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BRIEF COMMUNICATION

Combined Serotonergic-Cholinergic Lesions Do Not Disrupt Memory in Rats

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SAHGAL, A. AND A. B. KEITH. *Combined serotonergic-cholinergic lesions do not disrupt memory in rats.* PHARMACOL BIOCHEM BEHAV **45**(4) 995–1001, 1993.—Rats were trained on a delayed nonmatching to position task, divided into four groups and given the following lesions: (a) SHAM (vehicle injection into nucleus basalis magnocellularis (NBM) and raphé nuclei (RN), (b) RN (5,7-dihydroxytryptamine lesions of raphé, vehicle into NBM), (c) NBM (quisqualic acid lesion of NBM, vehicle into RN), and (d) COMB (lesions of both RN and NBM). RN lesions had no effect on performance measures including accuracy (percent correct), errors of omission, bias, latencies, and magazine response rate. NBM lesions produced delay-independent (nonmnemonic) disruptions, but performance improved over the 20 days' test. The effects of COMB lesions were no worse than NBM lesions alone. The results suggest that (a) the serotonergic system is not essential for performance in this task, (b) NBM lesions transiently impair nonmnemonic aspects of performance, and (c) serotonergic-cholinergic interactions may not be essential for some cognitive processes.

Memory Raphé Nuclei Nucleus basalis magnocellularis Serotonin Acetylcholine Rat

PREVIOUS studies have suggested that acetylcholine has a key role in the mediation of memory and other cognitive processes (5,10,11,19,23,25,26). Relatively speaking, far less effort has been directed at other neurotransmitter systems, even though many of them densely innervate areas of the brain known to be important for cognition, for example the cortex and hippocampus. Moreover, some of these neurotransmitter systems, for example, the indoleamine serotonin (5-HT), could be deficient in old age (27) and in dementing disorders such as Alzheimer's disease (6) and Lewy body dementias (7,31), where cognitive decline is a prominent feature. However, empirical work directed at elucidating the role of serotonin in cognition has been inconclusive. Thus, neurotoxin lesions of the dorsal hippocampal serotonergic innervation facilitated performance on a spatial task (4), and antagonist drugs enhanced the retrieval of a previously learned, aversive, habit in mice (3). Wenk and co-workers (44) found that raphé lesions impaired choice accuracy in a spatial delayed alternation T-maze task, but only when the cognitive burden was high. Others report that serotonergic lesions by themselves had no effect on spatial (watermaze) learning (29,34), or on operant continuous nonmatching to sample performance (40).

Some authors have studied the importance of serotonergic interactions with other, and especially cholinergic, systems: many of these studies have been reviewed elsewhere (8). In an early report, Swonger and Rech (41) proposed that serotonergic, adrenergic, and cholinergic systems interact in a dynamic manner, and Gray (22) has described a comprehensive, multitransmitter, (behavioral inhibition) system that is involved in cognition and other behaviors. More recently, Vanderwolf and his colleagues (42,43) have reported data from a number of maze learning and avoidance studies in which serotonergic and cholinergic mechanisms were manipulated, usually pharmacologically. They report that simultaneous blockade of these systems has much greater deleterious effects on performance than single transmitter disruption, and these findings have been confirmed (29,34). Interestingly, the effects of these manipulations were even more disruptive than combined lesions of the hippocampus and amygdala (12), which are known to produce severe impairments in primates (28). However, neurochemical evidence indicates that serotonergic neurones tonically inhibit cholinergic neurones in the cortex and hippocampus, but do not affect striatal cholinergic neurones (35). In other words, serotonergic depletion might enhance (cholinergically mediated) mnemonic processes, and there is evidence for this (4,30). Thus, serotonergic-cholinergic inter-

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actions appear to be complex and, as Decker and McGaugh (8) point out, require a careful task analysis.

The aim of the present study was to investigate the role of cortical cholinergic and serotonergic mechanisms, and their interactions, in cognition using relatively selective lesioning techniques (rather than the less specific pharmacological methods). The cortical, rather than septo-hippocampal, cholinergic pathways were lesioned since previous work involving toxin-induced lesions has demonstrated the importance of this area in cognition (5,19,26), and damage to this system is a prominent feature of Alzheimer's disease (32). A modified version of the nonmatching to position task (1,2,13,14,16, 38,39), a spatial test of working memory, was used. We investigated the effects of (a) neurotoxin lesions of the serotonergic raphé nuclei (RN), which project to cortical and hippocampal structures, (b) excitotoxin lesions of the nucleus basalis magnocellularis (NBM), which gives rise to the main cholinergic cortical projection, and (c) both areas lesioned together (COMB).

METHOD

Subjects

Thirty-one male PVG hooded rats (Bantin and Kingman, Hull, UK), weighing 240–255 g, were individually housed under diurnal conditions (lights on 07:00 to 19:00 hr). The final group allocations were SHAM:10; RN:7; NBM:8; COMB (RN + NBM):6. They had free access to water, but were food deprived to 85% of their free-feeding weights. All testing was done in a darkened room where white noise (70 db) was continuously present.

Apparatus

Ten rodent operant chambers (Campden Instruments, Loughborough, UK), each fitted with two retractable levers placed 15 cm apart, a centrally located illuminated food pellet dispenser fitted with a clear, hinged, Perspex® magazine flap connected to a microswitch, house light and three stimulus lights (one top centre, the other two 3 cm above each lever), were connected to two SPIDER® micro-computer systems (Paul Fray, Cambridge, UK).

The three stimulus and one house light bulbs in this equipment had been replaced by light emitting diodes to provide a lower, but adequate, level of illumination (see 38). The magazine illumination was provided by a 24 V, 2.9 W bulb.

Procedures

Animals were autoshaped (36) to respond to the two levers, after which training on nonmatching to position (NMTP) commenced. Each of 60 daily trials consisted of the following sequence of events. After a 10-s intertrial interval, either the left or the right hand lever would emerge; the stimulus light above it was also illuminated to provide an extra cue. The rat had to respond to the lever within (a limited hold of) 10 s, upon which the lever would retract and the magazine tray illuminate. The subject had to approach the tray and, within 10 s, operate the magazine flap. Both levers then immediately (0-s delay) emerged, and the stimulus lights above them were also illuminated. Rats now had to respond, for a food pellet, to the lever that had *not* appeared as the sample; these choice responses also had to be made within 10 s. The limited hold was introduced to encourage responding within a reasonable period of time; we have found that rats usually respond within 2 s. Incorrect responses, or a failure to respond, resulted in a "time-out" of 10 s, during which all lights in the chamber were off and levers retracted; these were the only occasions when the house light was off.

Delays between the sample and choice lever presentations were introduced once rats had learned the basic, 0-s delay, task. Now, magazine responses following the sample presentation were ineffective (but were recorded) until the appropriate delay interval had lapsed; the first response after this resulted in the choice levers being presented, providing this response occurred within 10 s of the end of the delay. At first, only short (up to 2 s) delays were programmed, and the duration was progressively increased to the final values (0, 2, 4, 8, 16, and 32 s) used in this study. Training continued until all animals were performing at stable levels, with little change in performance from day to day. Sixty daily trials were scheduled for each subject, with equal numbers of left/right stimuli at each delay, presented in different pseudo-random orders.

Surgery

All surgery was done under deep pentobarbital (45 mg/kg, intraperitoneally:IP) anaesthesia, and the rats had been treated with desmethylimipramine (Sigma, Poole, UK: 25 mg/kg, IP) 30 min prior to surgery. A stereotaxic instrument (David Kopf Instruments, Tujunga, CA) was used; three holes were drilled in each animal's skull (two for NBM and one for RN), and infusions made by a Schuco infusion pump (Schuco International, London, UK) via a 30-ga needle. The general health, food, and water consumption of all animals was carefully monitored following surgery.

The site and extent of NBM and RN lesions was histologically confirmed in a separate group of nine rats. All subjects received NBM and RN lesions as described below. Following a 9-day recovery period, the rats were deeply anaesthetised with sodium pentobarbitone (60 mg/kg) and perfused with 30 ml of phosphate buffered saline, followed by 60 ml of 10% formalin, through the left cardiac ventricle. Brains were removed and kept in formalin for 7 days before being double embedded in paraffin wax. Cut coronal sections were mounted on glass slides and stained with haematoxylin and eosin.

(a) RN lesions. The seven animals in this group received 2 μl infusions of 5,7-dihydroxytryptamine creatinine sulphate (5,7-DHT: Sigma, Poole, UK), 5.04 mg/ml in phosphate-buffered saline containing 0.2% ascorbic acid. Dorsal and medial raphé nuclei were lesioned. The injection coordinates from bregma were: *dorsal,* AP −7.3 mm; lateral 0.0; vertical 6.2 mm from the skull, *medial,* AP −7.3 mm; lateral 0.0; vertical 8.4 mm from the skull. The incisor bar was set 3.3 mm below the interaural line. The infusions were made over 9 min (25 nmoles), and the injection cannula was left in place a further 5 min to aid diffusion and prevent tracking by the neurotoxin on removal. All rats in this group received vehicle only injections into the NBM (see below) at the same time.

(b) NBM lesions. The eight rats in this group were given 1 μl bilateral infusions of 0.12 M quisqualic acid (Sigma, Poole, UK) dissolved in 0.9% saline. This excitotoxin has fewer nonspecific effects compared to ibotenic acid (15, 17). The coordinates were 1 mm caudal to bregma ± 3 mm from midline and 7.6 mm below dura with the incisor bar set 3.3 mm below the interaural line. Infusions were made over 3 min, and the cannula was left in place for a further 5 min to promote diffusion. These rats also re-

ceived infusions of vehicle into the RN (see above) during surgery.

(c) COMB lesions. Using appropriate neurotoxins, the six animals in this group were given bilateral lesions of *both* RN and NBM, as described above.

(d) SHAM lesions. The 10 control subjects were given bilateral infusions of vehicle into RN and NBM, as described above.

Neurochemical Determinations

At the conclusion of the experiment all animals were killed by decapitation and the brains rapidly removed and dissected on an ice-cold tray. Tissue samples were taken from temporal neocortex (in the region of the rhinal sulcus) and hippocampus, bilaterally. These samples were frozen over liquid nitrogen and then stored at −70°C.

These areas were chosen because the RN innervate the cortex and hippocampus, and the NBM projects to cortical areas. They should, therefore, provide a good indication as to the extent and locus of the RN and NBM lesions.

(a) Serotonin (5-HT). Samples from the two areas were sonicated in 0.2 M perchloric acid containing 1 mM EDTA and 4 mM sodium metabisulphite. After centrifugation, 20 μl aliquots were assayed in a Waters HPLC system (Millipore, Watford, UK) comprising a WISP 712 autosampler with refrigerated sample compartment, model 510 pump, model 460 electrochemical detector and a 15 cm Resolve C18 5 μ column, maintained at 35°C. The mobile phase consisted of 100 mM sodium acetate, 100 mM citric acid, 0.5 mM sodium octyl sulphate, 1 mM di-*n*-butylamine, 0.15 mM sodium EDTA and 3% v/v methanol at *p*H 3.7. Serotonin for standard solutions was purchased from Sigma, Poole, UK. All other HPLC chemicals were purchased from BDH, Poole, UK.

(b) Choline acetyltransferase (ChAT). Samples of neocortex were sonicated in 9 volumes of 0.32 M sucrose containing 0.5% Triton X-100. ChAT was then estimated on aliquots using the method of Fonnum (21). ^{14}C acetyl-CoA was purchased from Amersham International, Amersham, UK. All other chemicals were purchased from Sigma, Poole, UK and BDH, Poole, UK.

Performance Measures and Analyses

The performance measures included percent correct responses, number of missed trials (usually termed errors of omission), latency to respond to the sample lever, magazine flap, and choice lever and magazine response rate. The bias measure Index Y (I_y: ref. 37) was also calculated. This contrasts accuracy between the two levers, and provides an estimate of response-type biases towards one or other side of the operant box:

I_y = (absolute value of left minus right lever corrects)/(total number of corrects)

The data were transformed as appropriate (arcsin: percents, I_y; square-root: misses; logarithmic: latencies) and analysed by parametric analysis of variance (ANOVA) including three-factor (lesion, delay, day) mixed measures analysis (45). When the *F*-ratio was significant ($p \leq 0.05$), means were compared by appropriate *a posteriori* tests, including the Newman–Keuls statistic (45). The delay main effects are not discussed in detail, unless worthy of comment. These terms were, as in all our previous studies, almost always highly ($p > 0.001$) significant.

RESULTS

Preoperative Testing

Data were collected immediately prior to surgery, and analyzed after grouping the animals according to the lesions they were due to receive. None of the measures suggested any reliable difference; in other words, the different groups were performing, preoperatively, at similar levels.

Neurochemical and Histological Determinations

Serotonin depletions in the RN and COMB groups were satisfactory in all animals (Table 1). The ChAT reductions in the NBM and COMB groups were also acceptable (Table 2), and consistent with previous reports using quisqualate. All animals were therefore included in the behavioral analyses.

Representative sections of bilateral lesions, showing the extent of gliosis and necrosis of the NBM are shown in Fig. 1, and of the RN in Fig. 2. Histological examination confirmed that the lesions were satisfactory, damage being restricted to the NBM or RN, respectively.

Postoperative Testing

The animals were allowed 2 weeks to recover from surgery. On the first day following this period, they were rehabituated to the test apparatus. Testing commenced on the following day, and continued for 20 days thereafter. Data were recorded daily, but only day 5, 10, and 20 analyses are reported here; these days were arbitrarily selected prior to any data analyses.

TABLE 1

LISTING OF SEROTONIN LEVELS (SEROTONIN, ng/mg TISSUE, ± SEM: PERCENT DEPLETION IS GIVEN IN PARENTHESES

Group	*N*	Cort	%	HIPP	%
SHAM	10	1.642 ± 0.098	(-)	1.072 ± 0.078	(-)
RN	7	0.218 ± 0.031	(87)	0.126 ± 0.027	(88)
NBM	8	1.348 ± 0.079	(18)	1.149 ± 0.101	(+7)
COMB	6	0.049 ± 0.019	(97)	0.067 ± 0.018	(94)

All reductions were significant, including NBM CORT ($p < 0.04$ in the latter case, $p < 0.001$ for the rest). The elevated level (+7%) seen in NBM HIPP was not significant ($p = 0.55$). CORT, temporal neocortex; HIPP, hippocampus. Other abbreviations as in the text.

TABLE 2

LISTING OF ChAT ACTIVITY (nmol/h/mg TISSUE, ± SEM: PERCENT REDUCTION IS GIVEN IN PARENTHESES, AND ABBREVIATIONS ARE AS IN THE TEXT. TISSUE FROM TEMPORAL NEOCORTEX (CORT) WAS ASSAYED

Group	N	CORT	%
SHAM	10	2.83 ± 0.14	(-)
RN	7	2.10 ± 0.10	(26)
NBM	8	1.01 ± 0.19	(64)
COMB	6	1.20 ± 0.17	(58)

All reductions were highly significant ($p < 0.001$).

Percent Correct

The data indicated group differences [$F(3, 27) = 4.456$, $p < 0.02$]; subsequent analysis confirmed that this was due to a ($p < 0.05$) difference between the SHAM and NBM as well as COMB lesioned animals, but there was no difference between SHAM and RN. Moreover, the NBM and COMB groups did not differ from each other, and both of these were impaired relative to the RN group. The day effect was significant [$F(2, 54) = 7.402$, $p < 0.01$], performance on day 5 being worse than days 10 and 20 (the latter scores not differing from each other). As expected, the delay factor was highly significant [$F(5, 135) = 56.754$, $p < 0.001$], performance declining monotonically with increasing delay. The group × delay term was the only significant interaction [$F(15, 135) = 1.925$, $p < 0.05$]. This reflects the fact that, especially on day 5, the NBM and COMB groups' performance was deficient at the zero and shorter delay conditions, with a floor effect confounding any differences at longer (16; 32-s) delays. These data are illustrated in Fig. 3, and summarized across test day and delay in Table 3.

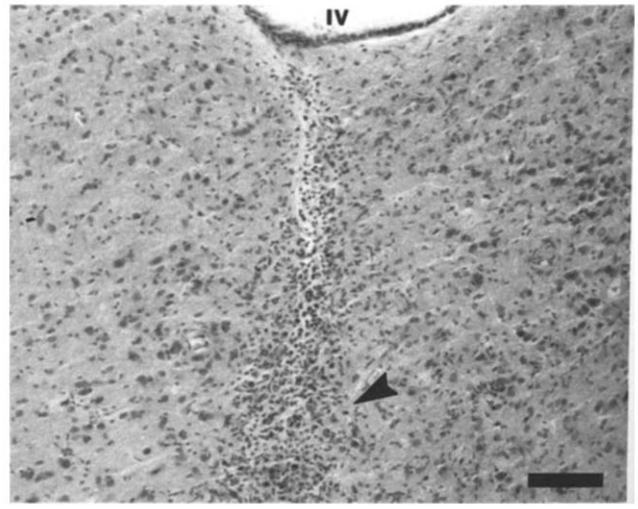

FIG. 2. Extent of neuronal damage in the RN, situated below the fourth (iv) ventricle. The bar represents 500 μ.

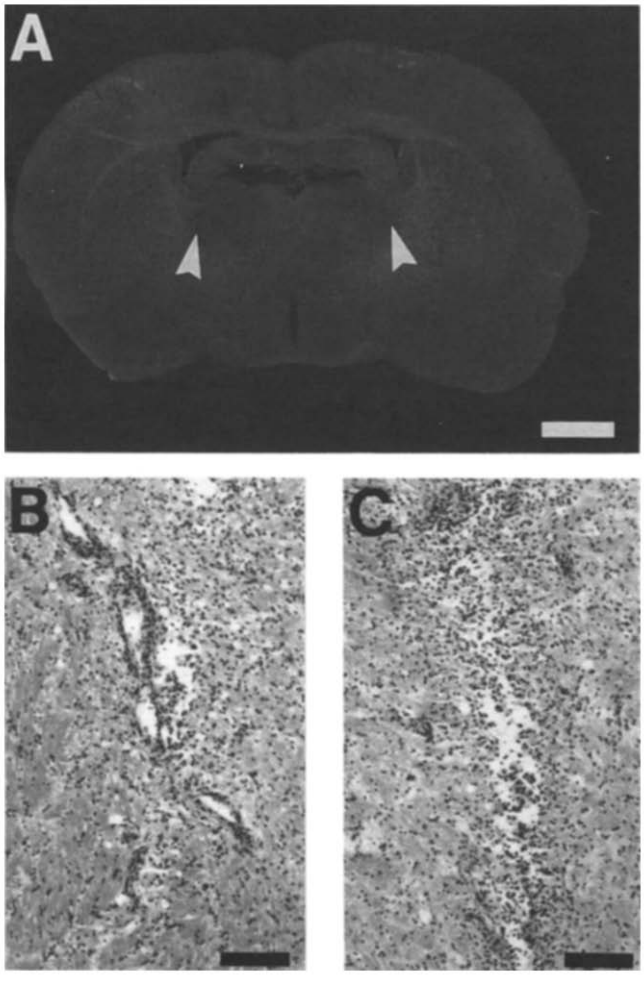

FIG. 1. Coronal section through the NBM. The top photomicrograph (A) indicates the lesion sites, which are shown under high power magnification in (B) and (C). The bar represents 500 μ.

Misses

NBM and COMB groups made more misses (errors of omission) than SHAM or RN, the latter two groups being no different from each other [$F(3, 27) = 6.970$, $p < 0.01$]. More misses were made at the longer delays, and towards the end of the (20-day) test period. However, the number of misses was small (<3% of total trials), therefore contributing little to overall performance: the data are summarized in Table 3.

Latency and Response Rate Measures

Although all three latency measures [sampling, $F(3, 27) = 4.772$, $p < 0.01$; magazine response, $F(3, 27) = 13.433$, $p < 0.001$; choice, $F(3, 27) = 6.819$, $p < 0.01$], and the magazine response rate measure [$F(3, 27) = 6.191$, $p < 0.01$] indicated group effects, the only reliable differences were between SHAM and NBM or COMB groups, the latter two being slower to initiate responses, as well as responding at a slower rate. The RN subjects were no different from SHAM; these data are shown in Table 3.

I_y

There was a significant group effect [$F(3, 27) = 4.075$, $p < 0.02$]. Subsequent analysis indicated that the COMB

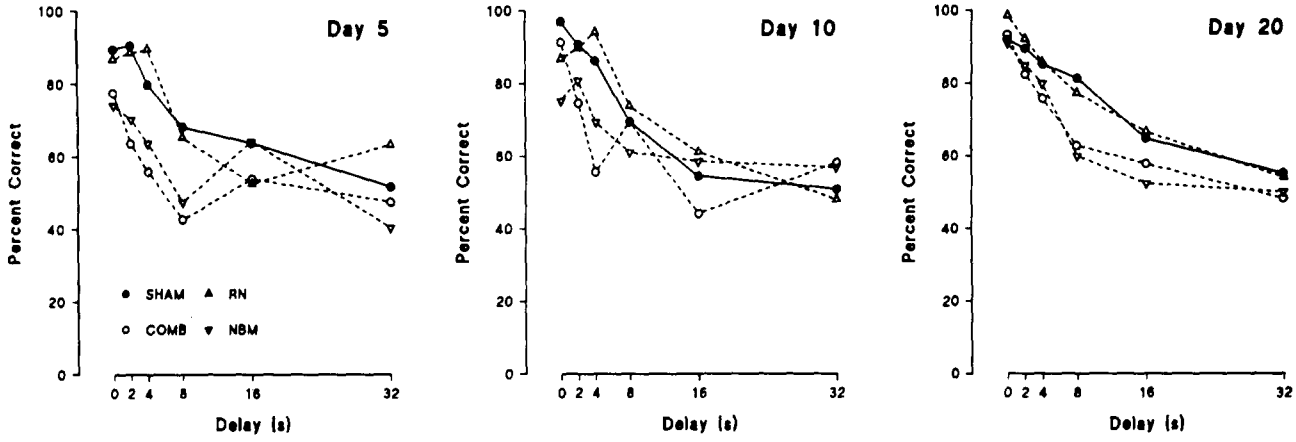

FIG. 3. Percent correct performance, day 5 (left), 10 (middle), and 20 (right panel); data from the remaining 17 days are not presented. The solid line represents control (SHAM) performance; note that "chance" accuracy is 50%. See text for lesion abbreviations.

group alone had a bias (Table 3), performing inefficiently with respect to both SHAM and RN groups. Although the NBM group's data also suggested a bias, this failed to reach significance; however, there was no significant difference between the NBM and COMB groups' bias indices (Table 3).

DISCUSSION

The main findings of this study are straightforward: neurotoxin lesions of both dorsal and medial raphé nuclei (RN group), which together provide the major serotonergic inputs to cortical and hippocampal targets, had no effect on the accuracy (percent correct), motivation (misses, latencies, bias) or motor (response rate) components of the spatial nonmatching to position memory task, in contrast to cortical cholinergic (NBM) lesions, which produced reliable, albeit transient disruptions of performance. The finding that combined serotonergic plus cortical cholinergic (COMB) lesions produced impairments that closely paralleled those seen with single NBM lesions also suggests that serotonergic mechanisms may not always be essential for efficient performance.

Several notes of caution must be sounded when interpreting these results. It has been pointed out that the effects of serotonergic manipulations may be situation- or task-dependent. Based on the observation that antiserotonergic agents by themselves had little or no effects on, for example, spontaneous alternation, Swonger and Rech (41) argued, over 20 years ago, that serotonergic pathways are not involved (in cognition) in a straightforward way. They proposed a hypothesis that predicts that deficits in serotonergic processes would be apparent only when "arousal" levels were inordinately high, because serotonergically mediated inhibitory functions may be necessary to restore an optimal arousal state. In other words, the well-learned nonmatching task used in this study may not place sufficient demands on serotonergic mechanisms. Nonetheless, it is difficult to explain why serotonergic manipulations affect performance on the relatively simpler avoidance (20) and watermaze (24, but see 29 and 34) tasks, unless it is assumed that these aversively motivated paradigms entail inefficiently high arousal levels from the outset.

Data from the NBM group, while suggesting that cortical cholinergic lesions disrupt performance, did not support the hypothesis that this pathway plays a central role in mnemonic processes (5,19,23,25,26). As such, the data support our previous (39) and other (30,33) findings, and are entirely consistent with recent reports that quisqualic acid, which reduces cortical cholinergic marker activity to levels substantially lower than the widely used (and behaviorally more disruptive) ibotenic acid, "paradoxically" produces fewer, if any, impairments on a variety of operant matching and maze learning tasks (15,18). This paradox, however, can be resolved quite simply. Ibotenic acid produces rather nonspecific lesions, which involve damage to other neurotransmitter systems. Dunnett and his coworkers (15) have argued that this nonspecific damage, involving disruption of cortico-striatal outputs passing through the dorsal and ventral globus pallidus, might be responsible for

TABLE 3

MEAN (± SEM) VALUES FOR THE VARIOUS INDICES OF PERFORMANCE, POOLED ACROSS DELAYS AND TEST DAYS

Group	*N*	PC	Misses	SL	ML	CL	MR/s	I_y
SHAM	10	75.6 ± 1.6	0.6 ± 0.1	1.8 ± 0.09	0.4 ± 0.03	0.5 ± 0.02	1.1 ± 0.03	0.27 ± 0.02
RN	7	76.3 ± 1.9	0.8 ± 0.1	1.8 ± 0.09	0.4 ± 0.02	0.4 ± 0.02	0.9 ± 0.03	0.27 ± 0.03
NBM	8	65.5 ± 2.0	2.3 ± 0.2	2.1 ± 0.19	0.7 ± 0.07	0.8 ± 0.05	0.8 ± 0.03	0.39 ± 0.03
COMB	6	64.1 ± 2.6	3.1 ± 0.3	2.8 ± 0.24	0.7 ± 0.08	0.7 ± 0.04	0.7 ± 0.04	0.41 ± 0.03

PC, percent correct; SL, sampling latency; ML, latency to make the first magazine response; CL, average (correct, incorrect) choice latency; MR/s, magazine response rate per second. All latencies are given in seconds, and the "misses" column refers to the mean number of misses per daily (60 trials) session.

the greater behavioral disruptions seen following ibotenate, but not quisqualate, lesions. Indeed, these authors have argued that cholinergic NBM projections to the neocortex may be important for processes involved in selective attention, rather than memory. However, prefrontal cortical projection areas, which escape damage following excitotoxin injection in the NBM, may be critical for mnemonic processing (14). The results of the present study are consistent with an attentional hypothesis: quisqualate lesions of the NBM were found to disrupt performance even at zero delay—when attentional, but not (working) memory, ability is still required.

Relatively few studies have considered the effects of combined serotonergic-cholinergic disruptions. It has been reported that response accuracy in an operant continuous nonmatching to sample task was not affected by *p*-chloroamphetamine (PCA) induced destruction of the serotonergic system. However, impairments produced by 0.1 mg/kg scopolamine were attenuated by PCA pretreatment (40). In contrast, Vanderwolf and colleagues (12,42,43) have published data that suggest that blockade of the two systems produces profound deficits on a range of behavioral, including cognitive, tasks, and have argued that these combined manipulations might provide a useful animal model of dementia. In similar vein, others report that 5,7-DHT lesions of the dorsal raphé, which by themselves had no effects on spatial learning in the watermaze, potentiated the effects of radiofrequency septal (29), or excitotoxin NBM (34) lesions. Our data do not support these findings. However, there are differences in the techniques used in those studies and our own, not least of which concern the site and nature of the intervention. The former (29) studies lesioned the septo-hippocampal projections using surgical techniques, whereas the latter (34) used ibotenate to lesion the NBM and this excitotoxin, unlike quisqualate, affects watermaze performance, probably via noncholinergic mechanisms (see above, and ref. 15). Unlike the more specific lesion methods employed in the present study, Vanderwolf's investigations (12,42,43) used the muscarinic antagonist scopolamine to block cholinergic activity; pharmacological methods were also used to disrupt serotonergic systems (42,43). In addition to having peripheral effects, such manipulations target all brain areas, including cholinergic cortical as well as hippocampal and thalamic sites. Some of these structures are known to be important for learning and memory (1,2,9,18,28), and "complete" blockade of the sort obtained by peripheral injection of antagonist drugs may well produce profound impairments.

In conclusion, selective hippocampal, prefrontal cortical or thalamic cholinergic damage, combined with serotonergic disruption, may produce substantial and specific impairments, which could model more closely the cognitive deficits seen in dementing disorders. Such investigations are currently being conducted in our laboratory.

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