 30 spontaneously active neurons satisfied the additional criterion of collision (Fig. 1). For seven spontaneously active neurons, spike heights were insufficient to permit collision testing and therefore only the first two criteria were satisfied. Twenty-six neurons were activated from frontal, 24 from rostral cingulate and 13 from caudal cingulate cortices. In two instances, a CPBF neuron was antidromically activated from both frontal and rostral cingulate cortices with different thresholds; no CPBF neuron was encountered which was antidromically activated from both the caudal cingulate electrode and either of the more rostrally placed cortical stimulating electrodes. Thirty-nine CPBF neurons were recorded with horseradish peroxidase filled electrodes and 24 with NaCl filled micropipettes.

Waveform characteristics

Extracellular action potentials recorded from CPBF neurons were generally small, whether recorded with NaCl or horseradish peroxidase filled electrodes. This made it difficult to make detailed study of such waveform characteristics as action potential duration and waveform morphology. However, when double pulses of high frequency stimuli were applied to the cerebral cortex, 8/63 (13%) CPBF neurons responded

Fig. 1. Tests of antidromicity demonstrated in a type I CPBF neuron. (A) Stimulation of rostral cingulate cortex (1.5 mA at arrowhead) 4.5 ms following spontaneous action potential (open arrow) results in an antidromic action potential with constant latency of 3.9 ms (superimposition of 5 sweeps). (B) Same stimulation parameters as in (A) delivered 3.5 ms followed spontaneous action potential. Absence of antidromic action potential is presumptive evidence of collision between orthodromic and antidromic action potentials. (C) High frequency double pulse stimulation at 200 Hz (arrowheads) produces two action potentials with constant latency and identical waveforms. Filtered AC recordings. Calibration: 2 ms, 50 μV.

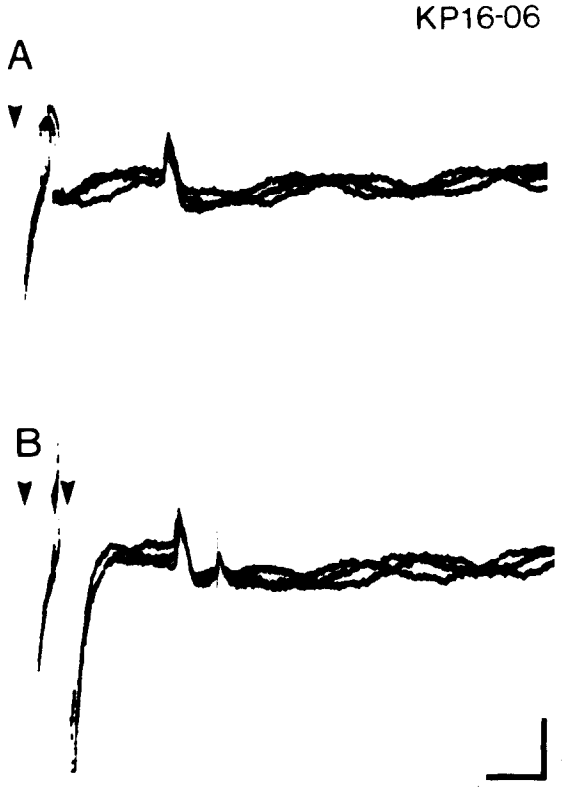

Fig. 2. Tests of antidromicity demonstrated in a type II CPBF neuron. (A) Stimulation of rostral cingulate cortex (1.0 mA at arrowhead) results in an antidromic action potential at constant latency of 14.2 ms (superimposition of 3 sweeps). (B) High frequency double pulse stimulation at 240 Hz (arrowheads) produces two action potentials with constant latency but different waveforms. The second action potential probably represents an antidromically activated initial segment spike alone, suggesting loss of the somato-dendritic segment of the antidromic action potential with high frequency stimulation. (This cell corresponds to cell 2 of the companion paper[49] and its morphology is shown in Fig. 2 of that paper.) Filtered recordings. Calibration: 2 ms 50 μV.

with two antidromic action potentials exhibiting constant latency but distinctly different waveforms (Fig. 2). In every instance, the second action potential was much smaller than the first. Although systematic study of the critical interstimulus interval required for diminution of the second spike was not attempted, whenever double pulse stimuli of 10 ms or less were applied this phenomenon was seen. In the remainder of the population (55/63 or 87%), the antidromic response to the double pulse paradigm consisted of two action potentials both of which displayed constant latency and apparently identical waveforms, even at interstimulus intervals approaching the refractory period. For ease of discussion, we have arbitrarily classified CPBF neurons exhibiting no change in action potential morphology with high frequency stimuli as type I CPBF neurons, and those displaying differing waveshapes in paired pulse trials as type II CPBF neurons.

Latency

Latency to antidromic activation of individual CPBF neurons varied from 0.78 to 34.0 ms (Fig. 3). Assuming average conduction pathways of 8 mm length,[16,25] conduction velocities varied between 0.2 and 10.2 m/s. Although latencies to antidromic activation from caudal cingulate cortex tended to be greater than either frontal or rostral cingulate cortices, this difference was not statistically significant (Kruskal–Wallis test).

The median latency to antidromic activation for all CPBF neurons was 5.9 ms. The median latency for type I CPBF neurons was 3.9 ms (range 0.78–34 ms), while that of type II CPBF neurons was 13.5 ms (range 12–29 ms), and this difference was statistically significant ($P < 0.005$, Mann–Whitney U-test, Fig. 3). No type II CPBF neuron exhibited a latency of less than 12 ms, while 80% of type I neurons had latencies shorter than 12 ms.

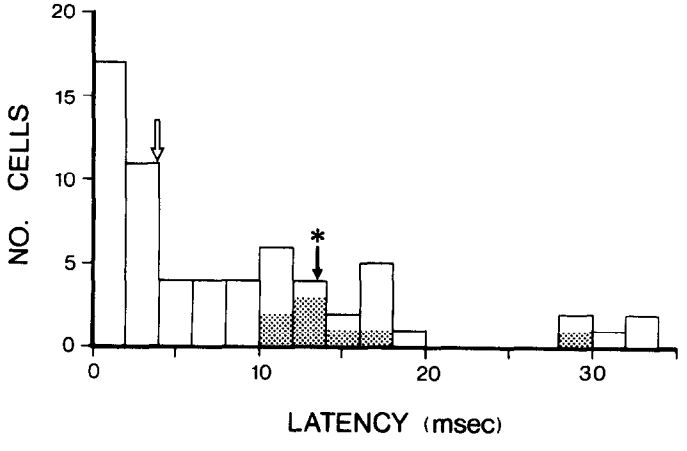

Fig. 3. Distribution of latencies to antidromic invasion of 63 CPBF neurons. The unshaded areas of the histogram represent type I CPBF neurons whereas the hatched areas represent type II CPBF neurons. The median latency for type I CPBF neurons (open arrow) differed significantly from that of type II CPBF neurons (filled arrow) (Mann–Whitney U-test, $P < 0.005$).

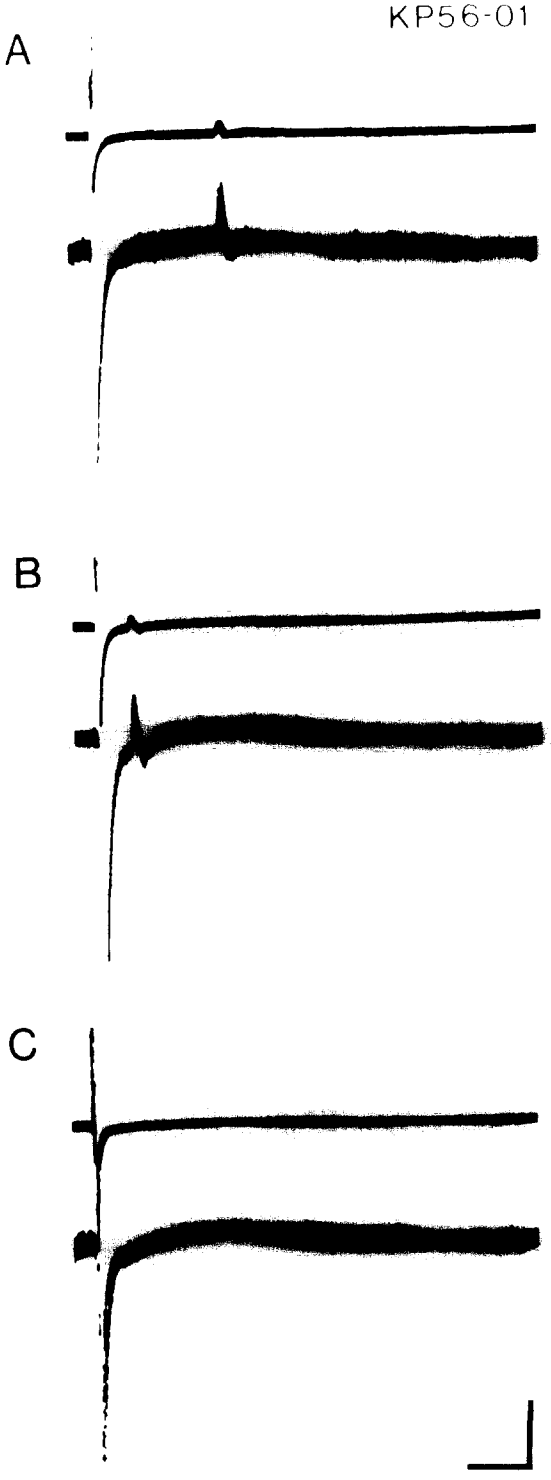

Fig. 4. Multiple discrete antidromic latencies. (A) Stimulation of frontal cortex (2.9 mA) results in an antidromic action potential with latency of 11.5 ms. (B) Increasing stimulation intensity to 4.3 mA produces an antidromic action potential with latency of 2.6 ms. (C) Collision of antidromic action potential in (B) by stimulation 2.4 ms following spontaneous action potential. (This cell corresponds to cell 4 of the companion paper[49] and its morphology is shown in Fig. 4 of that paper.) Calibration: 5 ms; upper traces (unfiltered DC recordings): 10 mV; lower traces (unfiltered AC recordings): 2 mV.

Multiple discrete antidromic latencies with different stimulation intensities were seen in 9/63 (14%) CPBF neurons (Fig. 4). In every case, gradually increasing stimulation intensity above threshold revealed a second threshold at which the antidromic latency "jumped" to a shorter latency. All 9 neurons displaying multiple discrete antidromic latencies were type I CPBF neurons. There was no significant difference between either the longest or shortest latencies of neurons exhibiting multiple discrete antidromic latencies and the latencies of the remainder of the population (Mann–Whitney U-test.).

Spontaneous activity

Spontaneous firing rates of CPBF neurons ranged between 0 and 49 Hz, with a median of 0 Hz and a mean ($\pm$SEM) of 5.8 ± 1.6 ($n = 46$) (Fig. 5); 26 (41%) CPBF neurons were silent. The firing rates of type I CPBF neurons were not significantly different from those of type II CPBF neurons.

Anatomical location of cortically projecting basal forebrain neurons

The locations of 57 CPBF neurons are shown in Fig. 6; the locations of 6 type I neurons could not be unambiguously determined. CPBF neurons were located within several nuclei of the basal forebrain, including the horizontal limb of the diagonal band, the magnocellular preoptic area, the ventral pallidum, and the globus pallidus (Fig. 6). The distribution of type II CPBF neurons overlapped those of type I, with the exception of the globus pallidus and its borders where no type II CPBF neurons were found. Neither firing rate nor antidromic latency varied between CPBF neurons in different areas of the basal forebrain. There was no obvious correlation between cortical stimulation site and location of CPBF neurons.

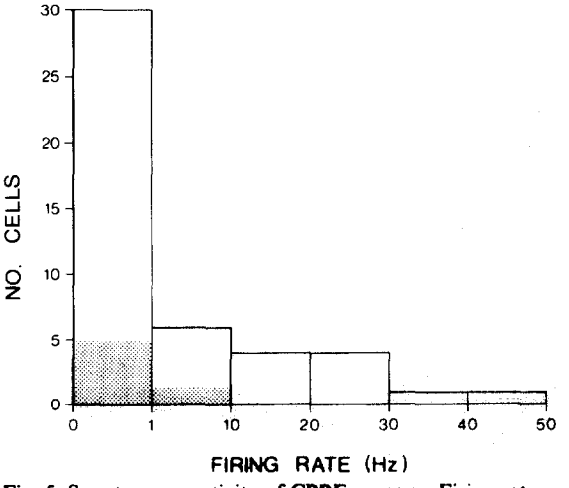

Fig. 5. Spontaneous activity of CPBF neurons. Firing rates of CPBF neurons ranged from 0 to 49 Hz. Of the 30 cells in the category between 0 and 1 Hz, 26 were silent. The hatching represents the firing rates of type II CPBF neurons, while the non-hatched regions represent the rates of type I CPBF neurons.

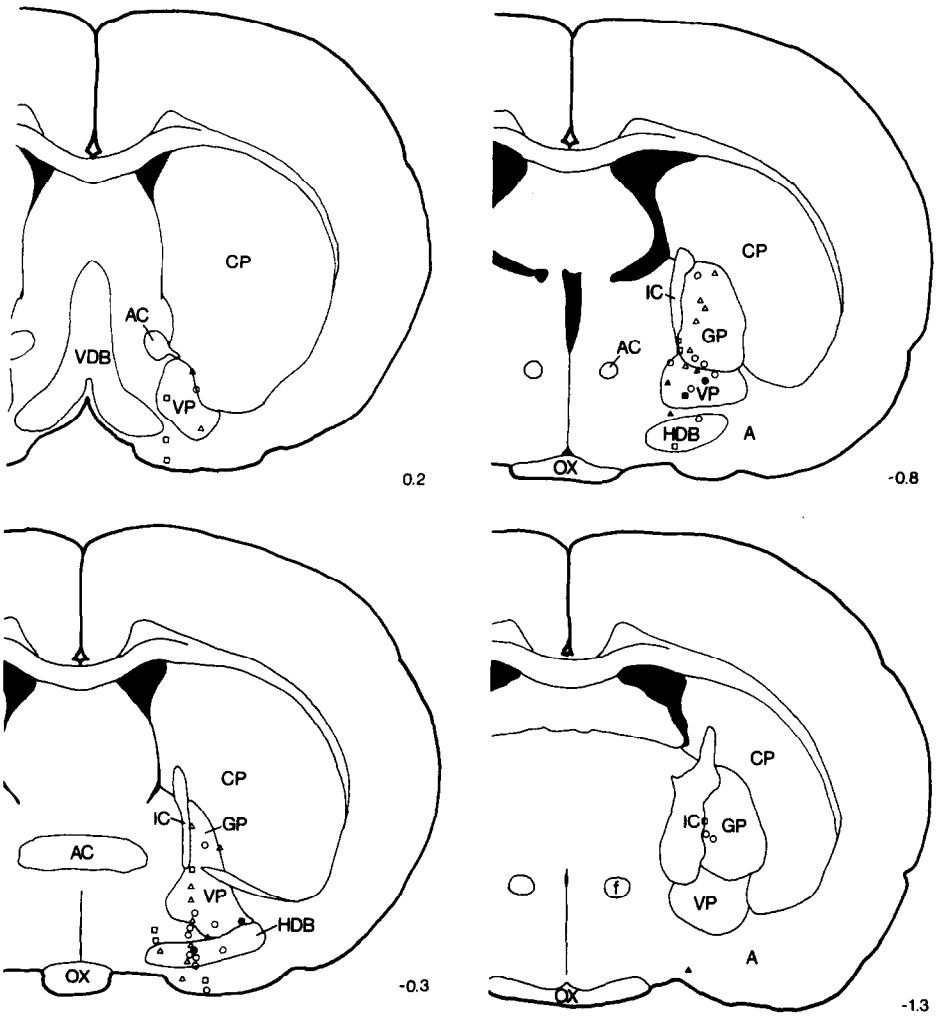

Fig. 6. Anatomical locations of CPBF neurons. The locations of CPBF neurons as reconstructed on standard coronal sections of rat brain (after Paxinos and Watson[36]). Rostro-caudal level (in mm) in relation to bregma is indicated in lower right. Open and closed symbols represent type I and type II CPBF neurons, respectively. Circles, triangles and squares represent neurons activated by frontal, rostral cingulate, and caudal cingulate cortical stimulation, respectively. Abbreviations: A, amygdala; AC, anterior commissure; CP, caudate putamen; f, fornix; GP, globus pallidus; HDB, horizontal limb of the diagonal band; IC, internal capsule; OX, optic chiasm; VDB, vertical limb of the diagonal band; VP, ventral pallidum.

DISCUSSION

Criteria for distinguishing type I and type II cortically projecting basal forebrain neurons

Type I and type II neurons were defined on the basis of their responses to high frequency (> 100 Hz) paired pulse cortical stimulation. The change in spike size of type II neurons following paired pulse antidromic stimulation probably represents loss of the somatodendritic portion of the antidromic action potential in the second spike of the pair, with the smaller spike representing the initial segment portion of the antidromic potential. Similar responses to paired pulses of antidromic stimuli have been reported in several other neuronal systems including motoneurons,[10,11] dopaminergic,[18,20] noradrenergic,[3,9,42,48] and serotonergic[46,58] neurons. These data have been interpreted as suggesting that there is a low "safety factor" for invasion of the somatodendritic portion of the neuron by the antidromically generated initial segment spike.[40]

In silent neurons, it can be difficult to determine if the antidromic action potential consists of both initial segment and somatodendritic action potentials, or initial segment spikes alone. Therefore, it is possible that some silent type I CPBF neurons displayed antidromic initial segment spikes only. Additionally, some CPBF neurons had spike amplitudes too low to be certain that the second spike was always a full amplitude action potential. Thus neurons were

classified as type II only when unambiguous and consistent changes in the amplitude of the second spike was seen; all other neurons were classified as type I.

Latency to antidromic activation

Heterogeneity of antidromic latency of CPBF neurons has been reported previously,[4-6,27] and our results confirm these findings with respect to the population as a whole. However, this heterogeneity was accounted for largely by the latencies of type I neurons, as type II neurons had relatively homogeneous latencies which were significantly longer than those of type I neurons. Although the latency of any given CPBF neuron could not be used to determine unambiguously to which subgroup it belonged, the overall differences between the groups support the hypothesis that at least type II neurons represent a physiologically distinct subpopulation of CPBF neurons.

These latency data also confirm the observation that CPBF neurons exhibit multiple discrete antidromic latencies.[4-6,27] It is unlikely that the small population of CPBF neurons in which this phenomenon was seen in the present study using fixed stimulating electrodes represents a distinct subpopulation of CPBF neurons for the following reasons: (1) neither latencies nor spontaneous firing rates of these neurons were significantly different from the remainder of the population; (2) using movable cortical stimulating electrodes, Aston-Jones *et al.*[4,5] have shown that virtually all CPBF neurons exhibit this phenomenon as the stimulating electrode is advanced through the cortex.

Spontaneous activity

CPBF neurons also displayed marked heterogeneity with respect to spontaneous firing rate in anesthetized rats, but firing rates were not different between type I and type II CPBF neurons. The distribution of firing rates of CPBF neurons encountered in the present study is consistent with the data of Aston-Jones *et al.*,[5,6] but at variance with that of Lamour *et al.*,[27] who reported a firing rate of 20 ± 1.6 Hz. Two observations may account for this difference. Lamour *et al.*[27] used urethan as anesthetic, whereas in this study and that of Aston-Jones *et al.*,[5,6] chloral hydrate was employed. Second, the level of anesthesia was apparently different in the present study as compared to Lamour *et al.*[27] They report that: "the level of anesthesia used in the present experiments was not deep enough to prevent arousal reactions during the noxious stimulation." Although they did not define arousal reactions precisely, in the present experiments animals were always anesthetized deeply enough to prevent flexion withdrawal to pinch of the hind paw. Furthermore, on several occasions a CPBF neuron was recorded while a supplemental dose of anesthetic was administered, and the result was generally a diminution of firing rate, as had been previously reported.[6] Taken together, these observations suggest that the spontaneous activity of CPBF neurons is particularly sensitive to level of anesthesia and/or the particularly anesthetic agent used.

Topographic relations between cortically projecting basal forebrain neurons and their cortical targets

Anatomical studies have shown that CPBF neurons project to the cerebral cortex in topographic fashion.[22,23,26,30-32,33,37,44] This topography could not be confirmed in the present study. However, the current intensities required for antidromic activation could be expected to activate relatively large areas of the cerebral cortex as well as fibers of passage in the subcortical white matter.

The degree of intracerebral axonal collateralization of CPBF neurons remains controversial, with some antomical studies demonstrating relatively widespread projections of individual CPBF neurons,[8,32] while others report restricted terminal fields.[7,37,57] Although we encountered two CPBF neurons which could be antidromically activated from different cortical stimulation sites, in both cases the stimulation sites were within 2 mm of each other. These data cannot be construed as evidence for collateralization of CPBF neurons as the precise extent of current spread[38] is unknown.

CONCLUSIONS

The major finding of the present study is that there exist at least two physiologically distinct subpopulations of CPBF neurons which can be readily distinguished on the basis of their responses to paired pulse stimulation of their cortical targets. With respect to the transmitter employed by these CPBF neurons, two hypotheses emerge. One of these would suggest that the observed differences between type I and type II CPBF neurons underscore the physiological heterogeneity of the basal forebrain cholinergic cortical afferent system. An alternative hypothesis derives from anatomical data which suggest that most, but possibly not all CPBF neurons are cholinergic.[33,43,55,59,60] Thus type I CPBF neurons may be cholinergic while type II CPBF neurons may be non-cholinergic. If substantiated, the latter hypothesis would permit cholinergic and non-cholinergic CPBF neurons to be distinguished using a simple test of antidromicity.

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