Effects of Complete Immunotoxin Lesions of the Cholinergic Basal Forebrain on Fear Conditioning and Spatial Learning

Karyn M. Frick,1* Jeansok J. Kim,1 and Mark G. Baxter2

ABSTRACT: Administration of muscarinic cholinergic antagonists such as scopolamine impairs the acquisition of contextual fear conditioning, but the role of the basal forebrain (BF) cholinergic system in consolidation is unclear. To test the hypothesis that BF cholinergic neurons are critical for acquisition and consolidation of fear conditioning, male Sprague-Dawley rats with 192 IgG-saporin lesions of the entire cholinergic BF made either before or after fear conditioning were tested for conditioned fear to context and tone by assessing freezing and 22 kHz ultrasonic vocalization (USV) responses. Spatial learning in a 1-day water maze task provided a comparison for effects of the BF lesions on fear conditioning. In the test phase, neither pre-training nor posttraining BF lesions affected freezing to the context or tone. During both training and testing, pre-lesioned rats were impaired in production of USVs associated with fear. Postlesioned rats emitted fewer USVs only during testing. Acquisition of a spatial water maze task was mildly impaired in lesioned rats, although probe trial and cued performance was unimpaired. Nevertheless, these data suggest that conditioned fear-induced USVs are more sensitive to the loss of BF cholinergic neurons than is conditioned fear-induced freezing. The failure of BF cholinergic lesions to impair contextual fear conditioning indicates that scopolamine-induced impairments in fear conditioning may not be mediated by affecting cholinergic input to the hippocampus and neocortex. © 2004 Wiley-Liss, Inc.

KEY WORDS: 192-IgG saporin; acetylcholine; hippocampus; neocortex; Morris water maze

INTRODUCTION

Basal forebrain (BF) cholinergic neurons have been regarded as critical to the acquisition of hippocampal- and cortical-dependent information, such as spatial memories. Although a role for these projections in attention is well established (e.g., McGaughy et al., 1996), recent work demonstrating that rats with selective removal of BF cholinergic neurons using the neurotoxin 192 IgG-saporin are unimpaired in hippocampal-dependent tasks, including the Morris water maze, calls into question the importance of BF cholinergic neurons in spatial learning and memory (for review, see Baxter and Chiba, 1999).

However, BF cholinergic neurons may be involved in other types of learning and memory. For example, the role of BF cholinergic projections to neocortex and hippocampus in another form of hippocampal-dependent memory, contextual fear conditioning (FC), has recently been the subject of debate. In FC, an initially neutral conditioned stimulus (CS; e.g., a tone or distinctive chamber) is contingently paired with an aversive unconditional stimulus (US; e.g., electric shock) that reflexively activates unconditioned fear responses. Through the formation of CS-US associations, the CS comes to elicit various conditioned fear responses that are similar to innate fear responses. A role for BF cholinergic activity in contextual FC is suggested by reports that hippocampal and neocortical acetylcholine release is increased by novel contexts and behaviorally relevant stimuli (Acquas et al., 1996; Nail-Boucherie et al., 2000). Correspondingly, pre-training systemic or intrahippocampal administration of scopolamine selectively impairs acquisition of context, but not tone, FC in rats, as assessed by the freezing response (Anagnostaras et al., 1995, 1999; Gale et al., 2001; Wallenstein and Vago, 2001; but see Rudy, 1996). Although evidence for a role of the cholinergic system in acquisition of FC is relatively consistent, its involvement in consolidation of FC memory is less clear. Posttraining injections of scopolamine (either systemic or intrahippocampal) are reportedly without effect on both context and tone fear conditioning (Anagnostaras et al., 1995, 1999), although other investigators report impairments in contextual fear (Rudy, 1996; Wallenstein and Vago, 2001) or tone fear (Rudy, 1996).

Although the effects of scopolamine on FC are assumed to result from disrupted BF cholinergic function, this has not been tested directly. Scopolamine infusion into the BF reduces hippocampal acetylcholine release (Gorman et al., 1994); however, its effects on learning and memory may not stem from disruption of BF cholinergic neurons. Both cholinergic and γ-aminobutyric acid (GABA)ergic BF neurons express cholinergic receptors (Bialowas and Frotscher, 1987; Onteniente et al., 1987), and activity of GABAergic, but not cholinergic, BF neurons is decreased by scopolamine (Alreja et al., 2000). Thus, scopolamine may not impair memory by blocking BF cholinergic function. The only way to test directly whether BF cholinergic neurons are required for the acquisition and consolidation of FC is to lesion these neurons selectively. To this end, rats in this study were given 192 IgG-saporin lesions of the entire BF; lesions
were made pre- or posttraining, to examine the contribution of these neurons to acquisition and consolidation of FC. FC was measured using two reliable indices of fear, freezing and 22-kHz ultrasonic vocalizations (USVs) (Blanchard and Blanchard, 1969; Fasenlow and Bolles, 1979; Blanchard et al., 1991; Lee et al., 2001). If scopolamine modulates FC by altering BF cholinergic function, then removal of these neurons should impair FC. If destruction of these neurons does not impair FC, then scopolamine may influence FC via another neuronal population or brain region. We also tested the rats in a challenging Morris water maze protocol in which spatial learning, spatial retention, and cued learning were tested in 1 day. The spatial water maze task is usually administered over the course of several days (e.g., Morris, 1984; Frick et al., 1995), resulting in gradual learning of a submerged platform position. Rats with 192 IgG-saporin lesions of the BF are generally not impaired using multi-day protocols (Baxter et al., 1996; Baxter and Gallagher, 1996). In addition, rats with restricted lesions of the medial septum/vertical limb of the diagonal band of Broca (MS/VDB) or nucleus basalis magnocellularis (nBM) are not impaired in a more challenging 1-day water maze protocol (Ricceri et al., 2000). However, it was unknown whether a lesion of the entire BF would affect spatial learning and memory in a 1-day task. Furthermore, the inclusion of this task permitted a comparison of lesion effects on another hippocampal-dependent task. Thus, all rats were tested in the hippocampal-dependent Morris water maze after completion of testing.

MATERIALS AND METHODS

Subjects

The subjects were 30 experimentally naive male Sprague-Dawley rats (weighing 294–341 g at surgery) purchased from Charles River (Raleigh, NC). They were individually housed in a climate-controlled vivarium with a 12:12 light/dark cycle (lights on at 7 AM). Surgery and behavioral testing was longer in this study to allow enough time for the interval between training and testing was longer in this study to allow enough time for the toxin to fully destroy BF cholinergic neurons (Waite et al., 1994). In addition, because all water maze testing occurred after surgery, the pre- and posttraining groups were combined and only two groups were analyzed: control (n = 10) and lesion (n = 20).

Surgery

The rats were randomly assigned to receive either a sham lesion or a BF lesion. A complete lesion of the entire BF, encompassing all three major nuclei projecting to the hippocampus and neocortex, was conducted. This complete lesion allowed us (1) to determine whether any part of the BF is involved in FC, and (2) to exclude the possibility that residual cholinergic projections mediate FC in the event that no lesion effect was observed. Lesion surgeries took place either prior to (pre-training) or after (posttraining) FC training. Rats were anesthetized with 80 mg/kg ketamine and 5 mg/kg xylazine (i.m.) and were then secured in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA). Lesion and sham injections were made using a motorized stereotaxic injector (Stoelting, Wood Dale, IL) and 28-gauge needle Hamilton syringe filled with either 0.15 µg/µl 192 IgG-saporin in sterile phosphate-buffered saline (PBS) (lesion surgeries) or sterile PBS (sham surgeries). The toxin 192 IgG-saporin consists of a ribosome-inactivating protein (saporin) coupled to an antibody against the low-affinity p75 neurotrophin receptor expressed by basal forebrain cholinergic neurons (Book et al., 1992). To destroy cholinergic neurons in the entire basal forebrain, lesions were made in each of the nuclei comprising this region, the MS/VDB, the horizontal limb of the diagonal band of Broca (HDB), and the nBM. To lesion the MS/VDB, two holes were drilled in the skull at stereotaxic coordinates anteroposterior (AP) = +0.45 mm and mediolateral (ML) = ±0.6 mm from Bregma. Injections were made at two depths at each site, dorsoventral (DV) = −7.8 mm and −6.2 mm from the surface of the skull. Solutions were delivered at a rate of 0.05 µl/min, injecting a total of 0.3 µl at each of the DV = −7.8 sites, and a volume of 0.2 µl at each of the DV = −6.2 sites. The syringe was left in place for 6 min after each 0.3-µl injection and for 4 min after each 0.2-µl injection, to limit diffusion of solution into the needle track. To lesion the nBM, four holes were drilled in the skull at coordinates AP = −0.75 mm and ML = ±2.3 mm (medial sites) and ML =
±3.3 mm (lateral sites) from Bregma. Injections were made at two depths from the skull surface: DV = −7.8 mm at the medial sites, and DV = −8.1 mm at the lateral sites. Solutions were delivered at a rate of 0.1 µl/min, injecting a total of 0.2 µl at each site. The syringe was left in place for 3 min after each injection. For the HDB, two holes were drilled in the skull at coordinates AP = −0.15 mm and ML = ±1.8 mm from Bregma. Injections were made at a depth of DV = −8.7 mm from the skull surface. Solutions were delivered at a rate of 0.1 µl/min, injecting a total of 0.2 µl at each site. The syringe was left in place for 3 min after each injection.

Fear Conditioning

All training and testing took place in two modular operant test chambers, each equipped with speaker modules (Coulbourn Instruments, Allentown, PA), located in a controlled acoustic room (Industrial Acoustic Company, NY). The two chambers differed in several aspects: chamber A was rectangular (27-cm width × 28-cm length × 30.5-cm height), whereas chamber B was octagonal (26.5-cm diameter × 25-cm height). Chamber A had front and back walls made of clear Plexiglas and two side walls made of metal plates, whereas chamber B had all eight walls constructed of clear Plexiglas. Chamber A was placed into a wooden isolation box (46-cm width × 53-cm length × 49-cm height) that was painted white, whereas chamber B was placed into a similar box that was painted black. The grid floor of chamber A was composed of 16 stainless steel bars (4.5-mm diameter) spaced 17 mm center-to-center and wired to a Coulbourn precision-regulated animal shocker. The grid floor of chamber B was composed of 17 stainless steel bars (5-mm diameter) spaced 15 mm apart and wired to a second, identical Coulbourn shocker. The floor grid and base pan of each chamber were washed thoroughly with tap water and dried completely before training and testing.

Posttraining rats were placed in either chamber A or B (day 1). Both cages were wiped with 5% ammonium hydroxide solution, and the overhead room light was on. After 2 min, rats were presented with 5 co-terminating tone-footshock pairings (tone: 2.9 kHz, 82 dB, 30 s; footshock: 1 mA, 2 s) with 1 min inter-trial intervals. Rats were removed 1 min after the last shock and returned to their home cages. The following day, the rats underwent surgery (Fig. 1). After recovery, the rats were placed in the same chamber as on day 1 for 8 min of context testing. The following day entailed a tone-retention test in a context shift. Rats trained in chamber A were tested in chamber B and vice versa. The floor of each chamber was replaced with Plexiglas, which was scattered with sawdust. In addition, the overhead lights were turned off, and each internal chamber wiped with 1% acetic acid solution. These changes produce a reliable context shift. The tone retention test consisted of 1-min baseline followed by 8 min of continuous tone. During training and testing, freezing (Blanchard and Blanchard, 1969) and USVs (Blanchard et al., 1991) were recorded as indices of conditioned fear (see below). After postoperative recovery, pretraining rats underwent identical FC training and testing as posttraining rats. The training-to-testing intervals were similar between the pre-training and postraining groups (Fig. 1).

The stimulus presentations were controlled and freezing data were collected by an IBM-PC computer equipped with the Coulbourn LabLinc Habtest Universal Linc System (Lafayette, IN). Although the collection of the vocalization and freezing data was fully automated, each session was recorded for video and audio analysis off-line, if necessary, using an infrared light source and miniature video camera (CB-21, Circuit Specialists, Mesa, AZ). A 24-cell infrared (IR) activity monitor that detects the movement of the emitted IR (1,300-nm) body heat image from the animals in the x, y, and z axes was mounted on top of each chamber and was used to assess freezing behavior (using a movement episodes setting with 400-ms sampling). In brief, the total time of inactivity exhibited by each animal was measured using a computer program, and freezing was defined as continuous inactivity lasting ≥3 s. Any behavior that yielded an inactivity of <3 s was recorded as general activity. Previous work (Lee and Kim, 1998) demonstrated that freezing scores obtained via IR monitoring and observer time sampling methods consistently correlated higher than r = 0.92. Also, IR freezing monitoring is sensitive to amygdalar manipulations, such as lesions (Kim et al., 2002) and drug infusions (Lee et al., 2001) in a manner similar to human observer scoring (LeDoux et al., 1988; Maren et al., 1996) or a videotrak image analysis system (Kim et al., 1993). For USVs, a heterodyne bat detector (Mini-3; Noldus Information Technology, Wageninge, The Netherlands) was used to transform high-frequency (22 ± 5 kHz) USVs into the audible range (cf. Lee et al., 2001). The output of the bat detector was fed through an audio amplitude filter (Noldus), which filtered out signals falling below an amplitude range that was individually adjusted for each animal. The resulting signal was then sent to an IBM-PC computer equipped with Noldus UltraVox vocalization analysis software. The software converted the signal into vocalization onset and offset times according to the following specifications: an onset was recorded if its duration was ≥30 ms, and the offset was recorded if the onset of the ensuing episode was ≥40 ms apart. If the interval was <40 ms, the two bouts were counted as a single episode.

Morris Water Maze

The Morris water maze protocol consists of three phases of testing: spatial acquisition, spatial retention, and cued acquisition. All testing was completed within 3 h. The 1-day water maze protocol was conducted as described below.

A white circular tank (180-cm diameter) was filled with water (24 ± 2°C) and was surrounded by a variety of extramaze cues. The tank was divided into four quadrants, and four start positions were located at the intersections of the quadrants. Data were recorded using an HVS 2020 automated tracking system (HVS Image, Hampton, England). Before water maze testing, all rats were habituated to the water using a four-trial shaping procedure in which a smaller ring (97 cm) was inserted inside of the tank to decrease the total swimming area. This procedure habituated the rats to the water and taught them to escape from the water by climbing onto a platform. Each rat was first placed on a visible red Lucite platform (15-cm diameter) for 10 s, and then placed at three progressively further distances from the platform where it was al-
lowed 30 s to escape onto the platform. No data were collected during this procedure.

**Spatial acquisition**

In the spatial acquisition phase, the rats learned to find a submerged platform using extramaze cues. A transparent Lucite platform (15-cm diameter) was submerged 2 cm underneath the water in the northeast quadrant of the tank, where it remained for all spatial trials. Each rat participated in twelve trials, which were organized into three blocks of four trials (one trial/start position within a block). Each block was considered a separate test session and blocks were separated by 30 min. For each trial, the rat was given a maximum time of 60 s to locate the platform, after which it remained there for 10–15 s. If it did not locate the platform within 60 s, it was placed on it by the experimenter. The next trial started immediately after removal from the platform. After completion of the fourth trial of the block, the rat was removed from the platform and placed in a temporary holding cage. Swim time (s), swim distance (cm), and swim speed (cm/s) were recorded.

**Spatial retention**

Thirty min after completion of spatial acquisition, one 30-s probe trial was conducted. During this trial, the platform was removed from the tank. The following measures were recorded during the probe trial: quadrant time (percentage time spent in the training quadrant) and platform crossings (the number of times the rat crossed the exact location of the platform).

**Cued acquisition**

In the cued acquisition phase, the rats learned to swim to a visible platform. This task was designed to examine nonmnemonic aspects of water maze performance such as swimming ability, motivation, and visual ability. The visible platform was the same used for shaping. This task was conducted 20 min after completion of the probe trial. Four cued trials were conducted, with start locations randomized and the platform located in a different quadrant during each trial. Swim time, swim distance, and swim speed were recorded. The intertrial interval was ~15 min.

**Immunohistochemistry**

Eight rats (six lesioned and two vehicle) were killed with an overdose of sodium pentobarbital and were transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brain was then removed from the skull, postfixed in paraformaldehyde for 2 h, and transferred to 20% sucrose where it was allowed to sink before sectioning. Brains were sectioned at a thickness of 60 μm on a freezing-sliding microtome. Sections were stored in a cryoprotectant solution (ethylene glycol/glycerol in phosphate buffer) at −80°C until processing for immunohistochemistry. Two adjacent one-in-six series of sections through the rostrocaudal extent of the basal forebrain were processed for choline acetyltransferase (ChAT) and parvalbumin immunohistochemistry to verify the location and specificity of the lesion. Parvalbumin immunostaining is found in GABAergic basal forebrain neurons (Freund, 1989), permitting an assessment of damage to noncholinergic basal forebrain neurons near the lesion site. Basal forebrain sections were processed for ChAT (Chemicon polyclonal goat anti-ChAT; Chemicon International, Temecula, CA) or parvalbumin (Sigma monoclonal mouse anti-parvalbumin; Sigma, St. Louis, MO) according to previously published avidin-biotin complex/diaminobenzidine visualization methods (e.g., Baxter et al., 1995). These methodologies have been described in detail previously (Berger-Sweeney et al., 2000; Cahill and Baxter, 2001).

**Radioenzymatic Determination of Enzyme Activities**

The remaining rats were sedated with CO2, a procedure that does not interfere with measurement of enzyme activity (Berger-Sweeney et al., 1994a), and decapitated. The frontoparietal cortex, hippocampus, and striatum were quickly dissected on an ice-cold metal plate and then stored at −70°C until assay. The samples were resuspended in 0.1 mM Tris, pH 7.4, and 0.02% Triton X-100, sonicated with a probe sonicator, and centrifuged for 10 min at 10,000g. The supernatant was diluted 1:5 with the same reagent and designated as the crude extract used for both assays. Activity of ChAT, which synthesizes acetylcholine, was measured by the formation of [14C]acetylcholine from [acetyl-1-14C]-acetyl-coenzyme A (55.7 mCi/mmol; New England Nuclear, Boston, MA) and choline (Fonnum, 1975). Activity of the enzyme glutamic acid decarboxylase (GAD), which synthesizes GABA, was measured from L-[1-14C]-glutamic acid (40-60 mCi/mmol, New England Nuclear, Boston, MA) using a [14C]CO2 trapping technique (O’Connor et al., 1988). Detailed descriptions of the assay procedure are provided elsewhere (Berger-Sweeney et al., 1994a; Frick and Berger-Sweeney, 2001; Frick et al., 2002). The protein content of the samples was measured using a Bio-Rad (Bio-Rad Laboratories, Hercules, CA) protein assay (Bradford, 1976). Enzyme activity was expressed as nmol of product/h/mg protein.

**Statistical Analysis**

FC data were analyzed using 2 (time of surgery, pre/post) × 2 (lesion status, sham/lesion) analysis of variance (ANOVA; Super-ANOVA; Abacus Concepts, Berkeley, CA) with repeated measures (freezing) or without repeated measures (USVs). For training, freezing during the postshock intervals and total USVs were analyzed. For context and tone testing, freezing in each trial and total USVs were analyzed. For training, spatial and cued acquisition measures were averaged for each block of four trials (spatial) or single trial (cued) and were analyzed using a one-way repeated-measures ANOVA (with lesion status as the independent variable). One-way ANOVAs without repeated measures were carried out on the spatial retention trial measures. For ChAT and GAD activity, separate one-way ANOVAs were conducted for each brain region for each enzyme.
RESULTS

Histological and Neurochemical Analyses

Immunohistochemical analysis of the BF lesions demonstrated cholinergic neuron loss throughout the rostrocaudal extent of the basal forebrain (including the MS, VDB, HDB, and nBM; Fig. 2A–F). In most cases, there was some slight sparing of cholinergic neurons in the most posterior nBM. Parvalbumin immunostaining was generally indistinguishable in the basal forebrain between control and BF-lesioned cases (Fig. 2G–I). In three of the six BF-lesion cases, there was a very small area of necrosis in the basal forebrain, just rostral to the decussation of the anterior commissure. In two cases, parvalbumin-immunoreactive neurons could be seen adjacent to the area of necrosis, although in the third case nonspecific damage appeared to extend beyond the area of necrosis. This is possibly due to the large amount of saporin infused intraparenchymally, to achieve a complete lesion of basal forebrain cholinergic neurons. In addition, some loss of striatal cholinergic interneurons was noted; this was slightly variable in extent among the BF-lesion cases but was most severe in the mid-ventromedial striatum (dorsal to the HDB).

Consistent with the immunohistochemical analysis, the BF lesions produced profound reductions of hippocampal ($F_{(1,20)} = 1087.2, P < 0.0001$) and neocortical ($F_{(1,20)} = 183, P < 0.0001$) ChAT activity (see Table 1 for mean values and percentage depletions). Reflecting the mild loss of striatal interneurons observed histologically, striatal ChAT activity was also slightly, but significantly, decreased in lesioned rats relative to controls ($F_{(1,20)} = 11.6, P = 0.003$). This damage likely resulted from diffusion of
toxin from the BF into the striatum. GAD activity was unaltered by the BF lesion in the neocortex ($F_{(1,20)} = 0.002$, $P = 0.96$) and striatum ($F_{(1,20)} = 0.02$, $P = 0.89$). However, hippocampal GAD activity was slightly, but significantly, decreased in lesioned rats relative to controls ($F_{(1,20)} = 13.2$, $P = 0.0016$). Because the hippocampus contains many GABAergic interneurons, it is impossible to determine whether this depletion is the result of nonspecific damage to basal forebrain GABAergic projection neurons or a downregulation of hippocampal GAD activity in response to cholinergic denervation.

**BF Lesions Reduced USVs But Did Not Affect Freezing**

**Training**

Neither time of surgery (pre- or post-FC training) nor lesion status (sham or lesion) affected freezing during training (Fig. 3A), as indicated by nonsignificant main effects of time of surgery and lesion status, and nonsignificant interactions of trial with these factors ($F_{(4,104)} < 1.2$, $P > 0.35$). Postshock freezing significantly increased over the course of shock presentation ($F_{(4,104)} = 20.8$, $P < 0.0001$). Similar to freezing, neither time of surgery nor lesion status significantly affected total USVs, although there was a trend for a reduction in lesioned rats ($F_{(1,26)} = 3.6$, $P = 0.067$). This effect was due to a reduction of vocalizations in rats operated before FC training; pre-lesion rats vocalized significantly less than pre-control rats, whereas postlesion and postcontrol rats emitted a similar number of vocalizations ($F_{(1,26)} = 9.1$, $P = 0.006$; Fig. 3B).

**Context test**

Rats operated after FC training (post groups) exhibited less freezing during the context test than rats operated before FC training (pre groups), regardless of lesion status (Fig. 4A; $F_{(1,26)} = 5.7$, $P = 0.025$). This effect is likely a general result of the surgical procedure, as anesthesia has been shown to impair memory in rats (Culley et al., 2002). There was no significant interaction of lesion status and time of surgery ($F_{(1,26)} = 0.046$, $P = 0.83$). Freezing decreased during the session in all groups ($F_{(7,182)} = 6.4$, $P < 0.0001$), including the pre-training control and lesioned groups whose % freezing decreased to 56 and 53%, respectively, during the last minute of testing. Time of surgery did not interact with any other effect ($F_{(7,182)} < 1.7$, $P > 0.11$). In contrast to freezing, USVs were affected by both time of surgery and lesion status (Fig. 4C). Pre-lesion rats vocalized less than postlesion rats ($F_{(1,26)} = 6.1$, $P = 0.02$). In addition, lesioned rats vocalized less than control rats, regardless of time of surgery ($F_{(1,26)} = 6.9$, $P = 0.014$). The time of surgery × lesion status interaction was not significant.

**Tone test**

Neither time of surgery nor lesion status affected freezing during the tone test (Fig. 4B), as indicated by nonsignificant main effects and interactions. Freezing decreased during the session in all groups ($F_{(7,182)} = 9.1$, $P < 0.0001$). Similar to freezing, USVs were unaffected by time of surgery and lesion status (Fig. 4D).

**BF Lesions Affected Acquisition of the Spatial, But Not Cued, Water Maze Task**

Lesioned rats performed more poorly on the spatial water maze task than control rats, as illustrated by increased swim times ($F_{(1,28)} = 5.4$, $P = 0.028$; Fig. 5A) and swim distances ($F_{(1,28)} = 5.9$, $P = 0.02$; data not shown) relative to controls. Swim speeds did not differ between the groups (data not shown). Swim times and distances decreased throughout testing in both groups ($F_{(2,56)} = 43.4$ and 55.8, respectively, $P < 0.0001$), but neither lesion status × trial block interaction was significant. In the absence of a significant interaction of lesion status and trial block, it is difficult to interpret the main effect of lesion as a learning impairment; it could also reflect a mild performance deficit. During the probe trial, the quadrant time measure of spatial retention did not differ between the groups (Fig. 5B; $F_{(1,28)} = 2.3$, $P = 0.14$), nor did the platform crossings measure (although there was a trend, $F_{(1,28)} = 4.1$, $P = 0.0519$; mean ±SEM, control = 1.5 ±

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ChAT, choline acetyltransferase.

GAD, glutamic acid decarboxylase.

$^a$Values represent the mean nmol product/h/mg protein ±SEM.

$^b$% depletion versus control.

$^*$P < 0.05 versus controls.
The present study examined the effects on FC of complete 192 IgG-saporin BF cholinergic lesions, given before or after FC training. Surprisingly, these lesions failed to affect retention of fear to context or tone, indexed by freezing. The lesions, however, did affect production of USVs associated with fear. They also mildly impaired Morris water maze performance.

**Pre-Training Lesion Effects On Fear Conditioning**

The failure of pre-training BF lesions to affect FC, as assessed by freezing, is surprising. Scopolamine administered before or after FC training systemically or intrahippocampally impairs contextual, but not tone, FC (Anagnostaras et al., 1995, 1999; Gale et al., 2001; Wallenstein and Vago, 2001; but see Rudy, 1996). These data suggested that BF cholinergic projections to hippocampus and neocortex are required for the acquisition of contextual FC. In the present experiment, nearly-complete depletions of hippocampal and neocortical acetylcholine were achieved, without effect on subsequent retention of contextual fear (as measured by freezing) in rats lesioned before FC training. Thus, our findings are inconsistent with the notion that BF cholinergic neurons are necessary for acquisition of conditioned fear.

What may account for the discrepancies between scopolamine and BF lesion effects on FC? One possibility is that the disruption of cholinergic transmission by scopolamine is fundamentally different from removal of cholinergic neurons. For example, whereas scopolamine may acutely disrupt cholinergic transmission involved in FC, the more permanent lesion may have some allowed compensation or stabilization of activity to occur in the neocortex, hippocampus, and/or BF during the postlesion recovery period that permitted normal acquisition of contextual fear. However, the lesion affected two other behavioral domains (USVs and water maze), arguing against a generalized recovery of function. Another possibility is that pre-training scopolamine impairs contextual FC by binding to cholinergic receptors on GABAergic or other neurons in the BF (Alreja et al., 2000). Systemic scopolamine injections might also impair acquisition of FC via different mechanisms, for example through blockade of muscarinic receptors in the amygdala (Power and McGaugh, 2002), olfactory bulb (Brashear et al., 1986), or anterior thalamus (Mitchell et al., 2002), brain regions that have also been implicated in FC. Because our lesions destroyed the entire BF, the cholinergic projections to these structures were likely reduced or eliminated. In addition, the effects of intrahippocampal scopolamine could be attributed to non-pharmacological effects of scopolamine infusion (e.g., local anesthesia; K. Gale, personal communication). Alternatively, these effects could be due to a deleterious effect of partially disrupted hippocampal function that is not observed with complete disruption of hippocampal dysfunction (Solomon et al., 1983; Baxter and Murray, 2001). For example, scopolamine severely impairs eye-blink conditioning, whereas a hippocampal ablation, which itself has no effect on eye-blink conditioning, blocks scopolamine impairment of eye-blink conditioning (Solomon et al., 1983). However, because complete hippocampal lesions reliably impair contextual fear (Kim and Fanselow, 1992; Phillips and LeDoux, 1992; Maren and Fanselow, 1997), we find this latter explanation unlikely.
Posttraining Lesion Effects on Fear Conditioning

Retention of FC, as measured by freezing, was also unaffected by posttraining BF lesions, suggesting that neocortical and hippocampal cholinergic innervation is not essential for consolidation or retrieval of fear memories established before the lesion. The effects of scopolamine on consolidation of FC are less clear, with some studies reporting no effect of posttraining scopolamine injections (Anagnostaras et al., 1995, 1999) and others reporting an impairment in subsequent context or tone testing (Rudy, 1996; Wallenstein and Vago, 2001). The apparent lack of effect of posttraining BF lesions on conditioned fear is interesting, given that BF cholinergic lesions can induce retrograde amnesia for other types of hippocampal-dependent learning (e.g., social transmission of food preferences; Vale-Martínez et al., 2002).

Lesion Effects on Ultrasonic Vocalizations

We have thus far neglected lesion effects on FC as measured by USVs, which were severely impaired by BF lesions. Rats lesioned before FC training did not exhibit USVs during training or testing. Rats lesioned after FC training were impaired in USV production during contextual fear testing and tended to emit fewer USVs in the tone test.

These data may indicate that USVs are a more sensitive index of fear memory than freezing. Although our data cannot address this possibility directly, it is noteworthy that all previous studies examining scopolamine effects on FC used freezing as the dependent measure. Hence, even if USVs are a more sensitive measure of conditioned fear than freezing, the absence of a cholinergic lesion effect on freezing during the retention test in our study suggests that the lesions, at best, produce a subtle effect on conditioned fear. Because the BF lesions were without effect on FC as measured by freezing, we are hesitant to suggest that the USV data reflect an impairment in conditioned fear, per se.

Different brain structures mediate different fear responses. Our cholinergic lesions may have disrupted the BF output pathway for USV production, rather than impaired associative learning. Infusion of the cholinergic agonist carbachol into the BF induces USVs similar to footshock-induced USVs (Brudzynski et al., 1991; Brudzynski, 1994). Infusions of glutamate into the laterodorsal tegmental area, which sends cholinergic fibers to the BF, also induce USVs (Brudzynski and Barnabi, 1996). In contrast, the lateral hypothalamus and the ventral periaqueductal gray mediate blood pressure and freezing conditioned responses, respectively (LeDoux et al., 1988). In addition, within the BF, different neuronal populations may mediate separate fear responses; perhaps the cholinergic neurons mediate USVs and GABAergic neurons mediate freezing, a possibility that invites direct experimental investigation.

Lesion Effects on Water Maze Performance, and the Question of Lesion Specificity

The BF lesions in this study impaired performance in the 1-day water maze protocol, indicated by main effects of lesion status on performance during spatial acquisition. In addition, lesioned rats performed worse during the probe trial relative to controls, al-
though these differences were not statistically significant. Because of the large number of lesioned rats tested, we had exceptional statistical power to detect lesion effects in this task.

The absence of a lesion status by trial interaction in spatial acquisition might suggest that the lesion-induced impairment is not one of spatial learning per se; similarly, the relatively intact performance on probe trials argues that any lesion-induced impairment in spatial navigation is not severe. Nevertheless, these findings, which differ from the results of other studies using intraparenchymal administration of 192 IgG-saporin (Berger-Sweeney et al., 1994b; Torres et al., 1994; Baxter et al., 1995; Baxter and Gallagher, 1996; Dornan et al., 1997), raise the question of lesion selectivity. More restricted lesions of the BF (limited to the MS/VDB or nBM) are without effect on the 1-day water maze task (Ricceri et al., 2000). In the present study, we found a small but statistically significant depletion of striatal ChAT activity and hippocampal GAD activity, and a loss of striatal cholinergic interneurons detected with immunohistochemistry. Effects of intraparenchymal 192 IgG-saporin on striatal cholinergic interneurons have been reported previously (Berger-Sweeney et al., 1994b). Effects of 192 IgG-saporin lesions on GAD activity have not been examined before in rats, although GAD activity has been used to examine nonspecific effects of a similar cholinergic toxin in mice (Berger-Sweeney et al., 2001). Because we detected some variable, very restricted nonspecific damage in some cases examined immunohistochemically, the loss of hippocampal GAD activity may reflect loss of GABAergic input from the MS/VDB. Alternatively, this loss could be a secondary consequence of cholinergic depletion, because other sources of GAD activity in hippocampus include GABAergic interneurons as well as glia. It might be difficult or impossible to achieve such severe damage to BF cholinergic neurons using intraparenchymal injections without causing some unintended damage.

It is unclear whether the water maze impairment is due to a severe depletion of cholinergic input to hippocampus and neocortex, unintended damage to noncholinergic BF neurons and striatal cholinergic interneurons, or a combination of these factors. We are agnostic as to which of these possibilities is correct. Most importantly, even in the face of a statistically significant impairment in water maze performance, there was no significant effect of the BF lesions on FC. Hence, regardless of any unintended damage caused by the lesions, the absence of effect on FC supports the notion that BF cholinergic neurons are not essential for acquisition or retrieval of conditioned fear.

**CONCLUSIONS**

Using a selective immunotoxin for BF cholinergic neurons, we removed cholinergic input to hippocampus and neocortex either before or after acquisition of conditioned fear. In neither case did lesioned rats exhibit an impairment in conditioned fear to tone or context measured by freezing in the retention test. The lesions impaired another measure of conditioned fear, USVs, perhaps suggesting a role for BF cholinergic neurons in USV production. Our findings suggest that BF cholinergic projections to hippocampus and neocortex are not necessary for the acquisition, consolidation, or retrieval of contextual fear. These findings also add to the growing compilation of data indicating a more restricted role for BF cholinergic neurons in learning and memory processes than has been commonly accepted (for review, see Baxter and Chiba, 1999;
Baxter and Murg, 2002. Future studies may examine more closely the involvement of BF cholinergic neurons in particular output pathways for conditioned fear (e.g., USVs or conditioned heart rate vs freezing), as well as different forms of FC that may require different aspects of forebrain function (e.g., trace fear conditioning).

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REFERENCES


