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Evidence for a cholinergic projection to neocortex from neurons in basal forebrain

(acetylcholine/basal nucleus/kainic acid/cortical afferents)

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ABSTRACT **Unilateral stereotaxic injection of 3.5 nmol of kainic acid into the ventral globus pallidus of rats reduced biochemical cholinergic neuronal markers by 45–50% and virtually eliminated histochemical staining for acetylcholinesterase in neocortex ipsilateral to the lesion. At the lesion site, the large, multipolar neurons that stain densely for acetylcholinesterase were absent when compared with the uninjected side. Kainate was as effective as electrocoagulation for reducing cholinergic markers although it did not affect aminergic projections ascending through the lesioned area. The conclusion that the cholinergic projection originated in neuronal perikarya at the lesion site was supported by the failure of kainate or electrolytic lesions in contiguous regions to produce similar effects. These studies provide strong evidence for a cholinergic projection to neocortex from neurons in the forebrain in the nucleus basalis.**

The mammalian cerebral cortex contains the neurotransmitter acetylcholine (AcCho) and moderately high levels of activity of its biosynthetic enzyme choline acetyltransferase (acetyl-CoA:choline *O*-acetyltransferase; EC 2.3.1.6) and its catabolic enzyme acetylcholinesterase (AcCho-esterase; acetylcholine acetylhydrolase; EC 3.1.1.7) (1, 2). AcCho and drugs that alter cholinergic neurotransmission influence cortical electrical activity, and under physiologic conditions AcCho is released from the surface of the neocortex (3). Neocortical neurons, especially in the deeper layers, are strongly excited by the iontophoretic application of AcCho (4); and muscarinic cholinergic receptors have been characterized in neocortex by neurophysiologic and neurochemical methods (5–7). Ligand binding studies with α-bungarotoxin have also demonstrated the presence of nicotinic receptors in cortex (8, 9). These findings suggest that the neocortex receives a substantial cholinergic innervation.

Previous studies on the possible source of cortical cholinergic innervation have, however, been inconclusive. Undercutting of the cerebral cortex to lesion cortical afferents results in a marked reduction in the activity of choline acetyltransferase, suggesting a subcortical source of cholinergic innervation (10); however, interpretation of the effects of this local lesion is confounded by the possible direct or retrograde damage to cortical intrinsic cholinergic neurons. Shute and Lewis, in a series of elegant experiments, used histochemical techniques to stain for AcCho-esterase activity in neuronal fibers and concluded that the neocortical cholinergic innervation arose from perikarya situated in the brain stem and diencephalon (11); however, the recent demonstration that many noncholinergic neurons are also cholinesterase-positive has undermined the specificity of their technique (12–14). In contrast, McGeer *et al.* (15), utilizing antiserum against choline acetyltransferase to stain for neurons containing this enzyme, which presumably are cholinergic, reported that choline acetyltransferase was confined to cortical intrinsic neurons; however, the specificity of their immunocytochemical technique has been questioned (16).

Because of the lack of specific histologic methods, mapping of central cholinergic pathways (for example, the septo-hippocampal projection) has depended primarily upon the assessment of the effects of focal lesions on the biochemical markers for cholinergic neurons in a proposed terminal field (17–20). These markers include the neurotransmitter itself, AcCho, its biosynthetic enzyme choline acetyltransferase, and the sodium-dependent high-affinity transport process for choline in nerve terminal (synaptosomal) preparations. Recent neurochemical studies of adult rat cortex markedly depleted of intrinsic neurons by fetal treatment with methylazoxymethanol suggested that a major component of cholinergic innervation to neocortex is derived from subcortical structures spared by the toxin (21). In the present study, we have examined the effects of discrete lesions made by electrocoagulation or direct injection of kainic acid in subcortical regions on the levels of the three presynaptic markers for the cholinergic terminals in the neocortex. We have demonstrated that a major cholinergic projection to the rat neocortex originates from cell bodies within and beneath the medial globus pallidus (GP) in the nucleus basalis.

MATERIALS AND METHODS

Placement of Lesions and Histology. Male Sprague–Dawley rats (160–190 g) were anesthetized with Equithesin (Jensen Salsbury Labs; Kansas City, MO; 0.6 ml, intraperitoneally) and placed in a David Kopf small animal stereotaxic apparatus. Unilateral high-frequency electrolytic lesions were made with an insulated electrode, which was bare 1 mm from the tip, with 10 mA delivered for 10–30 sec by a Grass LM4 lesion maker. The coordinates for electrolytic pallidal lesions are axial, 6.1 mm; lateral, 2.7 mm; ventral, 6.4 mm with the interauricular line, dura, and midline as 0. For kainic acid lesions, a 0.3-mm Hamilton cannula was lowered into the GP at coordinates: axial, 6.0 mm; lateral, 2.8 mm; ventral, 6.4 mm; kainic acid (Lot 47C-0074, Sigma), dissolved in artificial cerebrospinal fluid solution titrated to pH 7 at a concentration of 3.5 nmol/μl, was infused in a volume of 1 μl for 30 sec. Electrolytic lesions and kainic acid injections of 1–2 μl were placed in other subcortical regions described in *Results*. The electrode or cannula was then carefully withdrawn, and the scalp was apposed with sutures.

From 7 to 14 days after placement of the lesions, the rats were decapitated and cortex was carefully dissected away at 5°C.

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Abbreviations: AcCho, acetylcholine; GABA, γ-aminobutyric acid; GP, globus pallidus.

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The remaining forebrain was placed in cold buffered 10% (vol/vol) formalin. The sites of lesions were established by using 40-μm frozen sections stained for Nissl substance.

Two weeks after lesioning the GP with kainic acid, several rats were deeply anesthetized with pentobarbital and perfused with 20 ml of cold 0.9% saline followed by 100 ml of 10% buffered formalin. After additional fixation for 18 hr and immersion in 30% sucrose for 36 hr, 40-μm coronal frozen sections were prepared from forebrain and stained for AcCho-esterase by using the direct thiocholine method of Karnovsky and Roots (22) as described by Butcher *et al.* (23). Ethopropazine HCl (0.1 mM; Smith Kline and French Labs) was included in the incubation medium to inhibit cholinesterase (EC 3.1.1.8). As controls, adjacent sections were incubated in the same medium containing 0.1 mM BW 284C51 (Wellcome Research Labs; Beckenham, UK), a highly specific inhibitor of AcCho-esterase. Adjacent sections were stained with cresyl violet.

Preparation of Tissue and Biochemical Assays. Right and left cortical slabs were quickly dissected so that cingulate and occipital cortex and the cortex below the rhinal sulcus was discarded; thus, fronto-parietal sections containing areas 1–4, 6–8, and 40 of Krieg (24) were analyzed in these experiments. Biochemical markers for cholinergic, γ-aminobutyric acid (GABA)ergic, noradrenergic, serotonergic, and histaminergic neurons were measured in cortical slabs ipsilateral and contralateral to the lesion and in cortical slabs from unlesioned rats. For assays of choline acetyltransferase, glutamate decarboxylase, and tyrosine hydroxylase, tissue was homogenized in 20 vol of ice-cold 50 mM Tris·HCl, pH 7.4, containing 0.2% (vol/vol) Triton X-100. The activity of choline acetyltransferase was measured by the method of Bull and Oderfeld-Nowak (25); glutamate decarboxylase (L-glutamate 1-carboxy-lyase; EC 4.1.1.15) activity was measured by a modification of the method of Wilson *et al.* (26) with [1-^{14}C]-DL-glutamic acid as substrate. Tyrosine hydroxylase [L-tyrosine, tetrahydropteridine: O_2 oxidoreductase (3-hydroxylating); EC 1.14.16.2] activity was measured as described by Coyle (27) with the exception that DL-methyl-5,6,7,8-tetrahydropterine was used as the cofactor. Histidine decarboxylase activity (L-histidine carboxy-lyase; EC 4.1.1.22) was determined by the method of Ben-Ari *et al.* (28).

Synaptosomal uptake of L-[^{3}H]norepinephrine, [^{3}H]GABA, and [^{3}H]choline was determined in washed P_2 fractions as described (29, 30). Uptake of L-[^{3}H]norepinephrine (13 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) (New England Nuclear) was performed for 4 min at 37°C in the resuspended P_2 fraction with the equivalent of 5 mg of tissue and a substrate concentration of 0.05 μM in Krebs–Ringer phosphate buffer containing 1 μM nialamide and 0.01% (wt/vol) ascorbic acid. To determine nonspecific uptake, desmethylimipramine was added to the incubation medium to the final concentration of 1 μM. Uptake of [^{3}H]GABA (34 Ci/mmol; New England Nuclear) was measured in the resuspended P_2 fraction with the equivalent of 1.0 mg of tissue and 1 μM substrate for 2 min at 37°C. Nonspecific uptake was determined in sodium-free isotonic buffer. Uptake of [^{3}H]choline (6.4 Ci/mmol; Amersham) was determined in resuspended P_2 fraction with the equivalent of 5 mg of tissue and 0.05 μM substrate for 4 min at 37°C. Uptake in the presence of buffer containing 0.32 M sucrose substituted for NaCl was used to determine nonspecific transport.

Norepinephrine was measured by the radiometric enzymatic assay of Coyle and Henry (31). GABA was measured by the enzymatic fluorometric assay of Graham and Aprison (32), and acetylcholine was assayed by the radiometric enzymatic method of Goldberg and McCaman (33). Serotonin was assayed according to the procedure of Curzon and Green (34). Protein was determined by the method of Lowry *et al.* (35).

RESULTS

Effects of Subcortical Electrolytic Lesions. Large unilateral electrolytic lesions were placed in various subcortical regions thought to send projections to the neocortex. Seven days after lesion, ipsilateral, contralateral, and control cortices were assayed for choline acetyltransferase (a marker for cholinergic processes) and for glutamate decarboxylase (a marker for GABAergic neurons known to be intrinsic to the cerebral cortex) (36). Lesions that involved the capsule, the dorsal GP, the thalamus, the hypothalamus, and the zona incerta did not significantly alter the activities of choline acetyltransferase or glutamate decarboxylase in the ipsilateral or contralateral cortices (Table 1). However, electrolytic lesions that involved the ventral GP produced a consistent 45% reduction in the activity of choline acetyltransferase in the ipsilateral neocortex; notably, the activity of glutamate decarboxylase in the same cortical specimens was unaffected. These findings suggested that the nearly half the cholinergic innervation to frontal and parietal cortex was localized in neurons whose fibers traversed, or whose cell bodies were located in, the ventral GP.

Effects of Focal Kainic Acid Lesions. To determine whether the pathway originated in the pallidal region, several cholinergic and GABAergic neuronal markers were measured in the neocortex after direct injection of 3.5 nmol of kainic acid into the ventral GP. Kainic acid is a potent neurotoxin that selectively kills neurons with cell bodies near the injection site but generally spares axon terminals and axons of passage coming from distant neurons (37). Kainic acid lesions at the lower border of the caudal half of the GP produced neuronal loss within a radius of approximately 0.7 mm (Fig. 1) and reduced the activity of choline acetyltransferase, the level of endogenous AcCho, and the activity of the synaptosomal high-affinity uptake process for [^{3}H]choline by approximately 50% in the ipsilateral neocortex (Table 2). In contrast, three presynaptic markers for the GABAergic neurons intrinsic to the neocortex, L-glutamic acid decarboxylase, GABA, and synaptosomal uptake of [^{3}H]GABA were unaffected in the same cortical specimens.

The kainic acid lesion occurred in close proximity to the medial forebrain bundle, a major fascicle containing axons of

Table 1. Effects of unilateral subcortical electrolytic lesions on neurotransmitter enzymes in rat neocortex

Lesion area		Levels in neocortex: Choline acetyltransferase	Levels in neocortex: Glutamate decarboxylase
Ventral GP			
	Ipsilat	18.7 ± 1*	15.7 ± 1.3
	Contralat	34.0 ± 1	15.1 ± 1.4
	% change	−45	+4
Dorsal GP			
	Ipsilat	35.2 ± 2	15.4 ± 1.2
	Contralat	37.0 ± 2	15.6 ± 1.4
	% change	−5	−1
Thalamus or hypothalamus	Ipsilat	35.4 ± 1	ND
	Contralat	35.0 ± 1	
	% change	+1	

Unilateral electrolytic lesions were placed in rats held in a stereotaxic apparatus; location of the lesions is shown in Fig. 1. Seven days after lesioning, enzyme activity was measured in fronto-parietal cortex from both sides. Each value is the mean ± SEM of at least five separate preparations assayed in duplicate; results are expressed in nmol·mg of protein^{-1}·hr^{-1}. ND, not done. Ipsilat and contralat, ipsilateral and contralateral cortices, respectively.

* $P < 0.001$ ipsilateral vs. contralateral cortex (Student's t test).

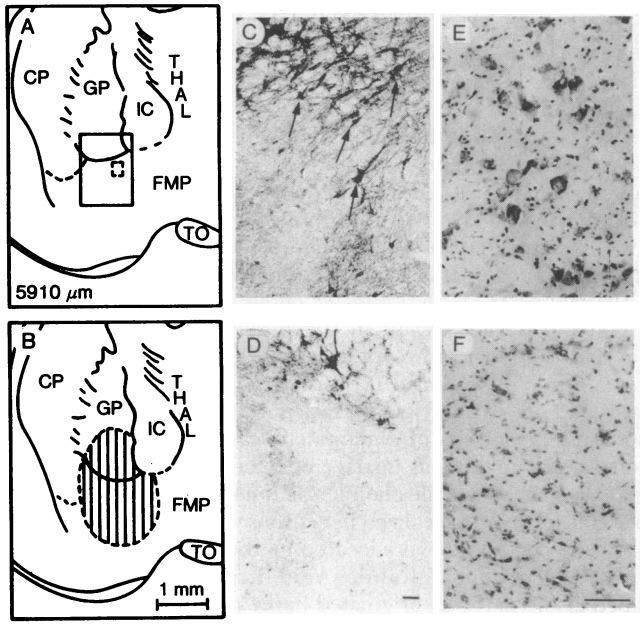

FIG. 1. Histological appearance of kainic acid lesion that was associated with a 45–50% fall in ipsilateral cholinergic markers. (*A* and *B*) Frontal sections (after ref. 38) with extent of typical lesion indicated by hatching in *B*. The box enclosed by the solid line in *A* indicates region of photomicrographs of tissue prepared to visualize AcCho-esterase (22, 23) shown in *C* (uninjected side) and *D* (injected side). AcCho-esterase-stained perikarya (some indicated by arrows) are seen in medial pallidal area and in substantia innominata in *C* but are absent on the injected side (*D*). *E* (control side) and *F* (lesion site) show higher power photomicrographs of Nissl-stained tissue at areas shown by broken box in *A*. Large neurons seen on the uninjected side were absent at the lesion site and mild glial reaction is present. Bars in *D* and *F* = 100 μm. THAL, thalamus; IC, ipsilateral cortex; FMP, forebrain medial perikarya; CP, caudate-putamen; TO, optic tract.

Table 2. Effects of unilateral kainic acid injections into ventral GP on neuronal biochemical markers in neocortex

Marker	Levels in neocortex: Ipsilateral	Levels in neocortex: Contralateral	% change
Choline acetyltransferase, nmol · hr^{-1} · mg $prot^{-1}$	20.8 ± 1.3*	42.0 ± 1	−50
Acetylcholine, pmol/mg	9.4 ± 0.9*	17.0 ± 0.9	−45
Choline uptake, fmol · mg^{-1} · (4 min)	54 ± 5*	98 ± 6	−45
Glutamate decarboxylase, nmol · hr^{-1} · mg $prot^{-1}$	11.6 ± 0.6	11.3 ± 0.7	+3
GABA, nmol/mg	1.4 ± 0.1	1.4 ± 0.1	0
GABA uptake, pmol · mg^{-1} · (2 min)	10.9 ± 1	10.0 ± 1	+9
Tyrosine hydroxylase, pmol · hr^{-1} · mg $prot^{-1}$	45.6 ± 4	44.2 ± 4	+3
Norepinephrine, pg/mg	575 ± 63	523 ± 43	+10
L-Norepinephrine uptake, fmol · mg^{-1} · (4 min)	60 ± 5	69 ± 6	−13
Histidine decarboxylase, dpm/μg prot	2.65 ± 0.18	2.94 ± 0.11	−10
Serotonin, μg/g	0.52 ± 0.04	0.54 ± 0.02	−4

Unilateral injections of kainic acid (3.5 nmol in 1 μl) were made into the ventral posterior GP of rats held in a stereotaxic apparatus; location of the lesions is shown in Fig. 1. Seven to 10 days after the lesion, markers for cholinergic neurons (choline acetyltransferase, AcCho, [^{3}H]choline uptake), GABAergic neurons (glutamate decarboxylase, GABA, [^{3}H]GABA uptake), noradrenergic axons (tyrosine hydroxylase, norepinephrine, [^{3}H]norepinephrine uptake), histaminergic axons (histidine decarboxylase), and serotonergic axons (serotonin) were compared in ipsilateral and contralateral neocortex. Noncholinergic markers are reported from groups of rats in which ipsilateral choline acetyltransferase was reduced by 50%. N = 6–24 in each group. Values are mean ± SEM. prot, Protein; 1 dpm = 16.7 mBq.

* $P < 0.001$, ipsilateral vs. contralateral (Student's t test).

subcortical serotonergic, noradrenergic, and histaminergic neurons projecting to the neocortex. To rule out the possibility that the kainic acid lesion might damage this pathway, biochemical markers for these neurons were assayed in the ipsilateral, contralateral, and control cortex after similarly placed lesions. The markers for the noradrenergic cortical terminals (tyrosine hydroxylase activity, endogenous norepinephrine, and synaptosomal uptake of L-[^{3}H]norepinephrine), cortical serotonergic terminals (endogenous serotonin), and cortical histaminergic terminals (histidine decarboxylase activity) were unaffected in cortical samples that exhibited an average 50% reduction in the activity of choline acetyltransferase.

Because kainic acid may cause a selective neuronal degeneration in areas distant from the primary injection site, it was necessary to determine that the reduction in cortical cholinergic parameters did not result from such distant cell loss (37). Kainic acid lesions of several areas close to the GP but not involving the lower border of the caudal half of the GP affected neither the activity of choline acetyltransferase nor that of glutamate decarboxylase in the neocortex; these regions included the thalamus, the dorsal GP, the pyriform cortex, and the entorhinal cortex.

Histology of the Pallidal Kainate Lesion. With the low dose of kainic acid utilized, the pallidal lesion was quite restricted, with a radius of 0.7 mm. Neuronal loss beyond the borders indicated in Fig. 1 was not evident; notably, the pyriform cortex and the lateral neocortex used for biochemical assays exhibited neither neuronal degeneration nor secondary gliosis. Histologic examination of the lesion site with Nissl-stained material revealed a virtual absence of neuronal perikarya, especially the large multipolar neurons just beneath the medial GP (Fig. 1). The affected area was involved in a moderate gliotic reaction. Examination of the same region in sections prepared by the histochemical method for AcCho-esterase revealed a marked loss of the large AcCho-esterase-reactive neurons within and beneath the medial GP on the injected side (Fig. 1). Furthermore, the AcCho-esterase-containing fibers, particularly prominent in the deep layers of the lateral neocortex, were severely decreased in neocortex ipsilateral to the pallidal kainate lesion (Fig. 2). Sections prepared under conditions to control for cholinesterase activity or nonspecific staining did not exhibit esterase-positive neuronal elements in cortical or subcortical regions.

DISCUSSION

The mapping experiments of Shute and Lewis indicated that the neocortex is innervated by AcCho-esterase-reactive fibers coming from neurons located in the region of the GP of the rat (11). They observed that these neurons have large cell bodies and exceptionally long dendrites. The location and appearance of these neurons was subsequently confirmed by other investigators (12). Shute and Lewis concluded that these neurons utilized AcCho as their neurotransmitter on the basis of their intense AcCho-esterase reactivity; this inference was premature because neurons that do not synthesize AcCho can also stain for AcCho-esterase (12–14). Thus, the neurotransmitter associated with this pathway has remained unknown. Nevertheless, their mapping of the proposed cholinergic pathway appears remarkably accurate in light of the present evidence.

Histologic evidence indicates that the large AcCho-ester-

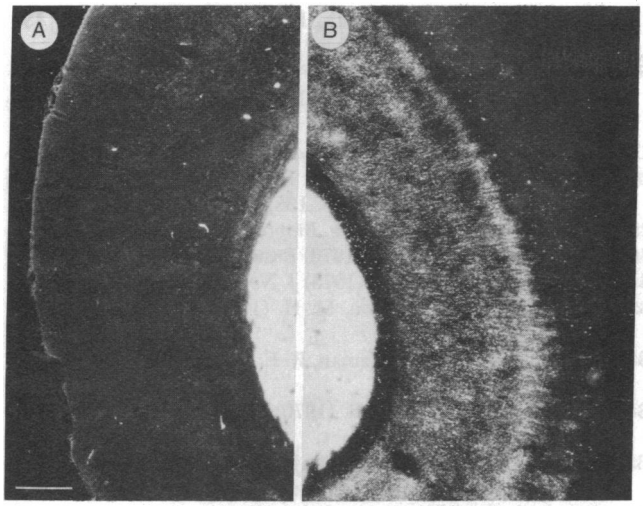

FIG. 2. Darkfield photomicrograph of lateral cortex stained for AcCho-esterase from the rat whose peripallidal lesion is shown in Fig. 1. Dense AcCho-esterase staining in deep layers of cortex ipsilateral to uninjected side (*B*) is absent on the side injected with kainic acid (*A*). Incubation of adjacent sections with medium containing inhibitor demonstrated that staining is related to AcCho-esterase. Bar, 500 μm.

ase-positive neurons in this region, although closely associated with the medial GP in the rat, are not truly part of the GP. Parent *et al.* (39) have pointed out the contrast between the large neurons in the medial GP–substantia innominata of rats and the smaller fusiform pallidal neurons, which react weakly for AcCho-esterase. Interspecies comparison of this region in the rat, rabbit, and monkey suggests that there is a marked similarity between the AcCho-esterase-positive neurons beneath and intermingled with the medial GP in rats and the more exclusively extrapallidal neurons in the basal nucleus of Meynert in the substantia innominata of primates (40). That the neurons are distinct from pallidal neurons is further supported by the fact that they project directly to neocortex. Thus, in rabbits, Das (41) observed degeneration of the large reticular, peripallidal neurons after cortical ablation; and Arikuni and Ban (42) found that horseradish peroxidase (EC 1.11.1.7) injected into the neocortex was retrogradely transported to these neuronal perikarya. Horseradish peroxidase retrograde transport experiments in monkeys have also demonstrated a direct, nonthalamic projection involving widespread areas of the neocortex from neurons in the substantia innominata analogous to those of the basal nucleus of Meynert (43–45); simultaneous staining of peroxidase-containing neurons for AcCho-esterase demonstrated high levels of activity of the enzyme (46).

Data from our experiments in which three presynaptic neurochemical markers for cholinergic neurons were measured strongly support the conclusion that a direct, ipsilateral peripallidal neocortical projection is a major source of cholinergic innervation to the parietal and frontal cortex in the rat. The results of electrolytic lesions in the GP agree with others recently reported (47). The fact that injection of small amounts of kainic acid at the base of the GP was as effective as total destruction of the area by an electrolytic lesion is good evidence that projection arises from the cell bodies in this region. Our results indicate that the decrease in cortical cholinergic markers was not related to direct damage to intrinsic cortical neurons because neuronal loss was not evident in Nissl-stained sections of the cortex, nor were GABAergic markers, a defined cortical intrinsic neuronal population, affected in the same samples. Measurement of cortical neurochemical markers for noradrenergic, serotonergic, and histaminergic neurons whose axons travel in the medial forebrain bundle adjacent to the lesion site indicated that these axons of passage were also spared. The results are unlikely to be related to diffusion of kainic acid into relevant contiguous structures such as the pyriform cortex or the thalamus because the direct injection into these areas did not affect cortical cholinergic markers. The ineffectiveness of thalamic lesions is of interest because neurophysiologic evidence has prompted the suggestion that AcCho released from cortex might be derived from thalamocortical pathways (3). Similarly, another putative cholinergic pathway to forebrain, the AcCho-esterase-positive ventral tegmental bundle (11, 48), which ascends from the brainstem through the region of the zona incerta, was not the site of kainate's action because an electrolytic lesion that included zone incerta also did not affect cortical cholinergic parameters. Although it is unlikely that kainic acid would damage internal capsule fibers, thereby producing a retrograde degeneration of intrinsic cortical neurons, this potential mechanism can be discounted because electrolytic lesions that largely spared the GP but severely damaged the capsule did not alter the cortical cholinergic markers. Thus, potentially confounding factors limiting conclusions from previous experiments that involved ablative techniques alone appear to have been adequately controlled. Additionally, potential pitfalls in the use of kainic acid appear to have been avoided.

The selective loss of biochemical markers for cholinergic neurons in the neocortex after kainate lesion was associated with a marked unilateral decrease in AcCho-esterase staining in the neocortex and loss of the large AcCho-esterase-positive neuronal perikarya in the medial GP and substantia innominata. The biochemical, histochemical and morphologic changes considered together with the results of previous studies provide compelling evidence that the origin of the ascending cholinergic pathway lesioned in our experiments is the large multipolar neurons situated in the medial GP and substantia innominata of the rat, neurons that are phylogenetically analogous to those of the basal nucleus of Meynert in primates. On morphologic grounds, this cholinergic pathway resembles other groups of reticular neurons dispersed in the brainstem, including the noradrenergic and serotonergic neurons that provide direct and widespread projections to the neocortex (49).

The demonstration of an ascending cholinergic projection to neocortex has considerable functional significance. Electrophysiologic evidence had suggested the existence of direct connections from the basal forebrain to neocortex prior to its morphologic description (50). Neuroanatomic studies of the large neurons in the peripallidal region have demonstrated that they receive fiber connections from many areas, including the mesencephalic reticular formation, the paramedian limbic areas, and amygdala (45, 46); in addition, they are contiguous to neurons of the lateral hypothalamus, GP, and axons traversing the medial forebrain bundle (51). Electrophysiologic studies in monkeys indicate that the activity of a high percentage of the basal nucleus neurons is modulated by some aspects of food reward after successful completion of a motor task (52). The ascending cholinergic projection to neocortex is also of potential clinical pharmacological and neuropathologic importance because blocking cholinergic neurotransmission with muscarinic receptor antagonists impairs human memory functions (53) and presenile "cortical" dementia (Alzheimer disease) is associated with marked reduction in cholinergic neurochemical markers in neocortex (54). The delineation of a major subcortical contribution to the cortical cholinergic innervation suggests that studies directed at understanding of the pathogenesis of such clinical disorders might profitably be di-

rected towards the cholinergic neurons in the basal forebrain.

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