Pharmacology Biochemistry and Behavior, Vol. 63, No. 1, pp. 159–165, 1999

0091-3057/99/$–see front matter

PII S0091-3057(98)00263-9

The Effects of Microinjection of *d*-Amphetamine Into The N. Accumbens During the Late Maintenance Phase of an Ethanol Consumption Bout

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Received 30 January 1998; Revised 13 October 1998; Accepted 27 October 1998

SAMSON, H. H., A. CHAPPELL, C. SLAWECKI AND C. HODGE. *The effects of microinjection of* d-*amphetamine into the n. accumbens during the late maintenance phase of an ethanol consumption bout.* PHARMACOL BIOCHEM BEHAV **63**(1) 159–165, 1999.—The microinjection of *d*-amphetamine into the n. accumbens of rats, prior to the start of an operant ethanol self-administration session, increases operant behavior and the amounts of ethanol presented as the reinforcer. Although this effect could result by blocking termination processes regulating a consummatory bout, it could also be a result of enhancing the stimulus control regulating the maintenance of a drinking bout. To explore this issue, rats were trained to self-administer 10% ethanol in an operant situation. Following establishment of stable behavior, they were surgically instrumented so that the n. accumbens could be microinjected with *d*-amphetamine during a drinking bout, without having to handle the animal. The microinjection of *d*-amphetamine in the rats self-administering ethanol at the late phase of the drinking bout resulted in a prolonged bout and increased self-administration. During extinction testing, a reinstatement of responding was found following the amphetamine microinjection. The data suggest the most likely action of the amphetamine microinjection was to alter stimulus control factors, which normally regulate the maintenance of drinking, thereby prolonging the bout and increasing intake. © 1999 Elsevier Science Inc.

Alcohol Ethanol Self-administration Amphetamine n. accumbens Rats

OVER the last several years, we have observed that the microinjection of dopaminergic agonists and antagonists into the nucleus accumbens (n. accumbens) prior to the start of an ethanol operant self-administration session can impact the pattern and amount of ethanol maintained responding [for a review, see (5,10)]. An interesting finding was that the microinjection of the indirect sympathomimetic, *d*-amphetamine, resulted in an increase in self-administration in the majority of animals (2,11). An analysis of the response patterns indicated that amphetamine microinjection slowed response rate during the middle and later parts of the ethanol drinking bout, while prolonging this slow rate of responding in some cases for up to 1 h (2). This occurred while having little to no effect on the latency to start responding at the beginning of the limited-access session. Also, response rates in the early portion of the bout did not appear to be greatly affected. Thus, while total ethanol reinforced responding increased, this was primarily a result of altered responding during a time in the drinking bout when ethanol would have been rising in the blood (i.e., at a time after the initial few minutes of drinking had occurred) (1).

We have postulated that there are several neurochemical and behavioral processes that regulate an ethanol consumption bout (11). First, there is an onset phase, which occurs prior to any ethanol consumption and is regulated by appeti-

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tive processes. Second, an early maintenance phase occurs consisting of consumption prior to the time of effective blood ethanol levels. We have hypothesized that both appetitive and consummatory processes (11) regulate this part of the drinking bout. Finally, the later maintenance and termination phases of the bout are related to continued ethanol consumption, with blood ethanol levels increasing to pharmacological levels. During this last phase of the bout, mixtures of consummatory processes along with the pharmacological actions of ethanol are proposed as the primary mechanisms regulating intake. However, the actual processes related to termination of a bout remain unclear.

We have postulated that dopaminergic receptors in the mesolimbic system influence both the early and late maintenance phases of responding. For example, increases in operant ethanol self-administration produced by intraaccumbens infusions of the DA $D_{2/3}$ agonist quinpirole are blocked by coadministration of the D_1 antagonist SCH 23390 (6). Similarly, decreasing DA transmission in the n. accumbens either through local infusions of the D_2 antagonist raclopride (12) or by quinpirole infusions in the VTA (4) reduces ethanol self-administration by producing early termination of responding. Although presession manipulations of mesolimbic dopamine transmission primarily influenced the late maintenance and termination phases of ethanol self-administration, possible effects on the early maintenance phase (i.e., control by conditioned stimuli) could not be ruled out.

It is well documented that microinjections of amphetamine into the n. accumbens potentiate responding maintained by conditioned reinforcers (2,7,15). We have suggested that the early maintenance phase of the ethanol consumption bout is in part maintained by conditioned reinforcement processes (11,13). Thus, presession injections of dopamine agonists and antagonists could affect both early and late maintenance processes of bout control. To examine how amphetamine might be affecting different drinking phases, microinjections into the n. accumbens at different times during a drinking bout, rather than before the start of the drinking session were undertaken. The study reported here used an in-session microinjection procedure in which amphetamine was injected into the n. accumbens during the late maintenance/termination phase of the drinking bout in an attempt to separate the effects of amphetamine on early vs. late maintenance phase processes.

METHOD

Subjects

Male Long–Evans rats, weighing between 200–225 g at the start of the experiment, were housed individually in standard hanging wire cages in an AAALAC-approved vivarium with ad lib food and water always available. The vivarium was maintained on a 12 L:12 D cycle, with the lights on at 0600 h. All experimental sessions were run during the light portion of the cycle. The rats were maintained in accordance with the NIH guidelines at all phases of the experiment.

Apparatus

Self-administration sessions were conducted in Plexiglas chambers (30 × 30 × 24 cm) housed inside of individual sound attenuating boxes (Med Associates; St. Albans, VT). The chambers were equipped with two automatic dippers, a house and stimulus light, and response levers on the front and back walls of the chamber. Only the front lever had any programmed consequences. Back lever responses were recorded as a measure of nonspecific lever-press responding. A fan inside each sound-attenuating box provided masking for external noise. To microinject the animals without having to remove them from the chamber, a head-mounted injection system was developed (EMDEC, Bellevue, WA) (Fig. 1). It consisted of a screw base attachment, which surrounded the implanted guide cannulae and was mounted on the rat's head with dental cement. A stainless steel hollow reticulating cable that mated to the screw base on the rat's head was attached to a screw cap. The other end of the cable was attached to a dual channel fluid swivel (Instech, Model 375 dual channel, Plymouth Meeting, PA). PE 20 tubing from the fluid swivel outputs was threaded through the cable and connected to the microinjectors, which were to be inserted into the guide cannulae. A 3-cm round hole in the Plexiglas top of the operant chamber and a 7 × 11-cm rectangular hole in the top of the sound-attenuating chamber allowed for the cable and tubing to connect to microinfusion pumps (Harvard Apparatus, Model 22, Holliston, MA) located outside the sound attenuating chambers. The dual liquid swivel was attached to a cantilevered drug arm mounted outside of the operant chamber, which allowed for free movement of the animal in the chamber while keeping the cable system suspended and out of the animal's reach. The microinfusion pump was interfaced with the computer, allowing for computer control of the time and duration of the microinjection.

Surgical Procedures

Each rat was anesthetized with Sodium Pentobarbital (IP, 50 mg/kg) and placed in a stereotaxic instrument (David Kopf Instruments, Model#960, Tujunga, CA). The nose bar was adjusted to −3.3 mm below the interaural line. Anatomical coordinates were taken from the Paxinos and Watson Atlas (9) and directed at the core of the n. accumbens (AP = +1.7 , ML = + 1.8, DV = −6.0). Bilateral stainless steel cannula guides (26 gauge) were attached to the skull using stainless steel screws and dental cement. Once the initial layer of dental cement–plastic was dry, the stereotaxic arms were removed and the screw base of the microinjection system attached to the head with additional dental cement. Sterile obturators (33 gauge solid wire) were placed in the guides to prevent blockage. The obturators were checked daily and replaced as needed.

Microinjection Procedures

Following recovery from surgery (1–3 days), the animals were attached to the microinjection apparatus during all subsequent operant sessions. To attach the apparatus, the rats were held outside the door of the operant chamber and the head set area cleaned with a damp gauze pad. The obturators were removed and replaced with either new obturators (no injection conditions), sham injectors (33 gauge) cut to the length of the guide cannulae, i.e., 15 mm with the attached PE tubing filled with artificial cerebrospinal fluid (ACSF) or injectors (cut to 16 mm to penetrate the brain 1 mm below the guide cannulae with the tubing filled with the drug to be injected). To hold the injectors in place during the session, a piece of silastic tubing was used to couple the injector to the cannula guide. A total volume of 1.0 μl total (0.5 μl per side) was injected during microinjections. The rate of infusion was 0.5 μl/min. On all days, the pumps were run to provide the same auditory stimuli independent of the occurrence of an active microinjection.

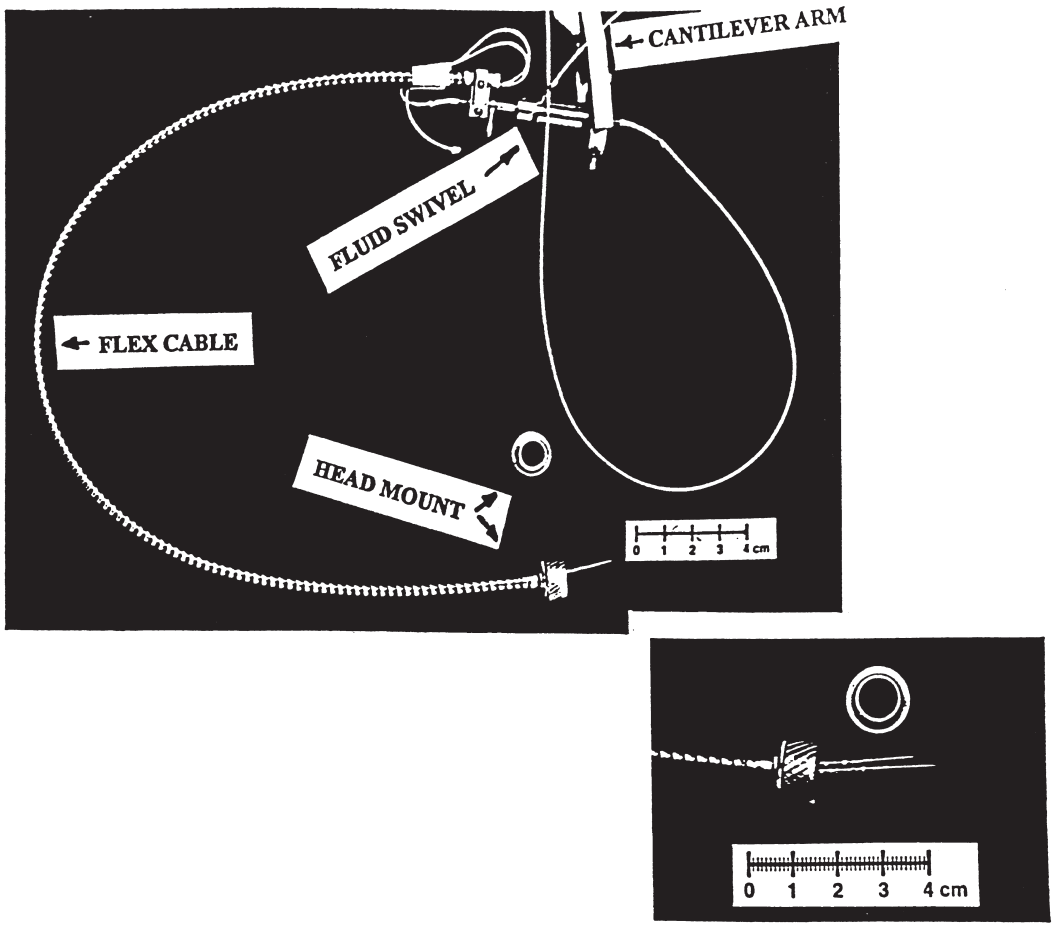

FIG. 1. In-session microinjection apparatus. The insert shows the head-mounted flange that allowed for the injector unit to be coupled to the animal during the session.

Drugs

d-Amphetamine sulfate (Sigma Chemicals, St Louis, MO) was prepared in artificial cerebrospinal fluid (ACSF, pH = 7.4) immediately prior to use.

Histology

After the last operant session the rats were deeply anesthetized with sodium pentobarbital (100 mg/kg) and transcardially perfused with 60 ml of phosphate-buffered saline (PBS) followed by 60 ml of 10% formalin in PBS. The brain was removed and kept in 10% formalin solution for at least 10 days. The brain was then cut into 90-μm sections using a freezing microtome and mounted on gelatin-coated slides. The brain sections were stained with cresyl violet and the locations of the microinjections determined by use of a light microscope.

Training Procedure

Upon arrival at the laboratory, the rats were individually caged, weighed, and handled for 3 days. On the fourth day the animals were given 3 days of forced ethanol exposure, with 10% ethanol (v/v) as the only fluid available in the home cage. This was followed with a 24 h/day, two-bottle 10% ethanol vs. water preference test on the home cage for 14 days.

Next, the rats were trained to lever press using a successive approximation procedure reinforced with presentation of a 20% sucrose solution in a single 30 min session in the operant chambers. Following this session, all rats received overnight sessions (12 h) for 3 consecutive days in which 20% sucrose was presented in the dipper according to a fixed ratio 1 (FR1) schedule of reinforcement. Following the overnight sessions, daily operant sessions began, 5 days/week (Monday through Friday), conducted at the same time each day. During the first 5 min of the session, the house light was off and lever pressing was not reinforced. This delay period was employed as a possible means for detecting if any drug leakage occurred during the insertion of the microinjectors, which might be indicated by increased responding on either lever during this time. After the 5 min delay, the house light was illuminated and lever presses on the front lever were reinforced with the presentation of 20% sucrose in the dipper. Responses on the back lever had no programmed consequences but were recorded. After three sessions with 20% sucrose, the rats began the sucrose-fading procedure (10). Briefly, over sessions, the concentration of the sucrose was decreased while ethanol was added to the solution until 10% ethanol in water was presented as the reinforcer. During the fading procedure, an FR1 was in effect. When responding was maintained by 10% ethanol in water, the FR requirement was increased to a variable

ratio schedule 2 (VR2) for five sessions, a VR3 for five sessions, and then to an FR4 for the remainder of the experiment. The rats received 3–4 weeks in this final condition of 10% ethanol reinforcement on an FR4 schedule to obtain a stable baseline (less than 20% daily variability in responding over five sessions) prior to surgery.

After 1 day of recovery from surgery, the rats were returned to the daily operant sessions. A 1% sucrose/10% ethanol solution on an FR1 was employed to facilitate the recovery of responding for one to three sessions. Then the rats were returned to the FR4 schedule with 10% ethanol reinforcement.

When baseline responding had been reestablished and 10 sessions of response patterns similar to the presurgery baseline were observed, the next phase of the experiment was begun. A computer program that determined when the later maintenance/termination phase of a bout had been reached was now employed to activate the infusion pumps. After examining the behavioral records of the animals, it was determined that the later maintenance phase could be approximated by the occurrence of a 5-min period of no responding (i.e., a 5-min interresponse time, IRT). While there were occasions in which one or two responses occurred at the start of a session and then a 5-min period occurred, these occurrences were rare. Therefore, in the final protocol, after a 5-min session delay during which time responding was not reinforced, the house light was illuminated, signaling the availability of the reinforcer. When the computer detected that the 5 min IRT had occurred, the pump was activated and turned off 1 min later. This time prior to the 5 min IRT was defined as the preinjection responding period (i.e., the time from the start of the session (house light on) until the activation of the infusion pump). The operant session was then continued for an additional 60 min, and this time was designated as the postinjection time. Because of differences in response patterns prior to the detection of the 5-min response pause, total session times (preinjection time + postinjection time) were variable.

After approximately five sessions in which the rats adapted to the new session parameters, the attachment of the microinjection apparatus was begun prior to the start of the session. The attachment procedure was then used before all remaining sessions. When behavior was stable (less than 15% daily variation in responding before and after pump activation), the rats received sham injections for five consecutive sessions. Then the drug injection portion of the experiment was begun.

Two injection protocols were followed. The first protocol varied the dose of amphetamine injected. For this part of the study, amphetamine doses of 5.0, 10.0, and 20.0 μg/μl were infused. Typically, one drug dose was tested per week, with the remaining four daily sessions consisting of sham controls. In a few cases (less than 10% of all sessions), sham controls were preformed on the Monday, Tuesday, and Thursday sessions, and drug injected on the Wednesday and Friday sessions.

The second protocol employed the use of extinction to examine if the amphetamine injection could reinstate responding in an extinction session. In this portion of the study, the rats had a normal sham session with reinforcer delivered on Monday. On Tuesday and Wednesday the rats were given sham sessions in which the dipper was never presented (extinction). In these sessions, the same 5-min pause in responding criteria was required to activate the pump and responding for the following 60 min after pump activation was recorded. On Thursday using the same extinction conditions, pump activation resulted in the microinjection of the 20 μg/μl dose of amphetamine. On Fridays, a normal sham session with reinforcer delivery was employed.

Data Analysis

The number of responses and reinforcements presented were recorded separately for preinjection and postinjection responding for both the active and inactive levers. Using within animal statistical procedures (Sigmastat, Jandel Scientific, San Rafael, CA), the effects of dose and extinction were analyzed for both the pre- and postinjection responding. As well, cumulative response records were visually inspected to determine changes in the patterns of responding resulting from the microinjections. Because there were multiple sham injection tests, they were first analyzed to determine if there were any significant differences between sham tests using a within-subjects ANOVA. No significant differences were found, so the data for sham injections were combined and used as single value for each animal for determinations of drug effects.

Because of the variable time period of the preinjection phase that occurred prior to the operation of the pump (i.e., the time from the start of the session until a five min pause in responding was detected), different amounts of "preinjection" session times resulted across the animals. Thus, comparing responding and intakes during the preinjection periods with those that occurred during the presurgery fixed time 30-min operant session required the use of a conversion procedure to equate for time. To approximate intake rate for the postsurgery data, we divided the prepump intakes (measured in grams intake/kg body weight, based on the number of reinforcers presented) by the time prior to pump onset, providing a measure of intake in terms of g/kg/min for this portion of the session. The same measure was calculated for the presurgery 30-min sessions, using 30 min as the divisor.

RESULTS

Thirty rats were used in this study. Nine animals developed an infection at various times following the surgery and had to be sacrificed prior to completing the experiment, two rats failed to regain baseline ethanol self-administration after surgery and were discontinued, six rats damaged their head stage connector mounts during the experiment and had to be discontinued with only partial data collected, and three rats were found upon histological examination to have incorrect bilateral cannula locations and were excluded from the data analysis. This resulted in 10 rats for which at least 95% of all conditions were completed that were included in the final data analysis.

Initial Ethanol Training

The sucrose-substitution procedure initiated ethanol self-administration at levels previously observed in this laboratory (30 min 10% ethanol intakes averaged 0.52 g/kg, range: 0.36 to 0.88 g/kg) (10).

Postsurgery Baseline Responding

Using the calculated rate values as described in the Data Analysis section, no differences between pre- and postsurgery intake rates were found. The average time of the preinjection portion of the session following surgery was 28.7 min. Calculating intake in terms of g/kg/min, prior to surgery resulted in a mean of 0.017 g/kg min (SEM = 0.0017), while after surgery prior to drug injection mean intake rates were 0.015 g/kg/min (SEM = 0.0022). There was no significant difference between these values. Thus, intake rates prior to surgery were similar to those occurring during the preinjection portion of the ses-

sions with the microinjection apparatus attached to the animals. Examination of the cumulative records indicated no major changes in the patterns of responding when comparing the presurgery 30-min sessions to the postsurgery preinjection portion of the session. Therefore, the animals did not appear to have altered their self-administration behavior because of either the change in the operant session protocol or because of being connected to the microinjection apparatus.

Responding for the next 60 min after the detected 5-min pause (pump operation) represented 41% of total session responding in the sham condition (Fig. 2). The general response pattern during this postinjection hour consisted of a broken pattern of responding with reinforcement deliveries spaced by response pausing (Fig. 3, sham). This indicates that the time of injection for most rats was during the late maintenance phase of the drinking bout.

Amphetamine Dose–Effect Determinations

On all days in which amphetamine was microinjected following the detected 5-min responding pause, responding before the injections was not statistically different from sham injection days (Fig. 2, left side of figure). The decrease in preinjection responding at the 10-μg dose was predominately a result of three rats who on this day varied from their normal preresponding pattern during this period. The within-subjects ANOVA did not find this decrease statistically significant. The nature of the altered pattern was not suggestive of a leak effect of amphetamine prior to the injection. Postinjection responding significantly increased across dose [within-subjects one-way ANOVA on dose, $F(3, 27) = 5.8904$, $p < 0.003$]. Multiple comparisons (Tukey test) found responding at the 20-μg dose was significantly greater than responding at the 5-μg and sham conditions (Fig. 2). Examination of the cumulative records indicated that for the 20-μg dose (Fig. 3), responding resumed almost immediately after the injection with a response pattern similar to maintenance stage responding previously observed when amphetamine was injected before the start of the self-administration session (3,6).

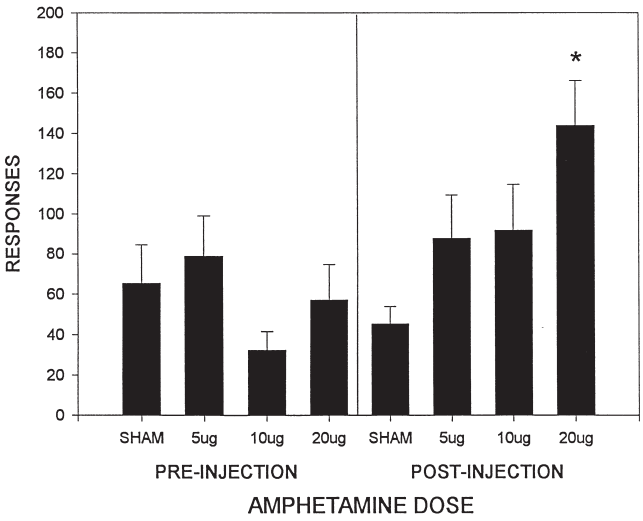

FIG. 2. Reinforced responses before (preinjection) and after (postinjection) the in-session microinjection of *d*-amphetamine. Values are means with error bars indicating the SEM. Asterisks indicates significantly different from sham.

Responding on the noncontingent lever increased after amphetamine microinjection [one-way repeated-measures ANOVA, $F(3, 27) = 3.3435$, $p < 0.0345$]. Post hoc comparisons found that the only significant difference was between the sham and 20-μg condition. This increase in noncontingent lever responding was from 4% of total responding in the sham condition to 10% of total responding after 20 μg of amphetamine. This increase in noncontingent lever presses compared to the sham condition, represented 17% of the total increased responding occurring at the 20-μg dose. Thus, 83% of the increased responding was observed on the active lever, suggesting that while some nonspecific increase in behavior occurred, the greater part of the increased responding was directed at the active lever.

Extinction–Reinstatement

Three of the 10 animals did not complete all of the extinction protocol and the data reported are for only the seven rats for which complete data were obtained. As expected, the amount of responding on the active lever prior to pump onset decreased over the days of extinction compared to the prior reinforced day, $F(3, 18) = 3.331$, $p = 0.043$ (Fig. 4). Post hoc multiple comparisons (Tukey test) found that responding on the last extinction day was significantly decreased compared to responding on the reinforced day. On the third day of extinction, following the amphetamine microinjection, a significant increase in active lever responding occurred compared to

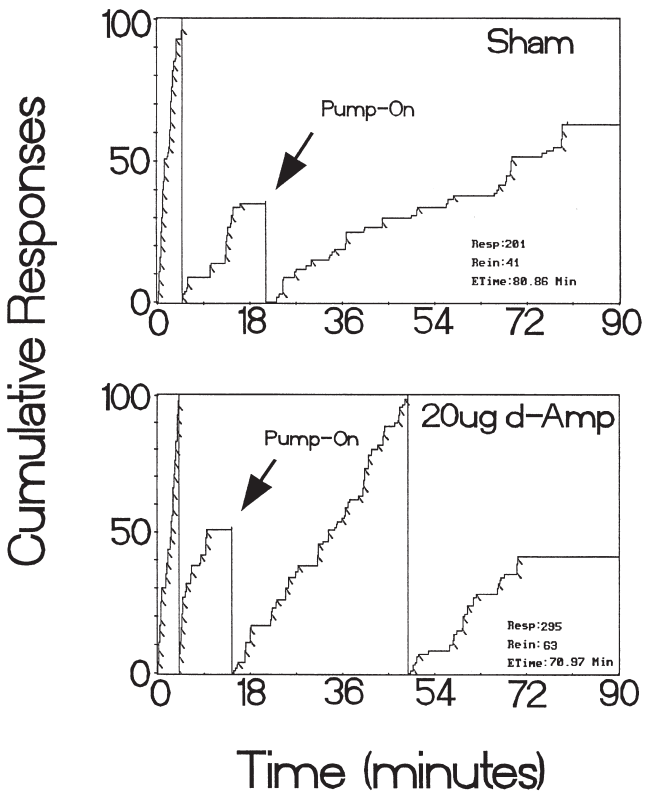

FIG. 3. Typical cumulative response records for either a sham or 20 μg *d*-amphetamine microinjection. Tick marks on the records indicate reinforcer presentation. The arrows indicate the time of the microinjection, with the record reset to zero at that time.

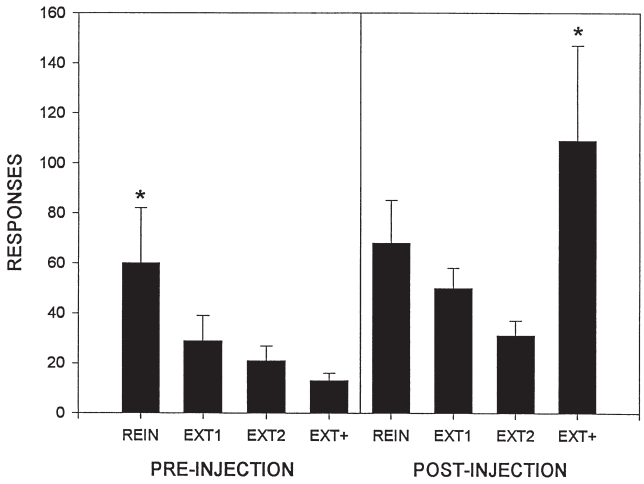

FIG. 4. Responding during extinction testing. (REIN—reinforced session prior to extinction testing; EXT1—first day of extinction; EXT2—second day of extinction; EXT+—third day of extinction plus amphetamine microinjection) Values are means and SEM. Asterisks indicates significantly different from extinction day 2.

the previous extinction day (Tukey test) [overall within-subject ANOVA across days was significant, $F(3, 18) = 3.294$, $p = 0.44$]. The level of responding on the amphetamine-extinction day was not significantly different from the last day in which reinforcement occurred (Tukey test), indicating a substantial reinstatement effect. Inactive lever responding did not change across extinction days 1 and 2 compared to reinforced days, but was increased on the amphetamine injection extinction session [within-subjects ANOVA, $F(3, 18) = 5.199$, $p = 0.009$; Tukey test]. On extinction day 2, the ratio of inactive lever to total responding across the entire session was 11%. On extinction day 3 when amphetamine was microinjected, the ratio increased to 19%. Thus, as noted above during the dose–effect curve experiment, there was a slight general activity increase noted following amphetamine, but 81% of the responding following amphetamine was directed towards the active lever.

DISCUSSION

The primary objective of this study was to determine if amphetamine microinjected into the n. accumbens during the later phases of an ethanol self-administration bout would produce an effect similar to that observed when the microinjection occurred prior to bout onset. Based on our regulatory model of ethanol bout control (10), it was hypothesized that a microinjection during the late maintenance phase would delay termination of responding and increase intake; an effect similar to that observed with presession microinjections (3,12). This prediction was based on the premise that amphetamine injections into the accumbens potentiates the efficacy of the discriminative and secondary reinforcing stimuli (2,7,8,15–17) associated with the control of consummatory behaviors. By enhancing this efficacy, bout maintenance would be prolonged. In support of this hypothesis, it has been observed that the conditioned reinforcing efficacy of stimuli paired with ethanol presentation can be facilitated by amphetamine microinjected into the n. accumbens (13). The hypothesis that n. accumbens dopamine is primarily involved in the maintenance of ethanol self-administration by facilitating conditioned stimuli is somewhat novel compared to the hypothesis that the levels of n. accumbens DA provides a signal of the amount of ethanol consumed and is, therefore, related to the termination of consumption (18). This termination hypothesis is based on the observed similarity between the time courses of the increase in extracellular DA as measured by microdialysis and the cessation of drinking. If this hypothesis regarding bout termination being a function of n. accumbens DA during the later maintenance phase is correct, the in-session amphetamine microinjection should have had less effect upon the remaining portion of the bout or even reduced consumption. However, because the bout was prolonged, the results from this study support the hypotheses that accumbens DA is involved in the maintenance of ethanol self-administration, possibly by enhancing the discriminative stimuli and conditioned reinforcers involved in the maintenance of drinking.

The increase in the responding found on the inactive lever following the amphetamine injections suggests that some nonspecific locomotor activation could have been involved in the continued responding observed. However, over 90% of the total increased responding was observed on the active lever, suggesting that most of the increased responding was directed towards the stimulus that was involved with the presentation of the ethanol. No specific stimuli were paired in this study with ethanol presentation, so no direct test of the discriminative stimuli hypothesis was possible. However, given the previous observation of the enhancement of responding by accumbens amphetamine to specific discriminative stimuli associated with ethanol presentation (13), it seems reasonable to assume that the active levers location in the chamber functioned to some degree as a discriminative stimulus.

Amphetamine injected into the accumbens can reinstate lever pressing during extinction (14), as was observed in this study. This finding also supports the hypothesis that accumbens DA activity is involved in the bout maintenance processes. The reinstatement would suggest that the discriminative stimuli present in the operant drinking environment that are salient to ethanol self-administration exert control over self-administration and can be potentiated by the actions of accumbens amphetamine injections. Although it is potentially possible that the amphetamine injection functioned as a reinforcer itself, it could be hypothesized that if this was the case, then responding should have decreased, not increased. Because there were limited numbers of amphetamine injections and they were given noncontingently with lever pressing, it is difficult to interpret the results as an effect of amphetamine reinforcement.

It can be argued that the amphetamine dose used in these studies would not result in enhanced extracellular DA levels similar to those observed in microdialysis studies of ethanol self-administering rats. Using electrochemical methods, we have found that following the microinjection of the 20-μg dose of amphetamine, local DA levels reach the micromolar concentration range (unpublished observations), a value 1000 times greater than that reported using dialysis in ethanol self-administering rats (18). Thus, it is conceivable that these very high DA levels, levels well above the normal physiological range, could result in actions very dissimilar to those that occur at the low increased levels observed in self-administering rats, The lower levels being more related to DA satiety functions, while the artificially high DA levels occurring after amphetamine injections result in a dissociation of accumbens function that potentiates maintenance type behavior. Although this explanation cannot be ruled out in the present

study, prior work with lower doses of amphetamine, doses that result in increases in n. accumbens DA to levels just slightly above those observed in dialysis experiments, failed to alter ethanol intake in any detectable manner (3,12). It would have been expected that these lower doses should have resulted in earlier termination, if low DA levels are involved in satiation.

In summary, the microinjection of amphetamine into the n. accumbens in the late maintenance phase of an ethanol drinking bout resulted in a resumption and/or extension of self-administration, in a pattern similar to that observed when amphetamine is injected prior to the drinking session. During extinction, responding on the lever associated with ethanol presentation was reinstated by the amphetamine microinjection. The results suggest that dopamine activation in the nucleus accumbens is involved with intake regulation by discriminative and/or conditioned reinforcing stimuli associated with the maintenance of ethanol self-administration throughout the entire drinking bout.

ACKNOWLEDGEMENTS

This research was supported by grants AA07404 and AA00142 from the National Institute on Alcohol Abuse and Alcoholism to HHS. Special thanks to Mr. H. Seibold of EMDEC, Bellevue, WA, for his assistance in the development of the microinjection equipment.

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