Neural Substrates of Olfactory Discrimination Learning with Auditory Secondary Reinforcement. I. Contributions of the Basolateral Amygdaloid Complex and Orbitofrontal Cortex

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The basolateral amygdaloid complex (BLA) and orbitofrontal cortex (OFC) share extensive reciprocal connections, and interactions between these regions likely contribute to both mnemonic and affective processes. The present study examined the potential differential contributions of the BLA and OFC to performance of an olfactory discrimination task that incorporates auditory conditioned reinforcement and to expression of immediate post-shock freezing behavior. Damage to the BLA had little effect on performance of the conditioned reinforcement task but abolished immediate post-shock freezing behavior. In contrast, damage to OFC resulted in both a mild but significant performance decrement in the conditioned reinforcement task and a significant attenuation of immediate post-shock freezing behavior. These findings suggest that immediate post-shock freezing behavior is likely critically dependent upon interactions between the BLA and OFC. However, although mnemonic processes underlying accurate performance of the conditioned reinforcement task might be supported by OFC in part, such processes are independent of either the BLA or interactions between these two regions.

BEHAVIORAL PARADIGMS THAT incorporate second-order schedules of reinforcement are particularly well-suited for assessing the acquired motivational value of initially-neutral stimuli (Mackintosh, 1974). In secondary reinforcement tasks, a stimulus first becomes associated with a primary reinforcer, such as food or water, and subsequently gains the ability to support behavior in the absence of primary reinforcement. With respect to the neural substrates of secondary reinforcement learning, aspirative amygdalectomy was shown to disrupt performance of a second-order visual discrimination task in non-human primates, leading to the suggestion that the amygdaloid complex is an integral component of the neural circuitry that supports maintenance of associations between secondary reinforcers and primary reward (Gaffan & Harrison, 1987). Additional findings from a number of laboratories suggest that the amygdaloid complex, and in particular its' basolateral components (BLA; lateral, basal, accessory basal nuclei), contribute to second-order learning in some behavioral paradigms in both rodents (Cador et al., 1989; Everitt & Robbins,

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1992; Burns et al., 1999; Hatfield et al., 1996; Whitelaw et al., 1996; Gewirtz & Davis, 1997) and non-human primates (Parkinson et al., 2001). By contrast, Malkova et al., (1997) found that fiber-sparing excitotoxic lesions of the amygdaloid complex fail to disrupt performance of a secondary reinforcement task. Thus a characterization of the precise role of the amygdaloid complex in secondary reinforcement remains elusive.

In order to evaluate factors that influence BLA contributions to secondary reinforcement, we have recently developed a concurrent-cue olfactory discrimination procedure for use with rodents that incorporates auditory conditioned reinforcement and that permits ready manipulation of a variety of task parameters. This task is in many respects analogous to the non-human primate visual discrimination procedure used by Malkova et al. (1997). Thus one aim of the present study was to evaluate, in rats, the effects of excitotoxic damage to the BLA on performance of this task following extensive pre-surgical training.

The BLA shares extensive reciprocal connections with numerous sensory and association cortical regions (Price et al., 1987), which ultimately contribute to the functions it performs. Specifically, direct connections between the BLA and both medial and orbital components of frontal association cortex have been identified in both primates (Porrino et al., 1981; Carmichael & Price, 1995) and rodents (Krettek & Price, 1977). Nevertheless, the information processing capacities of the BLA and frontal association cortex are likely quite distinct, as suggested by differences between the BLA and orbitofrontal cortex (OFC) in the time course over which stimulus specific rate modulation and alterations in functional connectivity can occur (Schoenbaum et al, 1998; 1999). With this in mind, a second aim of the present study was to compare the effects of post-training excitotoxic damage to OFC to that of damage to the BLA on performance of the conditioned reinforcement task.

Consistent with the finding that excitotoxic amygdaloid damage, which included damage to the BLA, failed to disrupt performance of a conditioned reinforcement task in non-human primates (Malkova et al, 1997), we predicted that BLA damage would not disrupt performance in the present study. Consistent with the finding aspirative lesions of OFC resulted in delay-dependent performance deficits in an olfactory delayed non-matching task when small stimulus samples were utilized, we predicted that damage to this region would disrupt task performance when long intertrial intervals (ITI) were utilized. To these ends, rats were trained to perform the conditioned reinforcement task, and then excitotoxic lesions were directed at either the BLA or OFC. Post-surgical performance was assessed first with varying intertrial intervals and then during extinction of the conditioned reinforcer. Finally, immediate post-shock freezing behavior was assessed as a separate behavioral index of lesion effect.

MATERIALS AND METHODS

Subjects

Male Sprague-Dawley rats weighing between 225g and 250g prior to training served as subjects and were housed individually in suspended wire mesh cages in a temperature— and humidity-controlled vivarium on a 12hr light schedule with lights on at 0700hrs. Three days prior to the onset of behavioral training subjects were placed on a water restriction schedule allowing 10min free access to water daily. This restriction schedule remained in effect throughout the study, except for an interval beginning two days prior to surgery and ending 10–12 days following surgery and for two days prior to the assessment of freezing

behavior. Subjects were provided continuous access to standard laboratory rat chow throughout the course of the study.

Apparatus

Olfactory discrimination training and testing procedures were conducted utilizing four identical custom-built plexiglass behavioral chambers, similar in design to those used previously in our laboratory to examine concurrent-cue olfactory discrimination learning (Otto & Garruto, 1997). A 28v houselight and a 2.7kHz pure tone generator were centered 7cm and 10cm from the ceiling, respectively, on one wall of each behavioral chamber. Two conical sniff portals (3cm diameter, 4cm from the floor) and a single water cup were situated below the houselight and tone generator. In order to encourage simultaneous odor sampling prior to response generation, the odor portals were closely juxtaposed, 4cm apart on center and angled toward each other. The water cup was centrally located and situated 3cm above the nose-poke portals. Nose-poke responses directed toward each odor portal and water cup responses were registered by photocells situated at the opening of each sniff portal and at the threshold of the water cup. Odors (mint extract [McCormick], orange extract [McCormick], amyl acetate, and pyridine [15% in propylene glycol]) could be independently delivered to the sniff portals utilizing an automated odor delivery system similar in design to that described previously (Cousens & Otto, 1998). Each chamber was housed within a sound-attenuating cubicle (60X60X40cm; Med Associates Inc., St. Albans, VT) equipped with a large capacity pole fan blower, which provided continuous ventilation of the inner behavioral chamber and background masking noise. All experimenter-imposed trial events, including odor delivery, tone delivery, and water delivery, were controlled by microcomputer.

Immediate post-shock freezing behavior was assessed in a separate behavioral chamber (10X25X30cm) with two opposing plexiglass walls, two metal walls, a metal ceiling, and a floor consisting of sixteen 6mm-diameter stainless steel rods spaced 1.7cm apart on center. Three 2cm wide black horizontal stripes lined two adjacent sides of the chamber. The chamber was contained within a sound-attenuating enclosure identical to that described above and was illuminated with a single 28v bulb positioned 6cm from the ceiling on one of the metal sides. A large capacity pole fan blower mounted on the side of the enclosure continually ventilated the inner behavioral chamber and provided masking noise. Shock delivery was coordinated by a microcomputer.

Procedures

Preoperative olfactory discrimination training

Preoperative training on the olfactory discrimination task was comprised of five successive phases, culminating in the performance of multiple-trial olfactory discrimination problems based on presentation of an auditory cue following each correct trial (see below *Phase 5*). Since access to primary water reward was only provided following four consecutive correct trials during this final phase, and since odor valence varied between problems, the auditory cue provided the only feedback regarding odor valence during performance of a given problem. Thus, reinforcing properties acquired by the auditory cue served to guide olfactory discrimination prior to access to primary reward. This task was modeled after a

visual discrimination conditioned reinforcement task originally described by Gaffan & Harrison (1987).

Phase 1. During an initial response-shaping phase, subjects were trained to make nose-poke responses to receive water rewards during a single 96-trial session. During this session, no odors were presented, and responses to either sniff portal were rewarded with the delivery of 0.025ml water. Each trial began with the illumination of the houselight, signaling that a positive response-reinforcer contingency was in effect. The first response made thereafter resulted in the onset of a pure tone, which would ultimately serve as the conditioned reinforcer, followed 2sec. later by the delivery of water. Only nose-poke responses of duration greater than 500msec had programmed consequences. Offset of both chamber illumination and tone coincided with cessation of water delivery and signaled onset of a 5sec. intertrial interval (ITI). This general trial structure was maintained throughout the remaining training phases, with exceptions as noted below.

Phase 2. Subjects were next trained on a series of concurrent-cue odor discrimination problems. Each daily session consisted of two blocks of 48trials, or 96trials per daily session. For each trial of the first session, mint extract was designated as the positive discriminative stimulus (S_D+) and was presented from either the left or right sniff portal at a rate of 0.75l/min. One of the remaining three odors was designated as the negative discriminative stimulus (S_D–) and was presented from the other sniff portal at the same rate. Odors were presented for 1sec. prior to chamber illumination in order to allow odor sampling prior to registration of a response. A single nose-poke response to the portal emitting the S_D+ resulted in offset of both odors and onset of the tone and water according to the trial structure of Phase 1. Alternatively, a single nose-poke response to the portal emitting the S_D– resulted in offset of both odors and the house light and immediate onset of the ITI. On each trial, both the spatial sequence of S_D+ presentation (left versus right odor portals) and the identity of the S_D– were determined by a pseudorandom sequence. With the exception of the initial session of this phase, the S_D+ was changed following attainment of a criterion level of 40 correct choices on the preceding block of 48 trials. When each of the four odors had served as the S_D+, subjects were advanced to the next phase.

Phase 3. Subjects were next trained to use the auditory stimulus to guide performance. During this phase, subjects were given 24 odor discrimination problems per daily session. Each problem was separated by a 2min. interproblem interval (IPI) and consisted of a series of trials similar to those described above, except that water reward was not delivered following each correct trial. Although the tone was presented following each correct trial, water was delivered only following the third consecutive correct trial. Although the S_D+ remained the same for each problem within a session, the S_D– varied between problems according to a pseudorandom sequence. Each problem could be terminated by three consecutive correct trials, three consecutive incorrect trials, or a maximum of 20 trials with neither previous occurrence. During this phase, the S_D+ was changed between sessions following attainment of a criterion level of performance of 20 correctly solved problems, and subjects were advance to the next phase only after each of the four odors had served as the S_D+.

Phase 4. The structure of the fourth phase of training was identical to that of the previous phase, with two exceptions. First, subjects were required to complete either four consecutive correct trials or four consecutive incorrect trials to terminate a problem. Second, unlike the previous phase in which a single odor served as the S_D+ during each of the

24 problems in a session, each odor served as the S_D+ for six of the 24 problems. Initially, the S_D+ was changed every six problems; however, once two consecutive sessions had been completed at the criterion level of performance of 20 correct problems per session, the S_D+ was changed every three problems. Thus, at the end of this phase of training, one odor served as the S_D+ on problems 1–3 and 13–15, and another served as the S_D+ on problems 4–6 and 16–18, and so on. Subjects were advanced to the fifth stage of training after completing two consecutive sessions with at least 20 correct problems per session.

Phase 5: Conditioned reinforcement. The fifth and final stage of training was identical to that of the preceding phase, except that the S_D+ on each problem was determined by a pseudorandom sequence. Each of the four odors served as the S_D+ and the S_D– for six problems, such that all possible positive-negative odor combinations were presented twice during each session. Correction trials were allowed during only the first session of this phase, preventing the termination of a problem following incorrect responses. Unlike the previous phases of training, the reinforcement valence of odors presented on a given problem was unrelated to reinforcement valence on previous problems; thus, accurate performance during this final phase required that odor selection be base on auditory feedback for correct responses or lack of auditory feedback for incorrect responses. Subjects continued to receive daily overtraining sessions until five consecutive sessions had been completed at the criterion level of performance of 20 correctly solved problems per session.

Surgery

Following accurate performance of the conditioned reinforcement task, subjects were permitted free access to water for 48hrs in preparation for surgery. Subjects were matched for presurgical performance and were assigned to groups slated to receive either excitotoxic lesions of the BLA (n=8) or OFC (n=8) or sham surgery (BLA sham-lesioned, n=6; OFC sham-lesioned, n=6). Subjects were anesthetized with sodium pentobarbital (50mg/kg; ip) and placed in a standard stereotaxic frame. During surgery, isoflurane was administered as an inhalant anesthetic as needed. For BLA-lesioned subjects, a microsyringe was lowered to a position within the BLA (3.0mm caudal to bregma; 4.9mm lateral to bregma; 8.5mm ventral to bregma) and was allowed to settle for 2 min. NMDA (0.4ul per hemisphere; 20ug/ul; pH = 7.4) was then infused at a rate of 0.1ul/min. Following a 1min interval to allow for diffusion, the microsyringe was raised to a second site (8.0mm ventral to bregma) and was left in place for an additional minute prior to a second infusion (0.2ul per hemisphere). Following the second infusion, the microsyringe was left in place for an additional 2min before its removal. For OFC-lesioned subjects, similar injections were made within OFC (0.5ul per hemisphere; 20ug/ul; pH = 7.4; 3.2mm rostral to bregma; 1.2mm lateral to bregma; 4.2mm ventral to bregma). BLA sham-lesioned and OFC sham-lesioned subjects were treated identically to their lesioned counterparts, except no infusions were made. Following removal of the microsyringe, the wound was sutured with surgical staples and cleaned.

Postoperative Behavioral Assessment

Discrimination performance with secondary reinforcement and varying ITIS. Subjects were provided a 7–10day postoperative recovery period, after which the water restriction schedule was reinstated. Two days thereafter, postoperative assessment of olfactory dis-

crimination performance began. In order to examine the effects of lesions on task performance with varying mnemonic demands, subjects received a series of daily sessions with systematically varying ITIs. Specifically, six sessions were conducted with the following ITI durations (sec): 5, 5, 10, 5, 20, 5.

Performance during extinction sessions. Because either BLA or OFC could potentially support behavioral adjustments following a change in reward contingency, we also examined the effects of lesions on behavioral extinction of the conditioned reinforcer by eliminating access to primary water reward following correctly solved problems. Subjects were given an additional four sessions following completion of the protocol described above. During these sessions only, if neither the S_D+ nor the S_D– were selected within 5sec of light onset, the trial was terminated and designated a 'null trial'. Following four consecutive null trials, the problem was terminated and designated a 'null problem'. Thus, according to this modified protocol, trials could be terminated by a correct response, an incorrect response, or failure to respond within 5sec of light onset, and problems could be terminated by four consecutive correct trials, four consecutive incorrect trials, four consecutive null trials, or a maximum 20 trials without four consecutive correct, incorrect, or null trials. In order to establish a performance baseline with additional null trial and null problem potential outcomes, water was available during the first of these four sessions. However, for the following three sessions, no water was delivered in the testing apparatus.

Immediate post-shock freezing. Immediate post-shock freezing behavior is critically dependent on the BLA (Maren, Aharonov & Fanselow, 1996; Cousens & Otto, 1998), and provides an independent behavioral index. Following the final extinction session, subjects were afforded free access to water in their home cage, and two days thereafter, immediate post-shock freezing behavior was assessed. Mild footshock (0.8mA) was delivered for 2sec, 4min after onset of a single 7min test session. Freezing behavior, defined as a crouching posture and an absence of any visible movement except associated with respiration (Bolles, 1970), was continually assessed throughout the session by an experimenter blind to lesion condition.

Histological Preparation

Following the completion of all behavioral procedures, subjects were administered a lethal dose of sodium pentobarbital (100mg/kg) and were perfused transcardially with normal saline followed by a 10% solution of pH buffered formalin. Brains were immersed in a formalin-sucrose solution (0.3g/l) for 48hrs and were subsequently sectioned at 50um. Sections were mounted on gelatin-subbed microscope slides and stained with cresyl violet. Lesion extent was evaluated with the aid of a light microscope.

RESULTS

Basolateral Amygdaloid Complex

Histological Assessment

NMDA infusions directed at the BLA resulted in substantial cell loss that generally encompassed the lateral, basal, and accessory basal nuclei (see Figure 1). With the exception of one subject excluded from behavioral analyses due to unilateral damage, all subjects sustained nearly complete bilateral destruction of these nuclei. Minor cell loss was

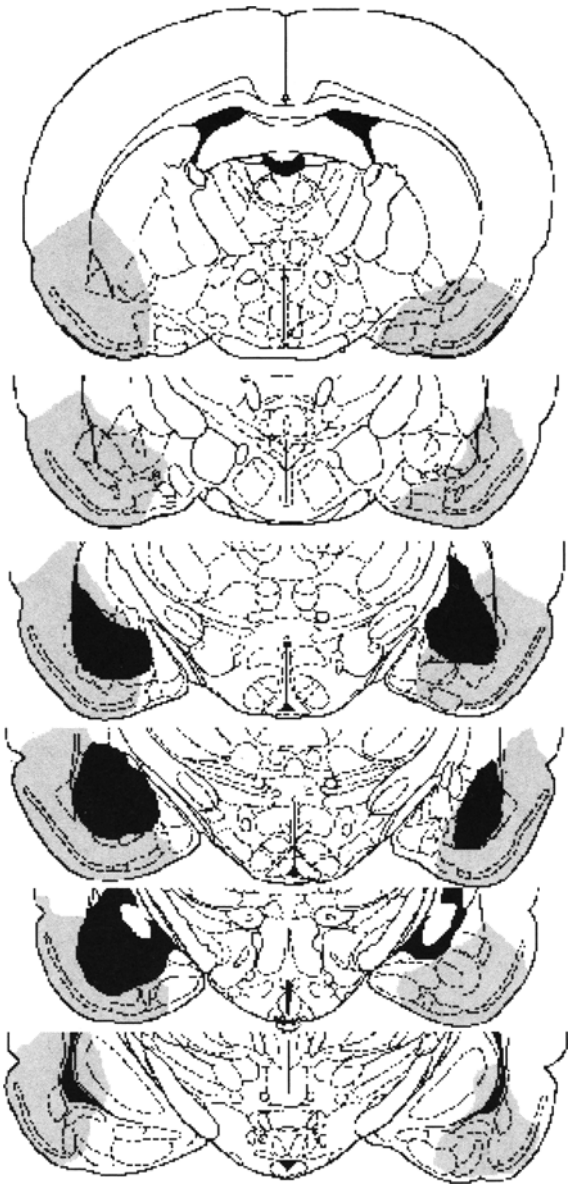

FIG. 1. Minimum extent (black) and maximum extent (gray) of NMDA-induced cell loss for BLA-lesioned subjects (n=7).

also observed within the central amygdaloid nucleus, periamygdaloid cortex, anterior piriform cortex, and the ventral bank of extreme anterior perirhinal cortex.

Postoperative Discrimination Performance

As illustrated in Figure 2, both BLA-lesioned and sham-lesioned subjects correctly solved a high percentage of discrimination problems during each of the six postoperative

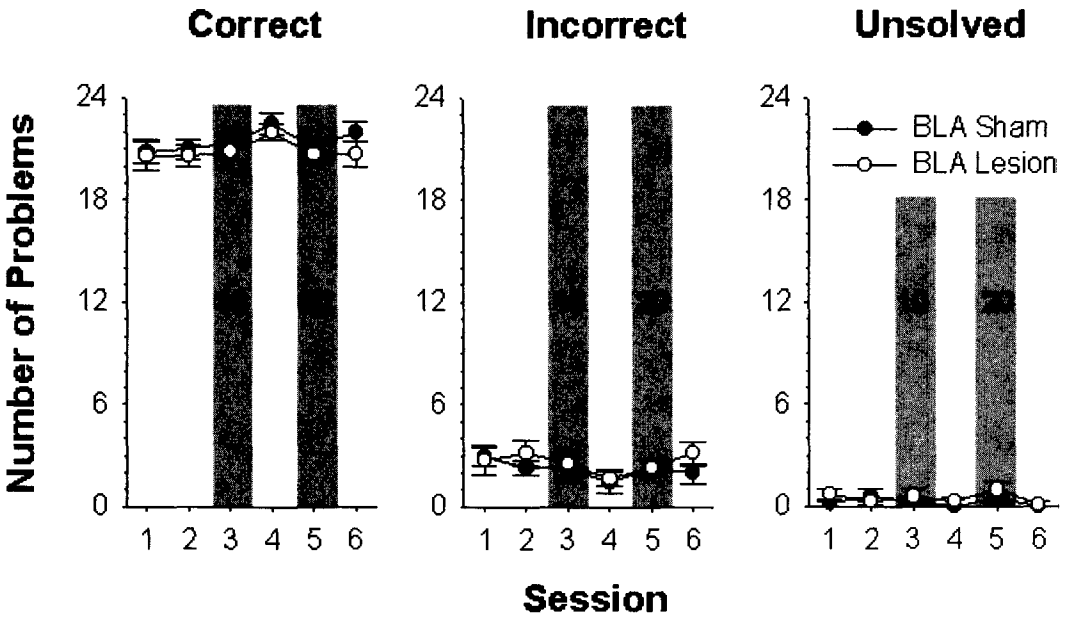

FIG. 2. Problem outcome frequency for BLA sham-lesioned subjects (filled circles) and BLA-lesioned subjects (open circles) across the initial six 24-problem post-surgical olfactory discrimination sessions. ITI duration was 5sec. during all sessions except during Sessions 3 and 5, when ITI duration was 10sec. and 20sec., respectively.

discrimination sessions. A two-way ANOVA with one repeated measure (session) conducted on correct problems per session failed to reveal significant main effects of lesion condition (F[1,11]=0.611, p=0.4510) or session (F[5,55]=2.039, p=0.0873) or an interaction between these factors(F[5,77]=0.205, p=0.9588). A similar ANOVA conducted on incorrect problems per session failed to reveal significant main effects of lesion condition (F[1,11]=0.325, p=0.5801) or session (F[5,55]=1.392, p=0.2416) and failed to reveal a significant interaction between these factors (F[5,77]=0.504, P=0.7719). Finally, an ANOVA conducted on unsolved problems per session also failed to reveal significant main effects of lesion condition (F[1,11]=1.102, p=0.3164) or session (F[5,55]=1.790, p=0.1301) and failed to reveal significant interaction between these factors (F[5,77]=0.815, p=0.5440). Thus, damage to the BLA failed to disrupt the ability of subjects to accurately solve discrimination problems, despite prolongation of the ITI to 10 and 20 seconds during Sessions 3 and 5, respectively.

Although BLA damage did not affect problem outcome, BLA-lesioned subjects required slightly more trials to complete each session compared to sham-lesioned subjects (see Figure 3). A two-way ANOVA with one repeated measure (session) conducted on total trials per session revealed a main effect of lesion condition (F[1,11]=6.727, p=0.0250) but neither a main effect of session (F[5,55]=2.965, p=0.193) nor an interaction (F[5,77] =8.22, p=0.5393). A similar ANOVA conducted on correct trials per session failed to reveal significant main effects of lesion condition (F[1,11]=3.262, p=0.0983) or session (F[5,55]=2.305, p=0.0568) and also failed to reveal significant interaction between these factors (F[5,77]=0.645, p=0.6662). Finally, an ANOVA conducted on incorrect trials per session revealed main effects of both lesion condition (F[1,11]=7.240, p=0.0210) and

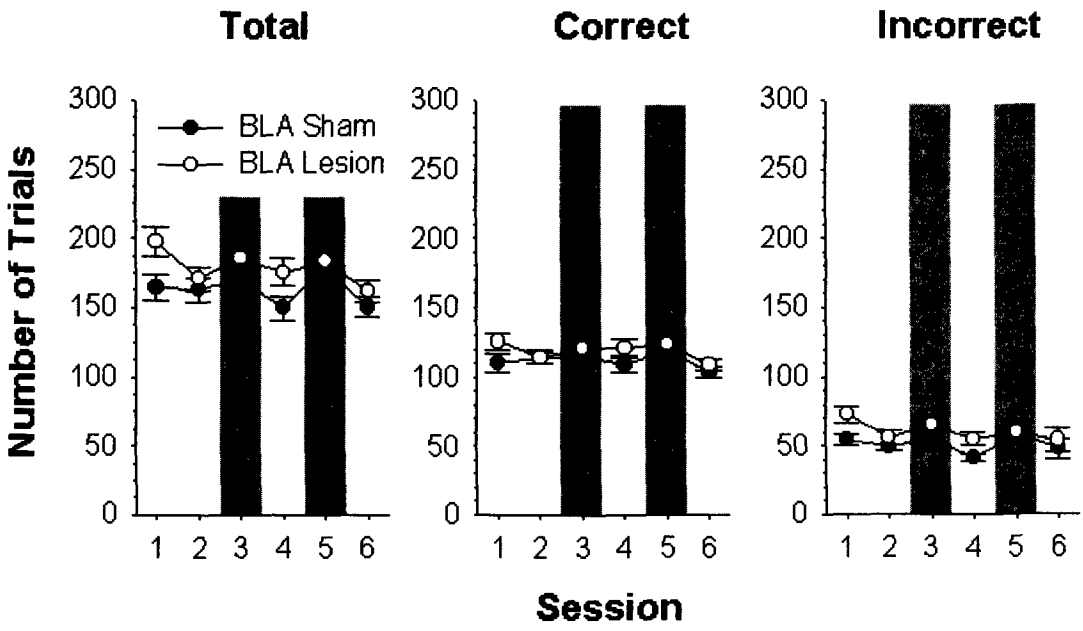

FIG. 3. rial frequency for BLA sham-lesioned subjects (filled circles) and BLA lesioned subjects (open circles) across the initial six 24-problem post-surgical olfactory discrimination sessions. ITI duration was 5sec. during all sessions except during Sessions 3 and 5, when ITI duration was 10sec. and 20sec., respectively.

session (F[5,55]=3.657, p=0.0063) but no interaction (F[5,77]=0.817, p=0.5424). Post-hoc comparisons (Student-Newman-Keuls) revealed that both groups of subjects exhibited a greater number of incorrect trials on Session 1 relative to Session 4. Thus, BLA-lesioned subjects exhibited more errors per session than did their sham counterparts, contributing to a greater total number of trials required to complete each session.

Behavioral Extinction of the Conditioned Reinforcer

Behavioral extinction was assessed by eliminating access to primary reward. BLA-lesioned and sham-lesioned subjects increasingly failed to complete discrimination trials as extinction sessions progressed (see Figure 4). A two-way ANOVA with one repeated measure (session) conducted on total trials per session revealed a significant main effect of session (F[2,22]= 16.270, P<0.0001) but failed to reveal either a significant main effect of lesion condition (F[1,11]=0.242, P=0.6327) or significant interaction between these factors (F[2,22]=0.525, P=0.5989). Post hoc comparisons conducted on session revealed that the total number of trials decreased significantly between successive sessions. A similar ANOVA conducted on correct trials per session revealed a main effect of session (F[2,22]=116.0896, p<0.0001) but neither a main effect of lesion condition (F[1,11]=.1387, p=0.7166) nor an interaction (F[2,22]=0.0628, p=0.9393), as did an ANOVA conducted on incorrect trials per session (F[2,22]= 32.656, p<0.0001; F[1,11]=0.129, p=0.7264; F[2,22]=0.892, p=0.4242). Post hoc comparisons revealed that the number of correct trials decreased significantly for both groups with each successive session and that the number of incorrect trials decreased between the second and third sessions. In contrast, the number of null trials per session increased as extinction sessions

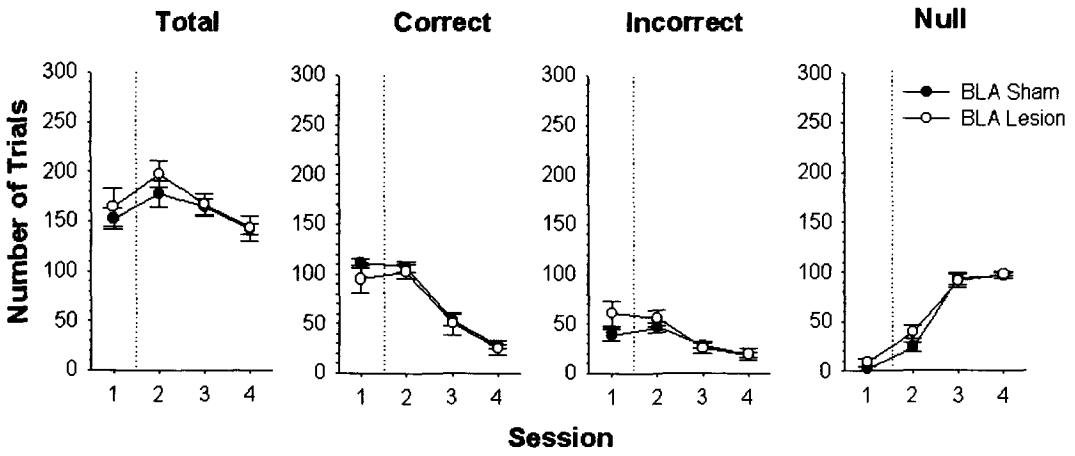

FIG. 4. Trial frequency for BLA sham-lesioned subjects (filled circles) and BLA-lesioned subjects (open circles) across the four extinction sessions. ITI duration was 5sec. during all sessions. Access to primary water reward was withheld on Sessions for each but the first session.

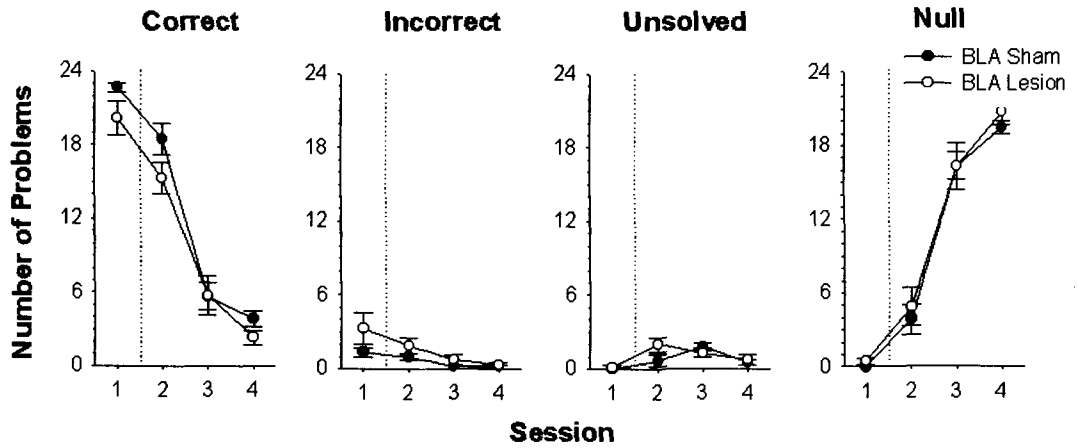

FIG. 5. Problem outcome frequency for BLA sham-lesioned subjects (filled circles) and BLA-lesioned subjects (open circles) across the four extinction sessions. ITI duration was 5sec. during all sessions. Access to primary water reward was withheld on Sessions for each but the first session.

progressed. An ANOVA conducted on null trials per session revealed a significant main effect of session ($F[2,22]= 113.052$, $p<0.0001$) but neither a main effect of lesion condition ($F[1,11]=2.869$, $p=0.1184$) nor an interaction ($F[2,22]=0.921$, $p=0.4131$). Post hoc comparisons revealed that the number of null trials increased significantly between each successive session for both groups.

The increase in null trials observed for both BLA-lesioned and sham-lesioned subjects corresponded with a decrease in correct and incorrect problems per session and an increase in null problems as extinction sessions progressed (see Figure 5). A two-way ANOVA with one repeated measure (session) conducted on correct problems per session across the three extinction sessions revealed a main effect of session ($F[2,22]=206.93$, $p<0.0001$) but failed to reveal either a main effect of lesion condition ($F[1,11]=1.24$, $p=0.2887$) or an

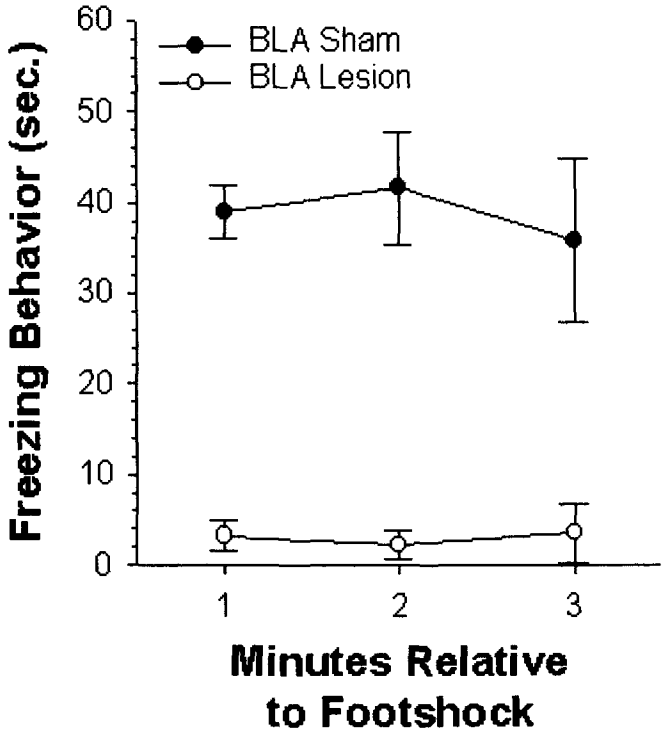

FIG. 6. Freezing behavior during the 3min. period immediately following footshock presentation (0.8mA; 2sec.) for BLA sham-lesioned subjects (filled circles) and BLA-lesioned subjects (open circles).

interaction (F[2,22]=2.55, p=0.1009). Post-hoc comparisons revealed that the number correct problems per session significantly declined for both groups between each successive extinction session. A similar ANOVA conducted on incorrect problems per session revealed a significant main effect of session (F[2,22]=10.33, p=0.0007) but failed to reveal either a significant main effect of lesion condition (F[1,11]=1.371, p=0.2664) or significant interaction between these factors (F[2,22]=0.863, p=0.4358). Post-hoc comparisons revealed that the number of incorrect problems significantly declined for both groups between extinction Sessions 1 and 2. Correspondingly, an ANOVA conducted on null problems per session revealed a main effect of session (F[2,22]=217.012, p<0.0001) but failed to reveal either a main effect of lesion condition (F[1,11]=0.193, p=0.6691) or an interaction (F[2,22]=0.371, p=0.6943). Post-hoc comparisons revealed that the number of null problems per session significantly increased for both groups between each successive extinction session.

IMmediate Post-shock Freezing Behavior

As described previously, freezing behavior was assessed for a 3min period immediately following delivery of a single footshock; freezing behavior during this period is illustrated in Figure 6. Damage to the BLA effectively abolished immediate post-shock freezing behavior. A two way ANOVA with one repeated measure (minute) conducted on seconds freezing per minute during the 1min period immediately preceding footshock and the 3min

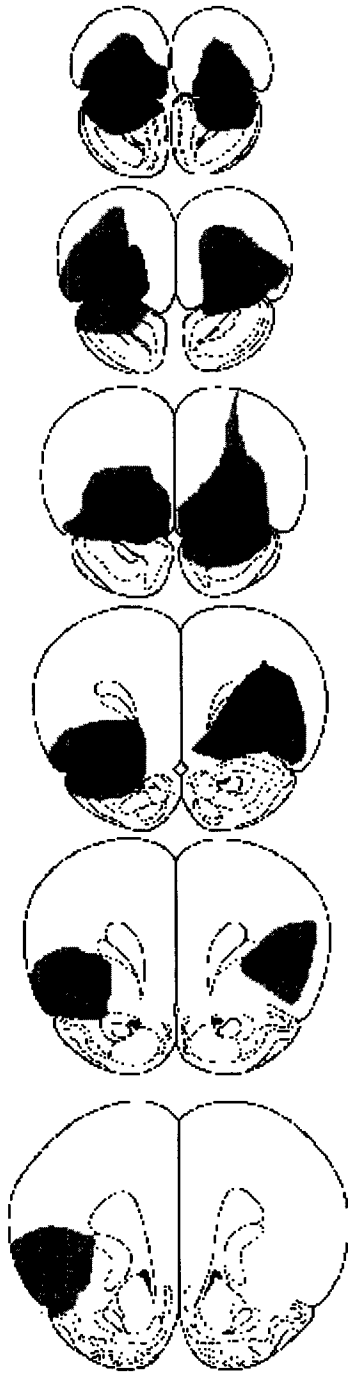

FIG. 7. Minimum extent (black) and maximum extent (gray) of NMDA-induced cell loss for OFC-lesioned subjects (n=7).

period following footshock revealed main effects of both minute (F[3,33]=23.4, p<0.0001) and lesion condition (F[1,11]=40, p<0.0001) and an interaction (F[3,33]=17.4, p<0.0001). Pairwise comparisons revealed that BLA-lesioned subjects spent less time freezing during each of the three minutes following footshock compared to sham-lesioned subjects and no more time freezing during this period compared to their own pre-shock level (0sec for both groups).

Orbitofrontal cortex

Histological Assessment

NMDA infusions directed at orbital PFC resulted in a pattern of cell loss that encompassed much of ventrolateral and lateral orbital cortices and ventral agranular insular cortex (see Figure 7). All subjects also sustained minor damage to piriform cortex, and several sustained damage to medial orbital cortex and the dorsal anterior olfactory nucleus. One subject was excluded from behavioral analyses due to unintended damage to the olfactory peduncle during histological preparation.

Post-operative Discrimination Performance

OFC-lesioned subjects completed fewer discrimination problems correctly than did their sham-lesioned counterparts during the six postoperative discrimination sessions; these data are illustrated in Figure 8. A two-way ANOVA with one repeated measure (session) conducted on correct problems per session revealed main effects of both lesion condition (F[1,11]=11.756, p=0.0056) and session (F[5,55]=6.289, p=0.0001) but no interaction (F[5,77]=0.729, p=0.6044). Post-hoc comparisons performed on session revealed a significantly greater number of correct problems on Session 4 relative to all other sessions. A similar ANOVA conducted on incorrect problems per session also revealed significant main effects of lesion condition (F[1,11]=14.464, p=0.0029) and session (F[5,55]=6.398, p<0.001), but failed to reveal interaction these factors (F[5,77]=0.929, p=0.4693). Post-hoc comparisons performed on session revealed a greater number of incorrect problems on Session 1 relative to Sessions 4, 5, and 6, and a greater number of incorrect problems on Sessions 2 and 3 relative to Session 4. An ANOVA conducted on unsolved problems per session failed to reveal a significant main effect of lesion condition (F[1,11]=2.88, p=0.1177) or significant interaction between lesion condition and session (F[5,77]=1.57, P=0.1845) but revealed a significant main effect of session (F[5,55]=2.44, P=0.0456). Post-hoc comparisons performed on session revealed a significantly greater number of unsolved probems on Session 5 relative to Sessions 1, 4, and 6. Thus, OFC damage mildly impaired the ability of subjects to accurately solve discrimination problems, but this deficit was not exacerbated by prolongation of the ITI.

As shown in Figure 9, OFC-lesioned subjects also required more trials to complete discrimination sessions compared to sham-lesioned subjects. A two-way ANOVA with one repeated measure (session) conducted on total trials per session revealed main effects for both lesion condition (F[1,11]=9.336, p=0.0109) and session (F[5,55]=5.322, p=0.0005) but no interaction (F[5,77]=0.915, p=0.4780). Post-hoc comparisons revealed a greater total number of trials on Session 5 relative to all other sessions for both groups. A similar ANOVA conducted on correct trials per session revealed main effects of both lesion condition (F[1,11]=5.224, p=0.0431) and session (F[5,55]=4.016, p=0.0035) but no

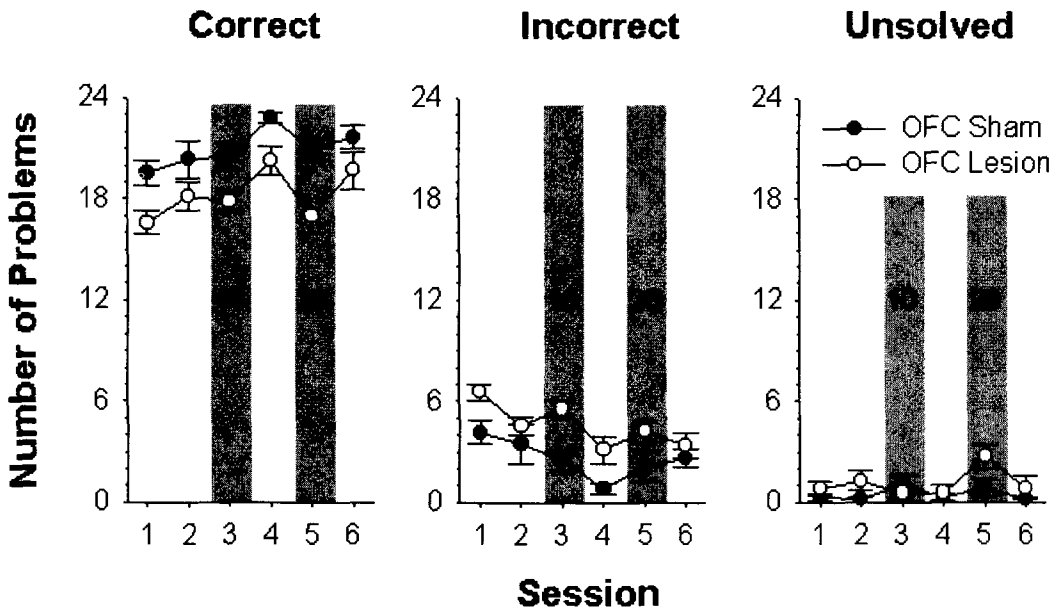

FIG. 8. Problem outcome frequency for OFC sham-lesioned subjects (filled circles) and OFC-lesioned subjects (open circles) across the initial six 24-problem post-surgical olfactory discrimination sessions. ITI duration was 5sec. during all sessions except during Sessions 3 and 5, when ITI duration was 10sec. and 20sec., respectively.

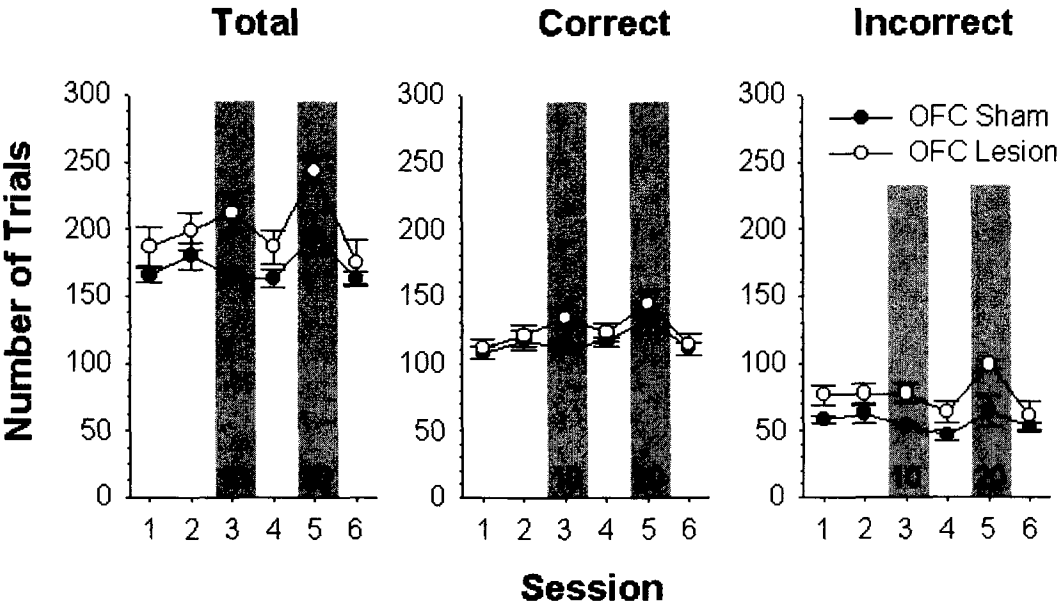

FIG. 9. Trial frequency for OFC sham-lesioned subjects (filled circles) and OFC-lesioned subjects (open circles) across the initial six 24-problem post-surgical olfactory discrimination sessions.

interaction (F[5,77]=0.735, p=0.6001), as did an ANOVA conducted on incorrect trials per session (F[1,11]=11.27, p=0.0064; F[5,55]=6.14, p=0.0001; F[5,77]=1.29, p=0.2836). Post-hoc comparisons revealed a greater number of correct trials on Session 5 relative to Sessions 1 and 6 and a greater number of incorrect trials on Session 5 relative to all other sessions for both groups. Thus, OFC-lesioned subjects exhibited a mild increase in both the number of correct trials per session and the number of incorrect trials per session, and these increases contributed to a greater total number of trials.

Because perseverative behavior is a hallmark of damage to frontal association cortex in humans and non-human primates, the extent to which OFC-lesioned subjects exhibited perseverative errors was examined. This analysis focused exclusively on performance during Session 3, the session with the greatest mean difference in total trials between OFC-lesioned and sham-lesioned subjects (211.9 vs. 164.7 trials). For this session, each discrimination problem except Problem 1 was classified as one of three types, distinguished by the identitiy of the S_D+ or the S_D– on the immediately preceding problem: unrelated (U), positive-negative (P-N), or negative-positive (N-P). On U problems, neither the S_D+ nor the S_D– had been encountered on the previous problem. On P-N problems, the S_D– had served as the S_D+ on the immediately preceding problem. On N-P problems, the S_D+ had served as the S_D– on the immediately preceding problem. Problems following correct problems were distinguished from those following incorrect problems; however, due to the low number of incorrect problems per session, only analyses of problems following correct problems were of sufficient number to be analyzed and reported. We reasoned that if the performance deficit observed of lesion subjects was due to enhanced perseverative responding, the performance of lesioned subjects would be worse on P-N problems and N-P problems relative to U problems.

Neither the probability of solving discrimination problems incorrectly nor the number of trials to complete discrimination problems was influenced by S_D valence on the immediately preceding problem (see Figure10). A two-way ANOVA conducted on the probability of solving problems incorrectly also revealed no effect of either lesion condition (F[1,33]=3.41; p=.0738) or problem type (F[2,33]=0.2614; p=0.7715); the interaction between these factors was also insignificant (F[2,33]=0.3951; p=0.6767). Consistent with the overall behavioral deficit exhibited by OFC subjects described previously, ANOVA conducted on the number of trials per problem revealed a significant main effect of lesion condition (F[1,33]=9.64; p=.0039) but failed to reveal significant main effect of problem type (F[2,33]=0.0854; p=0.9183) or a significant interaction between these factors (F[2,33]=0.1643; p=0.8492). These data suggest that, while OFC subjects performed more poorly than than sham-lesioned counterparts, this deficit cannot be attributed to an increased tendency to engage in perseverative responding.

Behavioral Extinction of the Conditioned Reinforcer

Following the elimination of primary reward, both OFC-lesioned and sham-lesioned subjects exhibited an increase in null trials as extinction sessions progressed (see Figure 11). A two-way ANOVA with one repeated measure (session) conducted on total number of trials per session revealed a significant main effect of session (F[2,22]=17.55, P<0.0001) but failed to reveal either a significant main effect of lesion condition (F[1,11]=1.66, P=0.2237) or a significant interaction between these factors (F[2,22]=1.62, P=0.2206). Post hoc comparisons revealed that the number of null trials increased significantly for both groups between the final two extinction sessions. A similar ANOVA

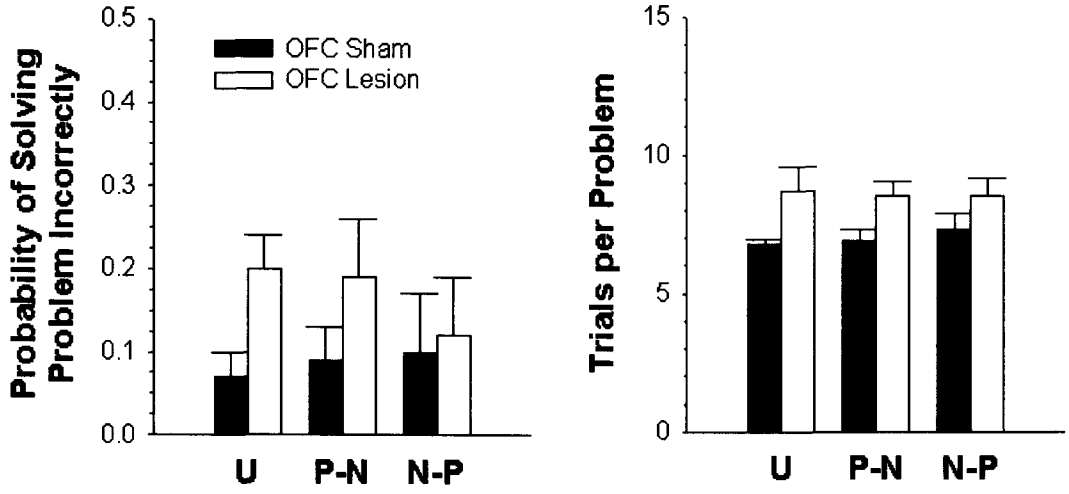

FIG. 10. Performance following correctly solved problems during Session 3 distinguished according to odor valence on the immediately preceding problem for OFC-lesioned subjects. A. Probability of solving problem incorrectly; B. Trials per problem. U, odor valence was unrelated to that of the previous problem; P-N, problems in which the S_D– served as S_D+ on the previous problem; N-P, problems in which the S_D+ served as S_D– on the previous problem.

conducted on correct trials per session revealed both a main effect of session (F[2,22]= 67.72, p<0.0001) and an interaction between session and lesion condition (F[2,22]=3.77, p=0.0391) but no main effect of lesion condition (F[1,11]=1.29, p=0.2804). Post hoc comparisons revealed that the number of correct trials diminished significantly for both groups between each successive extinction session. Pairwise comparisons revealed that OFC-lesioned subjects exhibited a greater number of correct responses relative to their sham-lesioned counterparts only during the first extinction session. ANOVA conducted on incorrect trials per session revealed a main effect of session (F[2,22]= 37.677, p<0.0001) but neither a main effect of lesion condition (F[1,11]=0.549, p=0.4743) nor an interaction (F[2,22]=0.957, p=0.3995), as did ANOVA conducted on null trials per session (F[2,22]=76.7618, p<0.0001; F[1,11]=0.0247, p=0.8779; F[2,22]=1.2471, p=0.3069). Post hoc comparisons revealed that the number of incorrect trials decreased significantly for both groups between the first two extinction sessions and that the number of null trials increased between these sessions.

OFC-lesioned and sham-lesioned subjects increasingly failed to complete discrimination problems as extinction sessions progressed; these data are illustrated in Figure 12. A two-way ANOVA with one repeated measure (session) conducted on number of unsolved problems per session across the three extinction sessions revealed a significant main effect of session (F[2,22]=4.7138, p=0.0198) but failed to reveal either a significant main effect of lesion condition (F[1,11]=0.0419, p=0.8415) or a significant interaction between these factors (F[2,22]=0.0434, p=0.9576). Post-hoc comparisons revealed that for both groups the number of unsolved problems was significantly greater on Session 1 relative to Session 3. A similar ANOVA conducted on correct problems revealed a main effect of session (F[2,22]=82.123, p<0.0001) but failed to reveal either a main effect of lesion condition (F[1,11]=0.112, p=0.7443) or an interaction (F[2,22]=0.235, p=0.7926). Post-hoc comparisons revealed that for both groups the number of correct problems significantly de-

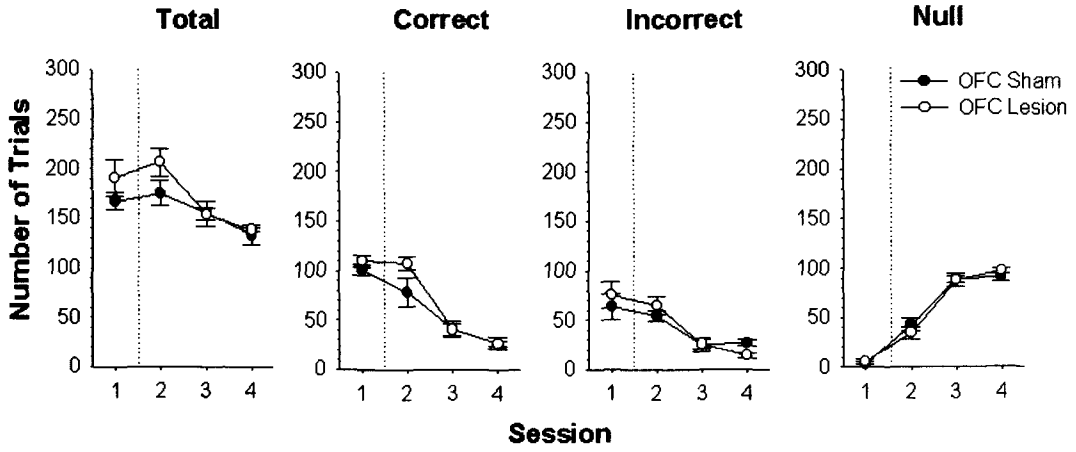

FIG. 11. Trial frequency for OFC sham-lesioned subjects (filled circles) and OFC-lesioned subjects (open circles) across the four extinction sessions. ITI duration was 5sec. during all sessions. Access to primary water reward was withheld on all but the first session.

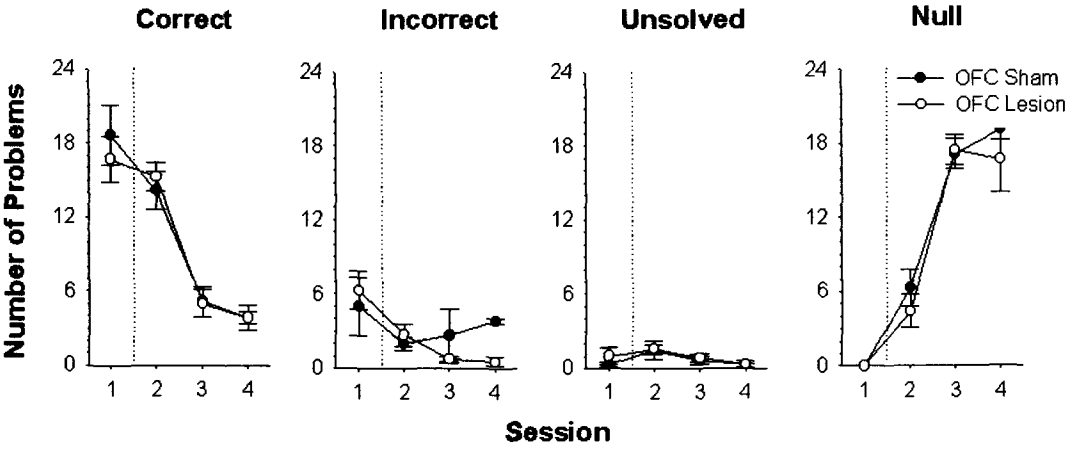

FIG. 12. Problem outcome frequency for OFC sham-lesioned subjects (filled circles) and OFC-lesioned subjects (open circles) across extinction sessions. ITI duration was 5sec. during all sessions. Access to primary water reward was withheld on all but the first session.

clined only between the first two extinction sessions. Analysis of the number of incorrect problems per session failed to reveal significant main effects of session ($F[2,22]=2.514$, $p=0.1039$) or lesion condition ($F[1,11]=0.119$, $P=0.7363$) or significant interaction between these factors ($F[2,22]=1.306$, $p=0.2912$). Correspondingly, ANOVA conducted on null problems per session revealed a main effect of session ($F[2,22]=38.632$, $p<0.0001$) but failed to reveal either a main effect of lesion condition ($F[1,11]=0.745$, $p=0.4065$) or an interaction ($F[2,22]=0.340$, $p=0.7156$). Post-hoc comparisons performed on session revealed that the number of null problems significantly increased only between the first two extinction sessions.

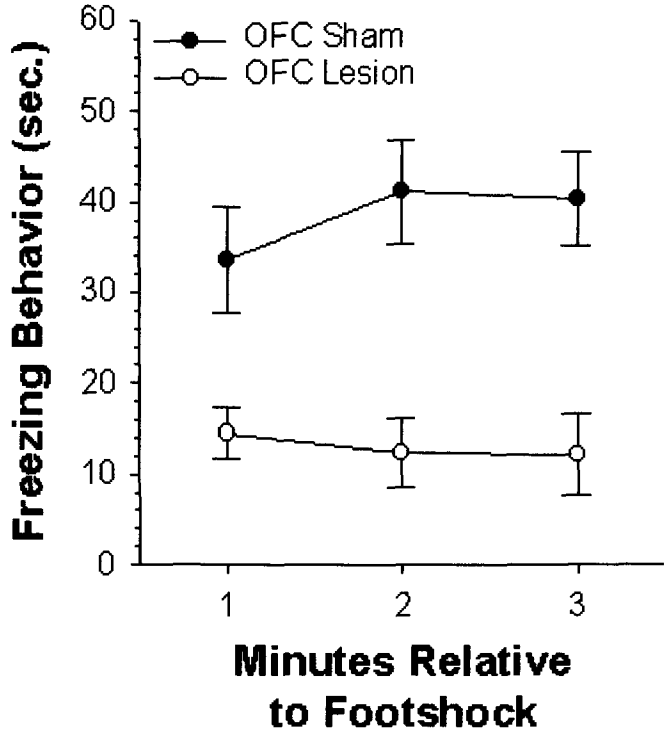

FIG. 13. Freezing behavior during the 3min. period immediately following footshock presentation (0.8mA; 2sec.) for OFC sham-lesioned subjects (filled circles) and OFC-lesioned subjects (open circles).

Immediate Post-shock Freezing Behavior

Unlike lesions directed at the BLA, damage to OFC attenuated, but did not abolish immediate post-shock freezing behavior (see Figure 13). A two-way ANOVA with one repeated measure (minute) conducted on seconds freezing per minute during the 1min period immediately preceding footshock and the 3min period following footshock revealed significant main effects of both minute ($F[3,33]=33.74$, $p<0.0001$) and lesion condition ($F[1,11]=20.63$, $p=0.0008$), as well as a significant interaction between these factors ($F[3,33]=9.11$, $p=0.0002$). Pairwise comparisons revealed that OFC-lesioned subjects spent less time freezing during each of the three minutes following footshock compared to sham-lesioned subjects, but more time freezing during this period compared to their own pre-shock level (0sec. for both groups).

DISCUSSION

Subjects with near complete excitotoxic damage to the BLA and significant cell loss in adjacent areas were largely unimpaired on performance of an olfactory discrimination task that incorporated auditory conditioned reinforcement. Although BLA-lesioned subjects exhibited a marginal increase in the number of trials required to complete discrimination problems during the initial six post-surgical sessions, these groups solved an equivalent number of discrimination problems correctly, despite prolongation of the ITI from 5sec. to

10sec. and 20sec. Subjects with excitotoxic damage to the OFC exhibited both a delay-independent increase in the number of trials required to complete discrimination problems and a delay-independent decrease in correctly solved problems. In contrast, both BLA-lesioned and OFC-lesioned subjects exhibited a sever disruption of immediate post-shock freezing behavior.

In the present task, the auditory cue provided information regarding the valence of concurrently presented olfactory cues and guided stimulus selection prior to access to primary water reward. Although a cue-omission procedure was not adopted in a control condition to determine whether the tone was critical for accurate task performance, it is difficult to conceive an accurate response strategy that is not critically dependent upon acquired motivational value of the auditory cue. For example, a strategy of odor selection based upon direct association with primary reward would fail to guide accurate performance, since such access was only provided following four consecutive correct odor choices on each problem and since primary reinforcement density was equivalent for all odors across preceding trials. Further, a strategy of consistently selecting a subset of odors regardless of odor-tone contingency would be expected to result correctly solving no more than half of odor discrimination problems. As demonstrated, sham-lesioned subjects maintained accuracy greater than 80%.

Neural Substrates of Conditioned Reinforcement

Basolateral Amygdala

Malkova et al. (1997) reported that excitotoxic damage to the amygdaloid complex in non-human primates failed to disrupt acquisition of novel visual discrimination problems under a second-order schedule of auditory reinforcement and suggested that the amygdala is not necessary for maintaining the motivational value of conditioned reinforcers once such value has been acquired. The present findings obtained with rodents are consistent with this suggestion and provide further evidence that the motivational value of conditioned reinforcers is not maintained by the BLA.

In apparent contrast to the data described here, several previous studies conducted with rodents have implicated the BLA in the performance of Pavlovian second-order learning tasks. For example, Gewirtz & Davis (1998) reported that intra-amygdaloid microinfusion of D,L-2-aminio-5-phosponovalerate (APV), an antagonist of NMDA-type glutamate receptors, enhanced first-order fear-potentiated startle responses elicited by a primary auditory conditional stimulus (CS) but disrupted second-order responses elicited by a conditioned visual CS that predicted the auditory CS. Hatfield et al. (1996) reported that although excitotoxic damage to the BLA failed to disrupt the acquisition of first-order appetitive responses elicited by a visual CS, such damage prevented the acquisition of second-order responses elicited by an auditory CS that predicted the visual CS. Hatfield et al. (1996) further reported that BLA damage rendered subjects insensitive to post-training US devaluation, suggesting that the BLA might be critical for a primary CS to gain access to the current incentive value of a US. This notion is not incompatible with the present findings, since BLA damage was produced well after the conditioned reinforcer had acquired the ability to support discriminative responding and the incentive value primary reward was not altered. It is possible that damage to the BLA would have rendered performance of the present task insensitive to devaluation of primary reward; however, for a variety of practical reasons, this procedure was not attempted.

The results of several other studies have further suggested that the BLA may be critically involved in the performance of tasks incorporating secondary reinforcement (Burns, Everitt, & Robbins, 1999; Cador, Robbins, & Everitt, 1989; Everitt, Cador, & Robbins, 1989; Everitt, Morris, O'Brien, Burns, & Robbins, 1991; Parkinson et al., 2001). Notably, Cador et al. (1989) reported that excitotoxic damage to the BLA selectively attenuated responding on a lever resulting in presentation of a compound stimulus that had been associated with food but did not affect responding on a second non-contingent lever. Providing a viable explanation for the apparent discrepancy between these findings and those reported in the present study and by Malkova et al. (2001), Parkinson et al. (2001) suggested that discriminative responding under a second-order schedule might be insensitive to amygdala damage if performance were supported by largely non-affective predictive attributes of the conditioned reinforcer, rather than by intrinsic incentive properties. The extensive pre-surgical training and relatively low response requirement described by Malkova et al. (2001) and adopted in the present study could have biased responding according to the predictive value of the conditioned reinforcer (Parkinson et al., 2001). Moreover, while the BLA appears to be involved in the expression of first-order CRs even after extensive training (Maren, 1999), Holland (1998) has demonstrated that the amount of training may interact with the acquisition of second-order CRs. Thus, it is now of interest to determine whether pre-surgical damage to the BLA would prevent acquisition of the present task or alternatively whether post-surgical damage produced without extensive overtraining would result in disruption of task performance. Consistent with a possible role for the BLA in acquisition of motivational value by the auditory conditioned reinforcer in the present task, Setlow et al. (2002) have recently suggested that the BLA is necessary for acquisition, but not expression, of motivational value by a first-order conditional stimulus in a Pavlovian second-order conditioning paradigm. This role would stand in stark contrast to an enduring role of the BLA in the expression of Pavlovian conditioned freezing behavior (Maren, Aharonov, & Fanselow, 1996; Cousens & Otto, 1998).

Orbitofrontal Cortex

OFC-lesioned subjects exhibited a mild but significant deficit in performance of the conditioned reinforcement task, characterized by a decrease in correctly solved problems and an increase in the number of trials per problem. These deficits are likely not due to disruption of non-specific motivational, motor, or sensory processes, since previous studies have reported that subjects with less selective, aspirative lesions of OFC were unimpaired in performance of an olfactory delayed non-matching task with short interstimulus interstimulus intervals (Otto & Eichenbaum, 1992). In further support of this notion, subjects with substantial damage to the frontal pole, including damage to orbitofrontal cortex were found to be unimpaired on olfactory threshold detection tests (Eichenbaum et al., 1980).

The results of several previous studies are consistent with the view that the performance deficits observed in the present study are due to a disruption of mnemonic processes. First, damage to OFC in rodents results in delay-dependent performance deficits in an olfactory delayed non-matching task when small stimulus samples are utilized (Otto & Eichenbaum, 1992), suggesting that this region might participate in the maintenance of olfactory representations during interstimulus delays. Thus, it is possible that the performance deficit observed in the present task was due to impaired retention of odor identity or odor valence during ITIs. Although performance deficits were independent of ITI duration in the present

task, the maximum ITI assessed was only 20sec., up to 70sec. less than interstimulus intervals producing maximum deficits in the delayed non-matching task (Otto & Eichenbaum, 1992). Thus, it is possible that greater performance deficits would have been observed in the present task if ITIs had been extended. ITIs were not extended beyond 20sec., however, since the results of pilot studies in our laboratory indicated that unoperated control subjects exhibited significantly impaired performance at longer delays. Second, as with damage to the BLA (Hatfield et al., 1996), Gallagher et al. (1999) found that OFC lesions rendered conditional responding insensitive to US devaluation, suggesting that this region might be critical for the CS to gain access to current US incentive value. Thus, it is possible that the performance deficit observed in the present task was due to loss of predictive or incentive value acquired through learned association with access to primary reward. We have undertaken an electrophysiological study in rodents performing the present task in order to determine whether orbitofrontal neurons exhibit firing rate modulation correlated to the transient retention of odor identity or odor valence or longer-term maintenance of conditioned reinforcer motivational value.

Schoenbaum et al. (2002) have recently demonstrated that damage to OFC resulted in a deficit in serial reversal learning in a successive cue, go/no-go olfactory discrimination task, suggesting that this region might contributes to behavioral adjustments following changing odor-outcome contingencies. Odor valence varied between problems in the present task, and an inability to adjust responding in light of variable reward contingencies would be expected disrupt discrimination. However, it is unlikely that the deficit observed in the present study was due to such an impairment because performance observed of OFC-lesioned subjects by Schoenbaum et al. (2002) recovered to a level that exceeded that of control subjects by the third reversal session. In the present study, OFC-lesioned subjects were consistently impaired across six post-surgical sessions.

Immediate Post-shock Freezing Behavior

Consistent with previous findings (Maren, et al., 1996; Cousens & Otto, 1998), excitotoxic damage to the BLA was associated with near complete abolition of immediate post-shock freezing behavior. This finding was wholly expected and provides a behavioral index of BLA damage separate from the olfactory discrimination task. Thus, it is unlikely that the lack of a performance deficit following BLA lesions was due to incomplete damage to the BLA and surrounding areas, a conclusion also consistent with histological examination. OFC lesions also severely attenuated immediate post-shock freezing, suggesting that the OFC participates in processing information with highly affective content or, alternatively, in generating freezing behavior.

Conclusions

Damage to the BLA largely failed to disrupt performance of the conditioned reinforcement task, suggesting that the BLA not critical for mnemonic processes presumed to underlie accurate task performance, including maintaining transient representations of odor valence within each discrimination problem and maintaining conditioned reinforcer motivational value across problems. In addition, it is unlikely that the BLA contributes to guiding goal directed behavior according to conditioned reinforcer value. In contrast, damage to OFC resulted in a mild but significant delay-independent impairment in task performance, consistent with the notion that this region contributes, in part, to processes underly-

ing accurate task performance. Damage to either the BLA or OFC resulted in substantial impairment of immediate post-shock freezing behavior, suggesting that both of these regions are critical for normal expression of immediate post-shock freezing behavior.

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