Cholinergic contribution to one-trial visual recognition in the monkey was first demonstrated in studies showing that this function could be enhanced and impaired, respectively, by systemic administration of the cholinergic agonist physostigmine and the cholinergic antagonist scopolamine (1, 2). Later, when the entorhinal/perirhinal, or rhinal, cortex was found to be a critical substrate for recognition memory (3–6), evidence was obtained that this cortex was also a critical site for the cholinergic contribution to such memory, based on the demonstration that (i) visual recognition performance was accompanied by efflux of acetylcholine in the rhinal cortex (7) and (ii) this performance was impaired by microinfusing scopolamine directly into the rhinal cortex (8). Replication of the latter finding in studies on rats (9, 10), together with evidence in rats that scopolamine blocks long-term synaptic depression in perirhinal neurons (9), has provided strong support for the notion that cholinergic modulation of rhinal synaptic activity is essential for the formation of new visual memories.

Missing from the above body of evidence, however, is a convincing demonstration in macaques that the formation of new visual memories can be disrupted not only by blocking cholinergic receptors in the rhinal cortex but also by eliminating the cholinergic input to this tissue. Indeed, even attempts to deprive the entire cortical mantle of acetylcholine by injecting an excitotoxin throughout the basal-forebrain sources of the cholinergic projections (11) were found to produce either only modest and transient impairment in visual recognition (12, 13) or none at all (14). Yet, for the following reasons, those studies cannot be considered definitive. On one hand, although many cortical areas showed significant reductions in acetylcholine levels, as indexed by postmortem choline acetyltransferase activity, this index was not assessed in the rhinal cortex itself, because the studies were conducted before the rhinal area’s essential role in recognition had been discovered. It is therefore possible that the cholinergic denervation of this critical area was insufficient to produce a substantial level of impairment. On the other hand, even the modest deficit that was obtained in one of the studies (13) may have been due to cholinergic denervation of areas other than the rhinal cortex or even to excitotoxic destruction of basal forebrain cell populations other than the cholinergic ones. Studies in rats similar to those described above for macaques have yielded similar results (15), attended by the same limitations of interpretation [see Dunnett et al. (16)].

To reexamine the issue in the present study, we adopted a different approach. In rats, injections of the immunotoxin 192 IgG-saporin into the lateral ventricle were shown to target the p75 neurotrophin receptor on cholinergic projection fibers and, after retrograde transport of the toxin into the basal forebrain cholinergic cell bodies, to destroy their ribosomal function permanently (17, 18). It was then found that the same selective destruction could be produced in rats by injecting the immunotoxin intracortically (19). Motivated by these findings, we infused a primate analogue of 192 IgG-saporin (ME20.4-SAP) directly into the rhinal cortex of monkeys. Our aim was to determine whether this procedure would selectively eliminate just those basal forebrain cholinergic neurons that project to rhinal tissue and, if so, to examine the effects of this manipulation on one-trial visual recognition memory.

Methods

Subjects. The subjects were six male rhesus monkeys (Macaca mulatta), all experimentally naive, with preoperative weights ranging from 4 to 8 kg. Animals were housed individually or in established pairs and fed a diet of primate chow (No. 5038, PMI Feeds, St. Louis) supplemented with fruit; water was available ad lib. Three of the animals were given infusions of the immunotoxin ME20.4-SAP, two were given control infusions, and one was left unoperated. Additional control animals of each type were planned but were not needed, inasmuch as the control monkeys of both types obtained nearly identical behavioral scores and so were combined into a single control group of three (see Results). All procedures were carried out in accordance with both the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the principles described in the Declaration of Helsinki and were approved by the Animal Care and Use Committee of the National Institute of Mental Health.

Test Apparatus. Behavioral training was conducted in a Wisconsin General Testing Apparatus inside a darkened room equipped with a white-noise generator that helped mask extraneous sounds. The test tray contained three food wells spaced 14 cm apart and aligned 14 cm from the cage front. A set of 1,200 distinctive objects was used for testing, and peanut halves served as rewards.

Preoperative Training. Animals were initially habituated to the transport cage and Wisconsin General Testing Apparatus, given a session of noncontingent access to food in the test wells, and then gradually shaped to displace objects from each of the wells to obtain the food. Once this preliminary phase was completed, which typically required 3–4 days, the animals began training with trial-unique stimuli on a rule for one-trial visual recognition memory, namely, delayed nonmatching-to-sample. Briefly, a

Abbreviations: AChE, acetylcholinesterase.

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baited sample object was placed over the central well, which the animal displaced for the reward. This sample presentation phase was followed 10 s later by the choice phase, in which the sample object (unbaited) and a novel object (baited) were presented simultaneously over the lateral test wells. This same two-phase procedure was repeated with a new pair of objects on each succeeding trial. The trials were presented at the rate of 20 trials per day, 5 days per week, and with intertrial intervals of 30 s. The left-right positions of the familiarized and novel choice objects on each trial were balanced pseudorandomly within each session, and there were no corrections for errors. After each of the 1,200 objects in the set had been presented once, they were reused in sequence (except for randomization within a session) to maximize the interval between exposures to a particular object (∼1 month). On attaining the criterion of 90 correct responses in 100 consecutive trials, animals were designated for immunolesioning or sham control procedures.

**Surgery.** An initial dose of atropine sulfate (0.04 mg/kg i.m.) was administered to reduce secretions, followed by ketamine hydrochloride (10 mg/kg i.m.) in preparation for intubation. Animals were then maintained on isofluorane anesthesia (1–2%, to effect). A solution of isotonic saline was given (i.v.) to maintain fluid levels, and mannitol (30%, 30 ml i.v. for 30 min) was administered to preclude brain edema. All surgical procedures were performed aseptically, and recordings of heart rate, respiration patterns, blood pressure, and body temperature were monitored throughout the surgery.

Bilateral operations were performed in one stage. The zygomatic arch was removed, followed by turning of a large lateral bone flap extending from the orbit to the caudal edge of the zygoma to expose the temporal lobe and ventrolateral portion of the frontal lobe. A dural flap was then turned to allow gentle retraction of the temporal lobe to gain access to the ventromedially located rhinal cortex. With the aid of an operating microscope, handheld microinfusions of the selective cholinergic immunotoxin ME20.4-SAP (0.01 μg/μl, Advanced Targeting Systems, San Diego, CA) were then performed in the animals designated for immunolesioning (n = 3), targeting both the perirhinal and entorhinal cortices. Unconjugated saporin was used for the two animals that were given control injections. As noted above under the heading “Subjects,” the third control animal was left unoperated. Gas-sterilized 25-μl Hamilton syringes equipped with 30-gauge needles beveled to a 45° angle were used for all infusions. Each 1.0-μl injection was administered in ∼2 s, and the needle was maintained in position for an additional period of several seconds before infusing the next location. The total number of injections per hemisphere ranged from 29 to 34.

For each hemisphere, once the infusions were completed, the dura was sutured, and the bone flap was reattached. Finally, the wound was closed in anatomical layers, and antibiotic ointment was applied. Dexamethasone phosphate (0.4 mg/kg) and cefazolin (15 mg/kg) were administered to all animals 1 day before surgery and for the week after surgery as prophylaxis for swelling and infection, respectively. Ketoprofen (2.2 mg/kg) was given for 3–5 days postoperatively for analgesia.

**Postoperative Testing.** Animals received a 2-week postsurgical recovery period and then returned to behavioral testing. The unoperated control animal was kept on rest for an equivalent period. Once animals regained criterion on delayed nonmatching-to-sample at the 10-s delay, performance testing was initiated. First, the delay interval between sample and choice phase was increased stepwise for five consecutive sessions per delay from the initial 10 s to 30 s and then was increased to 60 s and, finally, 120 s. Then, with both the intr trial and intertrial delays held constant at 20 s each, list lengths of the sample objects were increased stepwise for five consecutive sessions per list from the initial list length of 1 to lists of 3, 5, 10, and finally, 20 objects before the choice phase was presented. Each of these list-length sessions consisted of 30 trials, except for list lengths of 20, in which the number of trials per session was increased to 40.

**Histology.** Because both the immunolesioned and sham-lesioned animals were designated for additional experiments, two experimentally naïve monkeys were immunolesioned as described above, but in one hemisphere only. After an interval of 2 and 6 weeks, respectively, they were prepared for histological analyses; the latter interval was selected because it represented the midpoint of the postsurgical period during which the behaviorally trained animals were tested. To examine the effectiveness and selectivity of the immunotoxin, we processed the brain tissue for both cholinergic and GABAergic fibers by using acetycholinesterase (AChE) and parvalbumin immunoreactivity, respectively. We processed the brain tissue for other classes of fibers and cells, as well, by using standard staining methods.

At the postsurgical times noted, each of the two animals received an initial dose of ketamine for sedation (10 mg/kg i.m.) followed by an overdose of anesthetic (60 mg/kg i.v. of Beuthanasia-D Special, Schering-Plough); they were then perfused transcardially with buffered 0.9% saline and then by 4% paraformaldehyde in phosphate buffer. The brains were removed, incubated in cryoprotectant, photographed, immersed in isopentane, and then stored at −80°C. The frozen tissue was cut into 50-μm coronal sections with a sledge microtome and processed for immunoreactivity as described below. Adjacent sections were stained with thionine.

A protocol modified from one established by Tago et al. (20) was used to assess AChE-positive fibers. Initially, to quench endogenous peroxidase activity, sections were placed in 0.1% H₂O₂ for 30 min and rinsed in 0.1 M maleate buffer (pH 6.0). Sections were then incubated for 40 min in a solution of 15 mg acetylthiocholine iodide, 0.75 ml of 0.1 M sodium citrate, 1.5 ml of 30 mM cupric sulfate, and 1.5 ml of 5 mM potassium ferricyanide in 200 ml of maleate buffer (pH 6.0). Rinses in 30 mM Tris buffer (pH 7.6) preceded placement of the sections in a second incubation medium composed of 0.05 g of diamino-benzidine and 3.75 g of nickel ammonium sulfate in 125 ml of 30 mM Tris buffer (solution pH 6.4). After 10 min of incubation, 12 drops of 0.1% H₂O₂ were added to this solution, and sections remained in solution for 12 min before a final rinsing in 3 mM Tris buffer. The tissue was mounted on gelatin-coated slides, air-dried, dehydrated in ethanol, and placed in xylene before coverslipping.

Processing for parvalbumin immunoreactivity was conducted according to a protocol adapted from Melchitzky et al. (21). Sections were placed in 0.1% H₂O₂ for 30 min to halt endogenous peroxidase activity and then rinsed in 0.1 M PBS. Sections were subsequently agitated for 45 min in a blocking solution composed of 0.03% Triton X-100, 0.2% BSA, and 3% normal horse serum in PBS before placement in the primary antibody incubation (monoclonal mouse anti-PIV antibody diluted to 1:2,000 in blocking solution, Sigma–Aldrich) overnight at room temperature. Several PBS rinses were carried out, and sections were then placed in secondary antibody incubation (biotinylated horse anti-mouse antibody, 1:400 dilution) for 60 min. After another series of PBS rinses, sections were incubated for 90 min in avidin–biotin–peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories) before the visualization step, for which we used a Vector NovaRed kit (Vector Laboratories). The final reaction was stopped with 0.1 M phosphate buffer. Tissues were then mounted on gelatin-coated slides and prepared for coverslip-
ping. Sections were subsequently scanned at 2,400 dots-per-inch resolution with an Epson Perfection 2450 photo scanner. Optical density measurements were taken in white matter to calibrate for background and in the regions of interest (10 measurements per region per section) with NIH IMAGE software.

In addition to the series of sections prepared for AChE and parvalbumin immunoreactivity, two other series were processed: one for thionine and one for myelin staining, the latter following the method of Gallyas (22).

**Results**

**Histology.** In the two animals prepared for this purpose, comparison of the experimental and control hemispheres at either 2 weeks or 6 weeks after the infusions of ME20.4-SAP revealed dramatic and selective destruction of the cholinergic input to the rhinal cortex at both time points, with no significant difference in the optical densities collected between the two cases ($P > 0.5$). Specifically, AChE-positive fibers were significantly decreased in the immunolesioned hemisphere of each case (optical density in the control vs. experimental hemispheres, respectively (mean ± SE); perirhinal cortex, 167.40 ± 1.05 vs. 82.70 ± 0.71; entorhinal cortex, 177.90 ± 1.23 vs. 85.47 ± 0.80; one-way ANOVA, both $P < 0.0001$), whereas PV immunoreactivity was virtually unaltered (optical density in the experimental and control hemispheres, respectively: perirhinal cortex, 50.20 ± 1.34 vs. 49.93 ± 1.34; entorhinal cortex, 56.64 ± 1.51 vs. 57.30 ± 1.63; one-way ANOVA, both $P > 0.779$), suggesting survival of neighboring GABAergic neurons (Fig. 1). Moreover, inspection of the adjacent thionine- and myelin-stained sections revealed no signs of significant cell damage or generalized fiber damage as a consequence of the infusions in either hemisphere (Fig. 3, which is published as supporting information on the PNAS web site). Finally, although the cholinergic innervation of the rhinal cortex was dramatically reduced, there was no discernible loss of cholinergic fibers in the hippocampus, inferior temporal neocortex, or other nearby tissue.

**Behavior.** As already noted, the mean postoperative scores for the two sham-lesioned monkeys were nearly identical to those of the unoperated monkey: postoperative relearning at the 10-s delay, 40 vs. 40 trials, respectively; performance collapsed across the longer delays, 94% vs. 95% correct, respectively; performance collapsed across the list lengths, 90% correct for both. The two types of controls were therefore combined into a single control group of three.

The mean number of trials (±SE) taken by the control and experimental groups to attain criterion on 10-s delayed non-matching-to-sample preoperatively (control, 394 ± 85; experimental, 353 ± 14) did not differ significantly [Kruskal–Wallis test, $P = 0.827$]. Postoperatively, both groups showed substantial savings in relearning (control, 33 ± 24; experimental, 79 ± 35; Wilcoxon signed rank test, $P = 0.028$), and, although the immunolesioned group showed slightly less savings than the controls, the two groups did not differ reliably from each other [Kruskal–Wallis test, $P = 0.376$].

On the performance tests, however, the immunolesioned group demonstrated robust deficits (Fig. 2), scoring significantly below the control group on both the longer delays and list lengths. The mean scores, collapsed across the longer delays (30, 60, and 120 s) for the control vs. the experimental groups, were 94% vs. 83% correct, respectively; and the mean scores collapsed across the longer list lengths (lists of 3, 5, 10, and 20) were 90% vs. 67% correct, respectively. Statistical analysis of performance across all of the delays (10, 30, 60, and 120 s), assessed by a $2 \times 4$ repeated-measures ANOVA, yielded main effects of both group ($F = 9.919$, df = 1.4, $P = 0.035$) and delay ($F = 12.48$, $P = 0.025$), as well as a significant group × delay interaction ($F = 6.81$, $P = 0.014$), reflecting the ability of the immunolesioned animals to attain the same level of performance as the controls at the 10-s delay. Analysis of performance across the longer list lengths (3, 5, 10, and 20 samples), also assessed by a $2 \times 4$ repeated-measures ANOVA, yielded main effects of both group ($F = 54.265$, df = 1.4, $P = 0.002$) and list length ($F = 6.485$, $P = 0.025$), as well as a significant group × list length interaction ($F = 1.996$, $P = 0.224$).
Fig. 2. Effects of cholinergic immunolesions of the rhinal cortex on visual delayed nonmatching-to-sample. Although there was no difference in the response accuracy of animals before surgery, the animals receiving specific cholinergic lesions of the rhinal cortex \((n = 3)\) were significantly impaired in their ability to execute visual recognition memory tests involving increasing delays \((30–120 s)\) and list lengths \((3, 5, 10, \text{and} 20 \text{ samples})\) relative to control animals \((n = 5, \text{one unoperated})\). The performance values \(\text{mean \pm SE}\) of the control and immunolesioned animals are indicated by open and filled circles, respectively.

Discussion

Histological comparison of the immunolesioned and control hemispheres in the two animals prepared for this purpose indicated that administration of the immunotoxin ME20.4-SAP produced dramatic destruction of the cholinergic input to the rhinal cortex lasting for at least 6 weeks after the infusion, with no indication of any reinnervation up to that time point. The result implies that localized cholinotoxin infusion onto cholinergic projection fibers causes retrograde necrosis of the cells of origin, located in this case in the basal nucleus of Meynert \((11, 23)\), consistent with the evidence that the projection territory of each of these cells is quite limited in extent \((24, 25)\). Although there was a striking loss of cholinergic fibers in the rhinal cortex, there was no indication that other neurochemical systems or other classes of fibers in this cortex were damaged or that the cholinergic damage extended to adjacent areas.

This localized and selective cholinergic deafferentation was accompanied by substantial impairment of visual recognition memory. Indeed, the magnitude of the impairment produced by the cholinotoxin in the present study was comparable to the impairment produced by destroying perirhinal neurons with the excitotoxin ibotenic acid in the study by Málková \textit{et al.} \((6)\). The memory performance scores of the lesion groups in these two experiments were 85\% vs. 82\% correct, respectively, on the extended delays \((30, 60, \text{and} 120 s)\) and 70\% vs. 66\% correct, respectively, on the extended list lengths \((3, 5, \text{and} 10 \text{ samples})\); the Málková \textit{et al.} \((6)\) study did not include list lengths of 20 samples. It should be noted in this connection that an explanation of the current experimental group’s impairment in terms of mechanical destruction of perirhinal neurons incurred by the infusion process \textit{per se} is not only negated by the postoperative MRI scans but is ruled out by the high performance level attained by the animals that received the unconjugated saporin control injections.

The present results complement data from several lines of research pointing to the importance of the cholinergic system in memory, including not only the findings already cited that motivated this study, but also those dealing with the neuroanatomy of visual learning in monkeys \(\text{e.g.},\) Easton \textit{et al.} \((26)\), recognition memory in Alzheimer’s patients \(\text{e.g.},\) Sahakian \textit{et al.} \((27)\), and mnemonic effects of muscarinic receptor blockade in healthy human subjects \(\text{e.g.},\) Robbins \textit{et al.} \((28)\). More importantly, the results provide the most direct demonstration to date that cholinergic activation of the rhinal cortex is essential for the formation of new visual memories. Earlier evidence showing that acetylcholine is released during performance on recognition memory tests \((7)\) could have reflected a nonspecific arousal or attentional mechanism. Similarly, earlier intervention in the cholinergic system, namely, blockade of the muscarinic receptor by the administration of scopolamine, even when it was microinfused directly into the rhinal cortex \((8)\), left open the possibility that this pharmacological agent interfered in unknown ways with neurochemical events besides those dependent on acetylcholine. In light of the present results, however, it is safe to conclude that both steps in the sequence of local cholinergic events, i.e., efflux of acetylcholine into the rhinal cortex and binding of acetylcholine to muscarinic receptors located on rhinal cortical neurons, are prerequisites for the synaptic modification \((9)\) that underlies storage of the representations of new visual stimuli, enabling their later recognition.

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