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Effects of MDMA administration on scopolamine-induced disruptions of learning and performance in rats

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Abstract

Functional deficits following short-course high-dose administration of 3,4-methylenedioxymethamphetamine (MDMA) have been difficult to characterize despite evidence indicating that MDMA is neurotoxic in several species. Therefore, the present research used rats trained to respond under a complex behavioral procedure (i.e., a multiple schedule of repeated acquisition and performance of response chains), pharmacological challenge with scopolamine and neurotransmitter assays to examine the effects of MDMA neurotoxicity on learning. Prior to MDMA administration, 0.032–0.32 mg/kg of scopolamine produced dose-dependent rate-decreasing and error-increasing effects in both components of the multiple schedule. Administration of 10 mg/kg of MDMA twice per day for 4 days also produced rate-decreasing and error-increasing effects on these days, but responding returned to baseline levels several days after the final injection. In contrast to the recovery of responding, this regimen of MDMA in untrained rats significantly reduced levels of both serotonin and its major metabolite, 5-hydroxyindoleacetic acid (5-HIAA), for 13–14 days. Furthermore, the rate-decreasing and error-increasing effects of scopolamine were significantly attenuated after MDMA treatment. These results indicate that certain complex operant behaviors rapidly recover from the effects of short-course high-dose MDMA administration, despite the reduced levels of serotonin in the central nervous system (CNS), and that this MDMA-induced loss of serotonin may affect cholinergic transmission.

Keywords: MDMA; Learning; Rats; Scopolamine; Operant behavior; Serotonin

1. Introduction

A lack of consistent behavioral effects of repeated 3,4-methylenedioxymethamphetamine (MDMA) administration has been reported to date in either humans or animals (Frederick and Paule, 1997; Marston et al., 1999; McCann et al., 1999). These inconsistencies in the data on MDMA-induced functional deficits are surprising given the large amount of immunohistochemical and autoradiographic data indicating marked changes in the central nervous system (CNS) following short-course, high-dose exposure to MDMA. For example, as early as 1987, MDMA was shown to be selectively toxic to serotonergic nerve terminals in the rodent brain (Mokler et al., 1987; Ricaurte et al., 1993; Scanzello et al., 1993; Schmidt, 1987), and subsequent studies found comparable toxicity to serotonergic neurons in both old- and new-world monkeys (Insel et al., 1989; Kleven et al., 1989; Ricaurte et al., 1988; Winsauer et al., 2002). More recently, neuroimaging techniques have been used to show cortical changes in serotonin transporter binding in human MDMA users (McCann et al., 1998;

Abbreviations: 5-HT, 5-hydroxytryptamine; MDMA, 3,4-methylenedioxymethamphetamine; Epi, epinephrine; DA, dopamine; NE, norepinephrine; 5-HIAA, 5-hydroxyindoleacetic acid.

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Semple et al., 1999) and these changes have been associated with an increasing number of clinical studies in the literature indicating that MDMA users exhibit impairments in complex cognitive functions such as learning and memory (Bolla et al., 1998; Gouzoulis-Mayfrank et al., 2000; McCann et al., 1999; Morgan, 1999; Parrott et al., 1998). As noted in many clinical studies, however, clinical data are often complicated by a host of uncontrolled variables including retrospective reporting of drug use, possible impurities or contaminants of MDMA ("ecstasy") tablets, polydrug abuse (particularly marijuana use), and potential differences in intelligence, socioeconomic status and even nutrition.

Long-term behavioral deficits following MDMA administration have also been controversial because of the relative absence of effects reported in many prospective animal studies, even though the doses of MDMA administered were arguably higher than those used by humans (cf. Cole and Sumnall, 2003). This raises questions regarding both the sensitivity of the tests used to assess functional deficits in animals and the extent of the serotonergic depletion required to produce a deficit. Certainly, identifying behavioral tasks that can assay the same complex cognitive capabilities in multiple species is a significant challenge. For example, many of the MDMA studies involving rodents have used maze tasks in one form or another (Braida et al., 2002; Broening et al., 2001; Ricaurte et al., 1993; Robinson et al., 1993), but these tasks are hard to duplicate for higher order species. Therefore, some MDMA studies have used nonhuman primates and a battery of appetitively reinforced operant tasks (Frederick and Paule, 1997; Taffe et al., 2001) in order to overcome these limitations.

The present study examined the effects of a neurotoxic MDMA treatment in rodents while using an operant learning task that has been used with several species including humans (e.g., Bickel et al., 1990; Desjardins et al., 1982; Thompson, 1973; Thompson and Winsauer, 1985; Winsauer et al., 1999) and one that has been used previously to study the acute (Thompson et al., 1987) and chronic (Winsauer et al., 2002) effects of MDMA in old- and new-world monkeys, respectively. The operant learning task, which used a repeated-acquisition technique (Boren, 1963), required subjects to learn a different predetermined sequence of responses (of fixed length) each session and acquisition of the sequence was reinforced under a second-order fixed-ratio (FR) schedule. The advantage of this baseline behavior was that during a given session, learning was demonstrated by the within-session pattern of responding where the subject (provided the sequence was acquired) made fewer incorrect responses and an increasing number of consecutive correct responses as the session progressed. Across sessions, this "learning" curve was repeatedly demonstrated as the predetermined sequence changed each session. For comparison, responding on an invariant sequence also comprised performance components, which were combined with repeated-acquisition components under a multiple schedule (Moerschbaecher et al., 1979) as a behavioral control for the nonspecific effects of MDMA.

To examine the effects of repeated MDMA exposure, two separate neurotoxic regimens of MDMA were administered to rats trained under the multiple schedule. These subjects also received a muscarinic antagonist, scopolamine, before and shortly after each MDMA treatment because serotonergic lesions combined with a cholinergic lesion or a cholinergic receptor antagonist have been reported to produce disruptions in learning and memory that are greater than those produced by either type of blockade alone (Nilsson et al., 1988; Riekkinen et al., 1991; Vanderwolf, 1987). Finally, as a means of verifying the depletion of serotonin by MDMA and determining if the recovery of behavioral responding coincided with the recovery of serotonin levels in various brain regions, a single MDMA regimen was administered to additional, untrained rats for analysis.

2. Methods

2.1. Subjects

A total of 88 male Long-Evans hooded rats served as subjects for both the neurotoxicity studies and the behavioral studies. Of these, 19 rats served as subjects for the behavioral studies and 69 rats served as subjects in the toxicity studies. For the toxicity studies, the subjects were divided into two groups, one that received intraperitoneal MDMA administration (n=41) and another that received subcutaneous MDMA administration (n=28). The effects of the i.p. route of administration were of particular interest because both s.c. and i.p. routes have been used in previous studies, but direct comparisons between the neurotoxic effects of the two routes of administration are generally absent from the literature. Fewer subjects were investigated after s.c. administration because the vast majority of the studies in the literature have used this route of administration and the effects are well characterized. Within each of these groups the subjects were divided into four subgroups that were determined by the time of sacrifice after treatment: saline or MDMA, 3 days post treatment and saline or MDMA, 13 or 14 days post treatment (depending on the day of the week). Regardless of the study, rats were housed singly in a colony room maintained at 21±1 °C with 50±10% relative humidity on a 14-h light/10-h dark cycle, which began at 6 a.m. Rats involved in the behavioral studies earned 45-mg food pellets (Bioserv, Frenchtown, NJ) during the experimental sessions and, if necessary, were provided rodent chow (Labdiet, Brentwood, MO) in the home cage after the session in order to maintain them at 85% of their free-feeding weight. Subjects involved in the toxicity studies were also maintained at 85% of their free-feeding weight, but received only rodent chow. Water was available to all subjects ad libitum in their home cage. In all

situations, experiments were performed in accordance with the declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The experimental protocols were also approved by the Institutional Animal Care and Use Committee at Louisiana State University Health Sciences Center.

2.2. MDMA treatment

When rats were treated with 3,4-methylenedioxymethamphetamine (MDMA), the regimen consisted of either an i.p. or s.c. injection of 10 mg/kg twice a day (approximately 12 h apart) for 4 days. MDMA was obtained from the National Institute on Drug Abuse (Research Triangle Park, NC) and dissolved in saline. The injection volume was 0.1 ml/100 g. Rats in the control groups always received saline in a comparable volume via the same routes at the same intervals as the MDMA-treated groups. For the toxicity studies, all the treatment groups (MDMA or saline) received only one 4-day regimen prior to sacrifice by rapid decapitation, whereas for the behavioral studies, all of the rats received two 4-day regimens of MDMA (s.c.) separated by 24 days. The behavioral subjects were also tested throughout MDMA administration, approximately 6 h after the a.m. injection and a minimum of 4.5 h before the p.m. injection. This group also received an additional within-subject control condition prior to MDMA treatment where each rat received saline twice per day for 4 days and they were tested approximately 6 h after the a.m. injection on these days.

2.3. Neurotransmitter assay

Immediately after decapitation, the brains were chilled and dissected for analyses of biogenic amines by high performance liquid chromatography (HPLC). The medulla oblongata, hypothalamus, midbrain, striatum, hippocampus and cortex were separated using the procedure described by Glowinski and Iversen (1966). Each brain region was weighed to the nearest 0.1 mg, and homogenized in 10 ml in the case of the cortex, or 1 ml for the other brain areas, of chilled mobile phase buffer (0.1 M citrate, 10% ethanol, and 250 mg/l sodium octyl sulfate (SOS), pH 4). An internal standard of 3,4-dihydroxybenzylamine hydrobromide (DHBA) was added such that its final concentration was 1 ng/100 μl of the HPLC injectate. The homogenates were centrifuged in an RC2B Sorvall centrifuge at 12,000×*g* and the supernatants were frozen at −80 °C until assayed. The chromatographic system consisted of the following hardware and software: dual Rainin Rabbit HP pumps equipped with self-washing piston pump heads (capable of maximum flows of 10 ml/min) provided a flow rate of 1.5 ml/min. Injections of samples were accomplished automatically using an Alcott model 728 autosampler. This feature allowed as many as 40 samples to be analyzed overnight. The HPLC column consisted of a Rainin microsorb (5 μM) C18 column (25 cm×1.6 mm). A 1.5 cm×4.6 mm guard column packed with microsorb C18 preceded the analytical column. Chromatographically separated monoamines were assayed by electrochemical detection utilizing an ESA model 5100 Coulchem multielectrode array. This electrode array consisted of a model 5020 guard cell and a model 5010 dual analytical electrode cell. The guard cell voltage was +0.4 V. The two analytical cells were set at −0.04 V (Detector 1) and +0.32 V (Detector 2). Norepinephrine (NE), epinephrine (Epi), dopamine (DA), 5-hydroxytryptamine (5-HT, serotonin) and 5-hydroxyindoleacetic acid (5-HIAA) were determined in unknown and standard samples by comparison to retention times and integrated areas of peaks. Co-chromatography of spiked standards of each compound was initially analyzed with brain regions in order to make sure that the peaks identified as specific monoamines were indeed authentic. Levels of each neurotransmitter in each of the brain regions dissected were then analyzed for an effect of MDMA treatment at the two specified times of sacrifice.

2.4. Apparatus

Nine identical modular test chambers (Coulbourn Instruments, model E10-10TC) configured specifically for rodents were used. Located on the front wall of each chamber were a houselight, speaker, auditory feedback relay, pellet trough (5.5 cm above the floor and centered), and three response keys aligned horizontally (8 cm apart, center to center, and 14.5 cm above the floor). Each response key could be transilluminated by three Sylvania 28ESB indicator lamps, one with a red plastic cap, one with a yellow cap, and one without a cap (white). Response keys required a minimum force of 0.15 N for activation and correct responses produced an audible click of the feedback relay. Each chamber was enclosed within a sound-attenuating cubicle equipped with a fan for ventilation. All test chambers were connected to a computer programmed in MED-PC/MED-STATE NOTATION software (MED Associates, and Thomas A. Tatham, St. Albans, VT), and to cumulative recorders (Gerbrands, Arlington, MA) located within the same room.

2.5. Behavioral procedure

2.5.1. Multiple schedule of repeated acquisition and performance

Preliminary training for the repeated-acquisition task has been described previously (Winsauer et al., 1995) and included magazine training, shaping of the response (nose press) and reinforcing responses on individually illuminated keys after shaping. To train repeated acquisition in all the rats, all three response keys were illuminated with yellow light, but only one of the three response keys was chosen to be correct for a particular session and each response emitted

on that key resulted in the delivery of a food pellet. Responding on either of the other two illuminated keys was considered an error and resulted in a 5-s timeout during which the key lights were extinguished and responding had no programmed consequence. For each additional session during this stage of training, the position for the correct response was varied pseudorandomly. After rats reliably acquired this task, regardless of the key position, a second response was added to the sequence or chain such that two correct responses were necessary to obtain food pellets. This type of sequential responding is procedurally defined as a "chain" because each response except the last produces a discriminative stimulus controlling the response that follows (Kelleher, 1966). The key positions for the correct responses varied both within the two-response sequence and across sessions. The color of the key lights changed after each correct response. A third response was added to the sequence when stable responding was obtained under the two-response sequence. Training continued until response rates and the percentage of errors did not vary by more than ±20% or ±10% of the mean, respectively, for 10 consecutive sessions. A second component was then added to the schedule so that rats responded under a multiple schedule of repeated acquisition and performance of response chains.

During acquisition components, the three response keys were illuminated at the same time with one of three colors: white, red or yellow. Responding on the correct key in the presence of one color changed the color of the key lights as well as the position for the next correct response (e.g., keys white, center correct; keys red, left correct; keys yellow, right correct). When the response sequence was completed by emitting three correct responses (i.e., one correct response in the presence of each color), the key lights were extinguished and the stimulus light in the pellet trough was illuminated for 0.4 s. Subsequently, the response keys were illuminated with the first color (i.e., white) and the sequence was reset. Within a given session, the correct response that was associated with a particular color did not change, and the same sequence (in this case, center–left–right or C–L–R) was repeated during all acquisition components of a given session. Responding on this sequence was maintained by food presentation under a second-order fixed-ratio 2 schedule such that every second completion of the sequence resulted in the presentation of a 45-mg food pellet. When rats responded on an incorrect key (in the example, the left or right key when the white lights were illuminated), the error was followed by a 5-s timeout. An incorrect response did not reset the three response sequence (i.e., the stimuli and the position of the correct response were the same before and after a timeout).

To establish a steady state of repeated acquisition, the sequence was changed from session to session. An example of sequences for five consecutive sessions was: C–L–R, L–R–C, C–R–L, R–L–C, L–C–R. The sequences were carefully selected to be equivalent in several ways and there were restrictions on their ordering across sessions. Briefly, each sequence was scheduled with equal frequency and consecutive correct responses within a sequence were scheduled on different keys. Occasionally, a correct sequence position for a given color was the same for two consecutive sessions (in the list of sequences above, L–R–C and C–R–L).

During performance components, response keys and houselights were illuminated, and the sequence remained the same (R–C–L) from session to session. In all other aspects (colored stimuli for each response in the sequence, fixed-ratio 2 schedule of food presentation, 5-s timeout, etc.), the performance components were identical to the acquisition components. Experimental sessions always began with an acquisition component, which then alternated with a performance component after 40 reinforcers or 20 min, whichever occurred first. Each session terminated after 200 reinforcers or 100 min, whichever occurred first. Throughout testing, sessions were generally conducted 5 days per week, Monday through Friday.

2.6. Drug testing

Dose-effect curves for scopolamine HCl (Sigma, St. Louis, MO) were determined in all 19 rats involved in the behavioral studies after responding had stabilized under the multiple schedule and prior to the first MDMA or saline regimen. Scopolamine was dissolved in saline (0.9%) and administered i.p. in a mixed order on Tuesdays and Fridays throughout behavioral testing. Saline (control) injections were administered on Thursdays. The volume of injection for both drug and saline was 0.1 ml/100 g of body weight and injections were given 15 min before the start of the session.

Following each of the two regimens of MDMA or saline, the effects of the 0.1- and 0.32-mg/kg doses of scopolamine were redetermined in a mixed order several times in each subject during the testing days that occurred after each MDMA or saline regimen. These data were then compared statistically with data from the dose-effect curves collected prior to MDMA treatment. Doses of scopolamine administered after MDMA treatment were selected from the dose-effect curves established in all of the subjects prior to MDMA treatment. Although previous studies used large dosages of cholinergic antagonist in order to achieve a substantial blockade of cholinergic transmission (Robinson et al., 1993; Vanderwolf, 1987), such large doses in the present study would have eliminated responding and essentially created a "floor" effect that would have been prohibitive for characterizing the interaction.

2.7. Statistical analysis

The data for both components of the multiple schedule were analyzed in terms of: (1) the overall response rate (total responses/min, excluding timeouts), and (2) the overall

accuracy, expressed as the percentage of errors [(incorrect responses/total responses)×100]. However, data were excluded from the analyses for percent errors when the response rate was less than 5 responses/min because of the small number of responses involved. The mean data for each subject were grouped and analyzed statistically for an effect of scopolamine dose on responding in each component using a one-way ANOVA with repeated measures (SigmaStat Statistical Software, SPSS, Chicago, IL). Dunnett's tests with an alpha level equal to or less than 0.05 were used to compare drug sessions with control (saline) sessions. The effects of MDMA treatment over days and the effects of scopolamine dose after each MDMA or saline treatment were analyzed using a two-way ANOVA with repeated measures. Multiple comparisons were conducted with Tukey tests following the two-way ANOVA. In addition to these measures based on session totals, within-session changes in responding were monitored by a cumulative recorder (Gerbrands) and the computer. For example, acquisition of a response sequence was indicated by within-session error reduction; that is, a decrease in the number of errors between food presentations as the session progressed.

For the toxicity studies, the effect of MDMA treatment on the levels of neurotransmitter in each brain area at each time of sacrifice was determined using Fischer's protected LSD test, giving the exact *P*-value. All values are plotted as the group average±standard error of the mean (S.E.M).

3. Results

3.1. Behavioral studies

Stable responding in both components of the multiple schedule was evident for all subjects during the baseline sessions that preceded administration of scopolamine or MDMA. In addition, the daily pattern of responding in the acquisition components was characterized by a steady state in terms of within-session error reduction, which was generally indicated by a distinct decrease in the number of errors and a concomitant increase in consecutive correct completions of the response sequence. This response pattern at the start of the session in the initial acquisition component also accounted for the fact that the mean percentage of errors was larger for the acquisition components (i.e., 12.6) than for the performance components (i.e., 5.2) under baseline and control conditions. The mean response rates were comparable in both components (57.2 for responding in acquisition and 57.3 for responding in performance).

Compared to control conditions where saline was administered, scopolamine (0.032–0.32 mg/kg) produced dose-dependent decreases in overall rates of responding in both the acquisition ($F(5,90)=35.55$, $p<0.001$) and performance ($F(5,90)=32.17$, $p<0.001$) components (Fig. 1). As

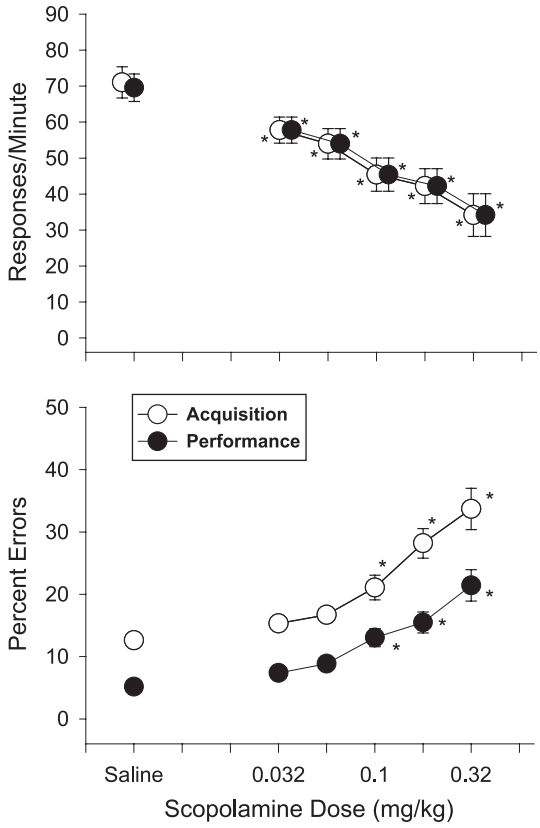

Fig. 1. Effects of scopolamine on the overall response rates and percent errors in 19 subjects responding under a multiple schedule of repeated acquisition and performance of response chains. The data from the acquisition components are represented by the open symbols, whereas the data from the performance components are represented by the closed symbols. Data points with vertical lines above "Saline" indicate the mean and standard error of the mean (S.E.M.) for control sessions in which saline was administered intraperitoneally (i.p.). The data points with vertical lines in the dose-effect curves indicate the mean and S.E.M. for sessions in which i.p. doses of scopolamine were administered in a mixed order. Any points without vertical lines indicate instances in which the S.E.M. is encompassed by the point. The asterisks reflect doses of scopolamine that were significantly ($p<0.05$) different from saline as determined by Dunnett's post hoc tests.

shown, the rate-decreasing effects were similar in both components with the 0.1 mg/kg decreasing responding to approximately 35% of control, and 0.32 mg/kg decreasing responding to approximately 50% of control.

Scopolamine also produced comparable dose-dependent increases in the percentage of errors in the acquisition ($F(5,88)=28.55$, $p<0.001$) and performance ($F(5,90)=25.43$, $p<0.001$) components at the same doses that decreased overall response rate to 35% of control or more (i.e., 0.1–0.32 mg/kg). For example, percent errors after 0.1 mg/kg of scopolamine increased from 12.6 to 21.1 and from 5.2 to 13.0 in acquisition and performance, respectively. Following the 0.32-mg/kg dose of scopolamine, mean percent errors peaked at 34.2 for the acquisition components, and 21.4 for the performance components.

Fig. 2 shows the effect of each MDMA regimen on overall response rate and percent errors during the 4 days of

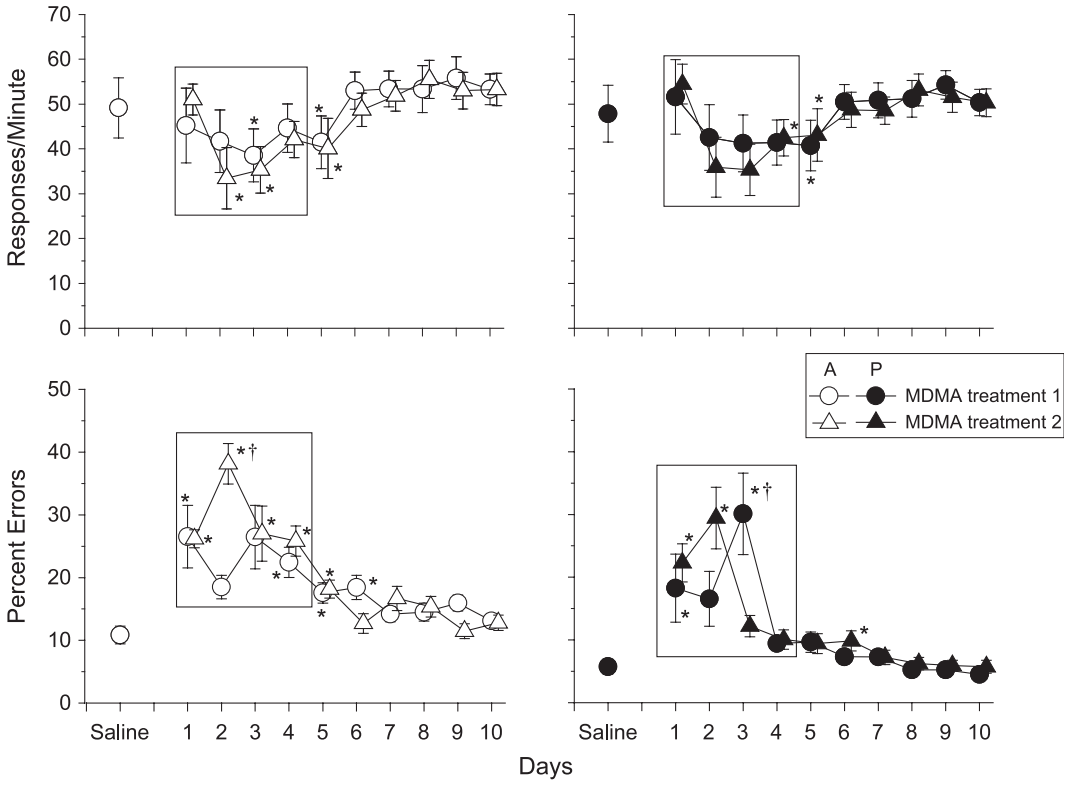

Fig. 2. Effects of two regimens of MDMA administration on overall response rate and percent errors over 10 days in rats (n=10) responding under the multiple schedule. Each regimen consisted of two daily s.c. injections of 10 mg/kg for 4 days (see data within box), and a total of 24 days separated the two toxic regimens of MDMA. The data from the first and second regimens are shown as circles and triangles, respectively. As a control, these same rats also received s.c. saline twice per day for days prior to MDMA treatment (data above Saline). For additional details, see legend for Fig. 1. * Indicate a significant difference from saline (p<0.05) and † indicate a significant difference between MDMA treatments as determined by Tukey post hoc tests.

treatment and a 6-day period subsequent to each treatment. As indicated by a two-way ANOVA, MDMA significantly decreased overall response rate in the acquisition components (F(2,16)=5.01, p<0.05) and increased percent errors in both the acquisition (F(2,16)=28.88, p<0.001) and performance (F(2,16)=7.92, p<0.01) components. Not surprisingly, as the effects of each MDMA regimen increased and then decreased over the 4 days, there was also a significant effect of day on both dependent measures in the acquisition (response rate, F(5,40)=7.25, p<0.001; error, F(5,40)=4.89, p=0.001) and performance (response rate, F(5,40)=7.54, p<0.001; error, F(5,40)=6.77, p<0.001) components, and a significant interaction of MDMA treatment and day for the acquisition components (response rate, F(10,76)=2.07, p<0.05; error, F(10,76)=5.61, p<0.001) and the performance components (response rate, F(10,76)=2.78, p<0.01; error, F(10,76)=4.61, p<0.001). When each day of each MDMA treatment was compared to mean data obtained after saline administration twice per day for 4 days (within-subject control), significant differences between the MDMA treatments were also evident. For example, on the second day of MDMA treatment, the second regimen of MDMA had a larger effect than the first treatment on percent errors in acquisition (F(2,16)=26.73, p<0.001), and on day 3, the first regimen had a larger effect than the second regimen on percent errors in performance (F(2,17)=9.57, p<0.01). In addition to these differences in the effects of MDMA on response rate and percent error, responding was also disrupted during the sessions that occurred on the day after the last injection. This was particularly true for responding in the acquisition components where both rate (F(2,16)=7.03, p<0.01) and accuracy (F(2,15)=6.76, p<0.01) were still disrupted after each regimen as indicated by multiple comparisons after a one-way ANOVA.

The effects of scopolamine on rate of responding and percent errors in the acquisition and performance components, before and after each MDMA (3a, n=10) or saline (3b, n=9) treatment, are shown in Fig. 3. For ease of comparison, the scopolamine dose-effect curve for each group is depicted, but only data for the 0.1 and 0.32 mg/kg doses were included in the statistical analyses. Similar to the overall effects of scopolamine obtained for the entire group (see Fig. 1), scopolamine in the MDMA- and saline-treated groups produced dose-dependent decreases in response rate and increases in percent errors in both components. This was indicated by significant main effects of scopolamine dose in the MDMA-treated subjects and saline-treated subjects (MDMA: acquisition response rate, F(2,18)= 167.28, p<0.001; acquisition error, F(2,18)=34.78, p<0.001; performance response rate, F(2,18)=153.55, p<0.001; performance error, F(2,18)=30.66, p<0.001; sal-

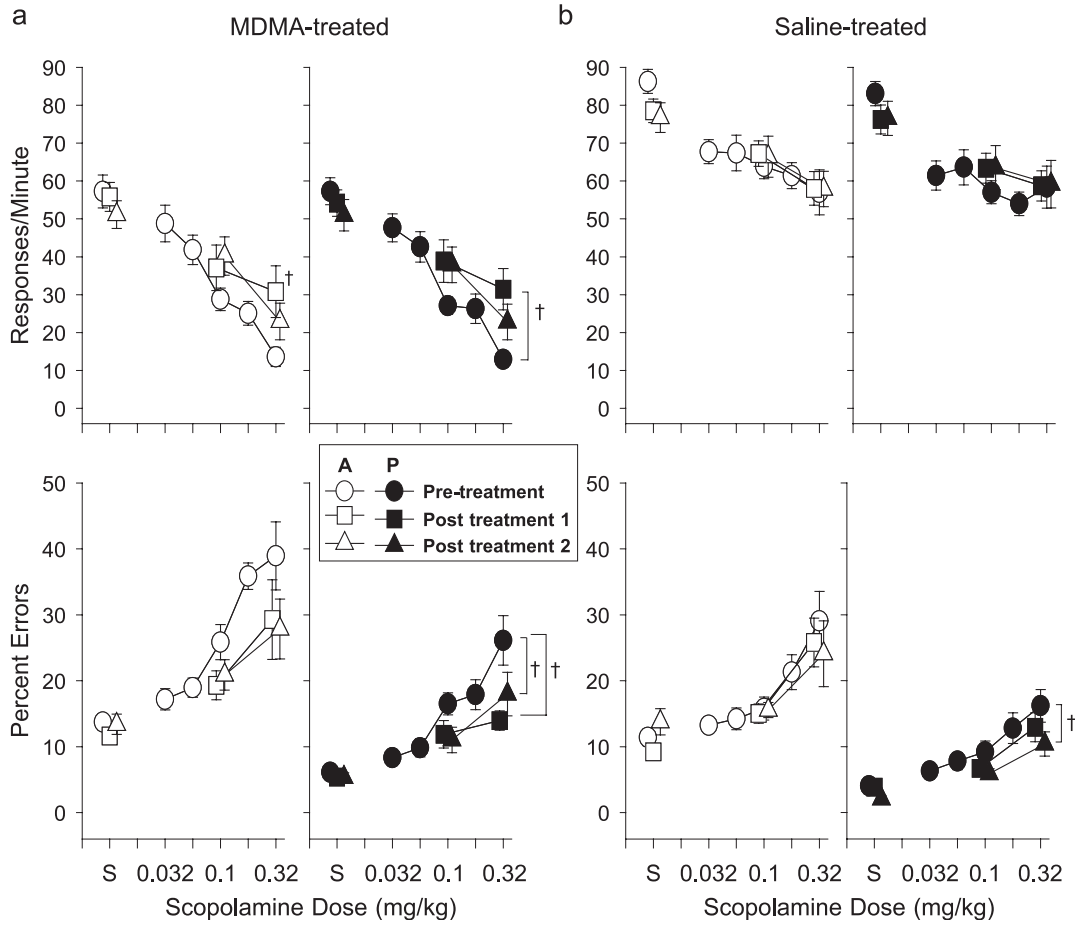

Fig. 3. Effects of 0.1 and 0.32 mg/kg of scopolamine on response rate and percent errors before and after two dosing regimens of MDMA (n=10) or saline (n=9) in rats responding under a multiple schedule. Circles (unfilled and filled) with vertical lines indicate the mean and S.E.M. for acute determinations of saline or scopolamine prior to the 4-day regimen of MDMA or saline injections, whereas the squares and triangles with vertical lines indicate the mean and S.E.M. after each MDMA or saline regimen. Crosses (†) indicate significant differences from scopolamine administration prior to MDMA administration, whereas crosses next to a bracket indicate main effects of treatment as indicated by a two-way ANOVA.

ine: acquisition response rate, $F(2,16)=23.01$, $p<0.001$; acquisition error, $F(2,16)=20.39$, $p<0.001$; performance response rate, $F(2,16)=16.22$, $p<0.001$; performance error, $F(2,16)=25.92$, $p<0.001$). In the MDMA-treated subjects, there was also a main effect of MDMA treatment on response rate ($F(2,18)=3.77$, $p<0.05$) and percent errors ($F(2,18)=6.41$, $p<0.01$) in the performance component and a significant interaction between scopolamine dose and MDMA treatment for response rate in both the acquisition and performance components (acquisition: $F(4,36)=3.70$, $p<0.05$; performance: $F(4,36)=4.70$, $p<0.01$). The interaction indicating that the effect of dose on response rate depended on the level of MDMA treatment in these two components. Post hoc analyses also revealed that this interaction was not due to a permanent change in baseline response rate or accuracy for the group as these indices of responding did not differ significantly during control sessions after each MDMA regimen (e.g., note comparability of data above S in each graph). There was no effect of MDMA treatment on percent errors in acquisition. In the saline-treated subjects, two 4-day treatments with saline did not alter the determinations for scopolamine dose except in the performance component where percent errors were significantly decreased after the second saline treatment ($F(2,16)=6.56$, $p<0.01$).

Fig. 4 depicts representative cumulative records of the within-session effects of 0.32 mg/kg of scopolamine in one subject before and after MDMA treatment. In the control record (top row), the number of errors made in the acquisition components decreased as the session progressed, indicating that the subject acquired the response chain. Very few errors were made in the performance components, which alternated with the acquisition components throughout the session. Note the high rate of correct responding obtained in both the acquisition and performance components. In contrast to the pattern of responding obtained under control conditions, 0.32 mg/kg of scopolamine (second and third rows) produced a large disruption in overall response rate and accuracy in both components, as indicated by the substantial reduction in correct responding and large increase in the frequency of errors. Moreover, acquisition of the response sequence did not occur until the

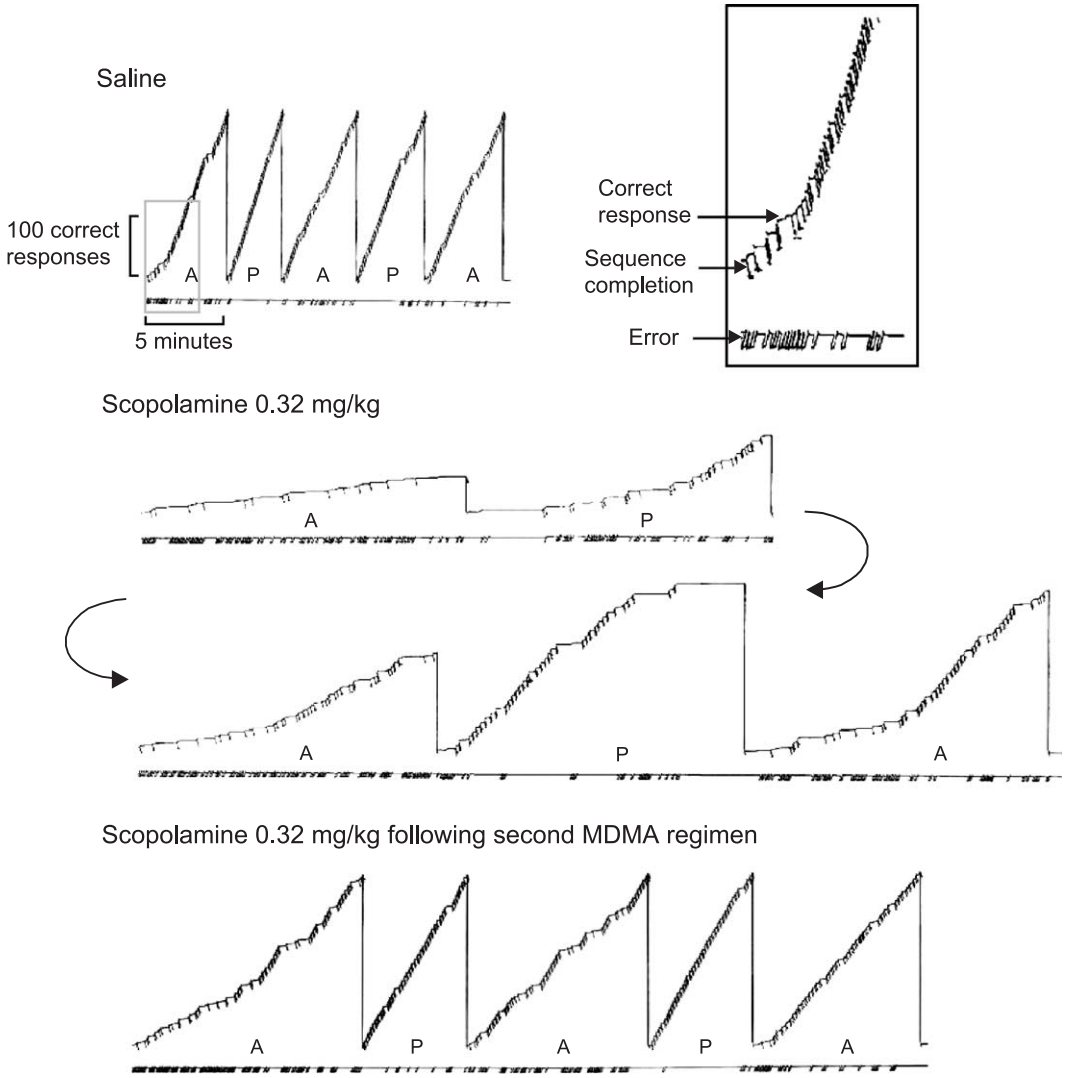

Fig. 4. Cumulative records for subject PR 273 showing the within-session effects of a 0.32-mg/kg dose of scopolamine on responding in the acquisition (A) and performance (P) components of the multiple schedule, before and after two regimens of MDMA. The record in the top row represents responding obtained when saline was administered 15 min prior to the session. In each record (see inset), the response pen stepped upward with each correct response and was deflected downward each time the three-response sequence was completed. Food pellets were delivered after every two completions of the sequence. Downward deflections of the event pen (below each record) indicate incorrect responses in both components. The response pen reset at the completion of each component. Each session began with an acquisition component, which alternated with a performance component after 40 reinforcers or 20 min, whichever occurred first. Each session terminated after 200 reinforcers or 100 min, whichever occurred first.

third acquisition component when errors gradually decreased and the number of consecutive correct responses increased. Although the rate of correct responding did increase somewhat as the session progressed, especially in the second performance component, control levels had not recovered by the end of the session.

When 0.32 mg/kg of scopolamine was administered after the second regimen of MDMA, the disruptive effects of this dose on the within-session pattern of responding were greatly attenuated (bottom row). In the acquisition components, the rate of correct responding was notably higher than that observed prior to MDMA treatment, but it remained lower than the saline control. The number of errors emitted in the acquisition components also remained elevated when compared to control; however, acquisition was evident early in the session as indicated by the comparatively rapid increase in correct responding and decrease in the error rate. In the performance components, 0.32 mg/kg of scopolamine decreased response rates in comparison to saline administration, but this dose produced substantially smaller rate-decreasing effects than those obtained before MDMA administration.

3.2. Neurotransmitter studies

3.2.1. IP administration

Three days after a single 4-day regimen of MDMA, serotonin levels were significantly decreased in the hippocampus ($p<0.05$), striatum ($p<0.001$), midbrain ($p<0.05$), hypothalamus ($p<0.005$) and cortex ($p<0.001$)

when compared to the levels in a time-matched saline-treated group of subjects (Fig. 5a). On day 14, serotonin levels were reduced by MDMA treatment in all brain regions assayed ($p<0.05$). The levels of the serotonin metabolite 5-HIAA were decreased by MDMA treatment on day 3 in the hippocampus ($p<0.005$) and the cortex ($p<0.001$) only. However, 14 days after i.p. MDMA treatment all brain areas except the medulla contained lower levels of 5-HIAA when compared to saline-treated animals ($p<0.05$; Fig. 5b). In contrast, MDMA treatment had comparatively small effects on other levels of neurotransmitter, although dopamine levels were significantly increased in the midbrain 3 days following the last injection ($p<0.05$) and decreased in hippocampus on day 14 when compared to the time-matched saline control group ($p<0.05$; Fig. 5c). The levels of epinephrine were also significantly lower than control levels in the midbrain 3 days following the last injection of MDMA, and in the hypothalamus 14 days following the last injection ($p<0.05$; Fig. 5d). Norepinephrine levels were not

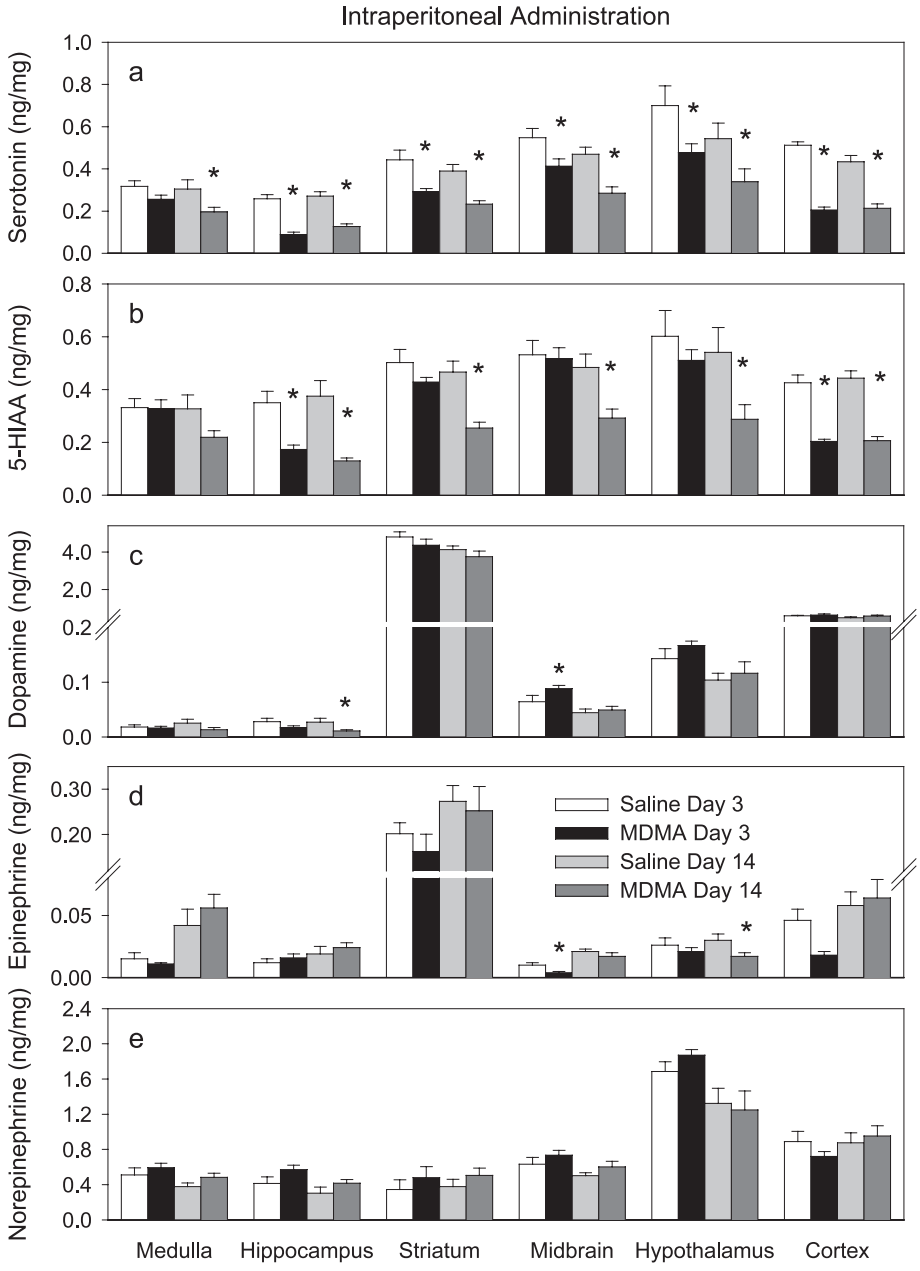

Fig. 5. Bar graphs depicting the levels of serotonin (a), 5-HIAA (b), dopamine (c), epinephrine (d) and norepinephrine (e) in rat brain after a single 4-day regimen of intraperitoneal MDMA. The first and third bars in each grouping represent the mean and S.E.M. for neurotransmitter levels in saline-treated rats, 3 (unfilled bars, $n=10$) or 14 (light gray bars, $n=11$) days after the last injection. The second and fourth bars in each grouping represent the mean and S.E.M. for neurotransmitter levels in MDMA-treated rats on day 3 (black bars, $n=10$) or 14 (dark gray bars, $n=10$). The asterisks indicate significant ($p<0.05$) differences between levels in MDMA-treated rats and time-matched saline-treated rats as determined by Fischer's protected LSD test.

significantly affected by MDMA treatment in any brain area at either day 3 or day 14.

3.2.2. SC administration

The effects of subcutaneous MDMA injections on brain neurotransmitter levels are shown in Fig. 6. On day 3, serotonin levels were significantly reduced when compared to time-matched saline-treated group in the hippocampus ($p<0.001$), midbrain ($p<0.05$), hypothalamus ($p<0.001$) and cortex ($p<0.001$; Fig. 6a) and 5-HIAA levels were reduced in all brain areas but the medulla ($p<0.01$; Fig. 6b). Fourteen days after the last injection of MDMA, serotonin and 5-HIAA levels were notably reduced in all the brain regions ($p<0.05$; Fig. 6a and b). Similar to i.p. MDMA administration, the largest effects obtained were on serotonin and 5-HIAA; however, s.c. MDMA administration was unlike i.p. administration in that it did not significantly affect the levels of DA, Epi or NE in any of the brain regions assayed (Fig. 6c–e).

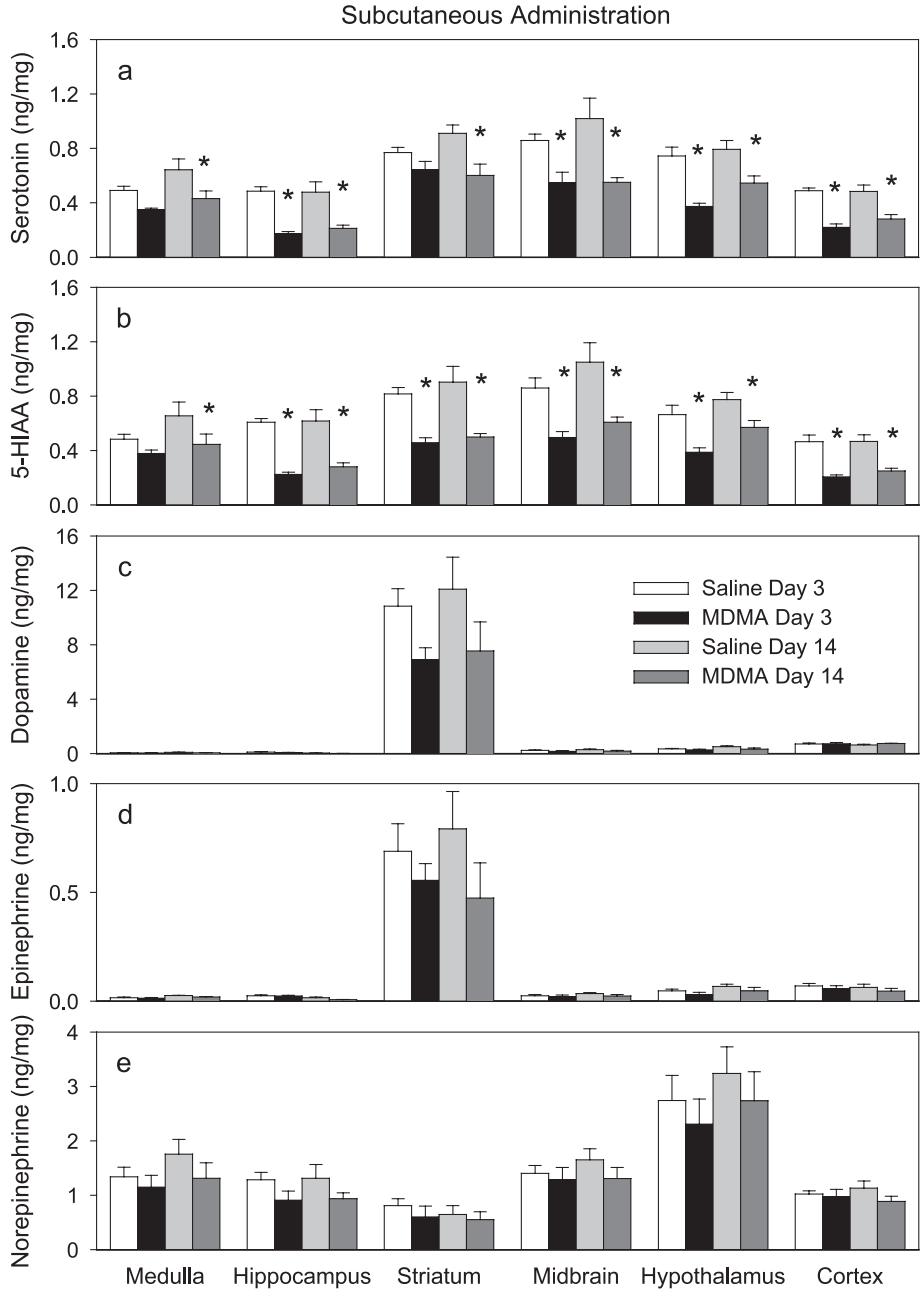

Fig. 6. Levels of serotonin (a), 5-HIAA (b), dopamine (c), epinephrine (d) and norepinephrine (e) in rat brain after a single regimen of subcutaneous MDMA. The first and third bars in each grouping indicate the mean and S.E.M. for neurotransmitter levels in saline-treated rats, 3 (unfilled bars, $n=7$) or 14 (light gray bars, $n=7$) days after the last injection. The second and fourth bars in each grouping represent the mean and S.E.M. for neurotransmitter levels in MDMA-treated rats on day 3 (black bars, $n=7$) or 14 (dark gray bars, $n=7$). The asterisks indicate significant ($p<0.05$) differences between levels in MDMA-treated rats and time-matched saline-treated rats as determined by Fischer's protected LSD test.

4. Discussion

In the present study, the muscarinic receptor antagonist, scopolamine, produced dose-dependent rate-decreasing and error-increasing effects in both the acquisition and performance components of the multiple schedule prior to two separate dosing regimens of MDMA. Following each regimen of MDMA, however, several redeterminations of two doses of scopolamine (0.1 and 0.32 mg/kg) indicated a significant attenuation of its effects in each component of the multiple schedule. Importantly, this attenuation of scopolamine's effects following MDMA administration could not be attributed to changes in the overall ability of subjects to respond under each behavioral task or to shifts in their baseline levels of responding as MDMA-induced decreases in rate and accuracy were mostly limited to the days on which they were receiving MDMA injections. The recovery of responding following MDMA administration was also remarkable in that it occurred at a time when both serotonin and 5-HIAA was significantly below control levels in multiple brain areas.

Although the behavioral effects of MDMA have been examined in multiple species using a variety of operant tasks, there is still a paucity of data on MDMA-induced functional deficits, partially due to the different questions addressed in those studies. In general, behavioral studies involving MDMA can be divided into three broad categories: those studies that have examined the acute dose effects of MDMA immediately prior to behavioral testing (e.g., Braida et al., 2002; Frederick et al., 1995; Sabol et al., 1995; Thompson et al., 1987), those that have administered a neurotoxic regimen of MDMA and then used behavioral procedures for the purposes of characterizing subsequent functional deficits (e.g., Gurtman et al., 2002; Ricaurte et al., 1993; Robinson et al., 1993; Taffe et al., 2001; Winsauer et al., 2002) and those that have done both (e.g., Byrne et al., 2000; Frederick et al., 1998; LeSage et al., 1993; Li et al., 1989; Marston et al., 1999). With few exceptions, the data from the first category of studies indicate that MDMA is disruptive to most complex behavioral tasks, whereas data from the second category of studies is less consistent and strongly suggests that the long-term behavioral toxicity of MDMA may be highly variable, and age and task dependent. For example, Robinson et al. (1993) showed that significant depletion of brain serotonin levels by MDMA did not produce any disruptions in rats performing a spatial navigation task in a Morris water maze. Similarly, Ricaurte et al. (1993) found that neurotoxic MDMA treatment did not affect choice accuracy in rats performing three appetitively reinforced variations of a spatial alternation task, although depletion of serotonin by 5,7-dihydroxytyptamine/desmethylimipramine treatment did affect this measure. In contrast to these findings, a neurotoxic regimen of MDMA in rat pups on postnatal days 11–20 produced disruptions in sequential and spatial, but not cued, learning approximately 1 month after the regimen (Broening et al., 2001).

Similar to many behavioral studies examining the acute effects of MDMA, the present study found that administration of a large dose (10 mg/kg) decreased response rate and increased errors during the acquisition and performance of an operant response chain. Somewhat surprising, however, was the fact that these effects were obtained approximately 6 h after the a.m. injection of MDMA. In general, most acute studies have examined much shorter presession administration intervals. In one study, for example, fewer than half of the rats trained under a differential reinforcement of low rates (DRL) schedule responded 20 min after 8 mg/kg (Sabol et al., 1995), whereas 5.6 mg/kg of MDMA in another study disrupted acquisition of water-reinforced lever responding by producing a 100-min pause 15 min after injection (Byrne et al., 2000). Interestingly, rates of responding during this 8-h acquisition session were close to baseline levels or higher after an initial pause, which suggested that the effects of MDMA during this session were limited to 1 or 2 h. The present data indicate that MDMA can exert marked effects on responding for as long as 6 h after s.c. injection and that this effect was not a cumulative effect of drug administration because the rate-decreasing and error-increasing effects were apparent on the first day of the regimen. In fact, even though rate-decreasing and error-increasing effects were present for several days immediately following the 4-day regimen of MDMA, the effects obtained during the regimen peaked on the second day of treatment and tended to decrease thereafter, suggesting the development of behavioral tolerance.

Another interesting aspect of the behavioral recovery that followed each regimen of MDMA was that it occurred at a time when both 5-HT and 5-HIAA levels were still significantly lower than those for the respective control animals. MDMA administration has been shown to produce considerable depletions in regional brain serotonin and 5-HIAA in rats (Boot et al., 2002; Mokler et al., 1987; Ricaurte et al., 1993; Schmidt, 1987) and nonhuman primates (Insel et al., 1989; Kleven et al., 1989; Winsauer et al., 2002). In rats, s.c. administration of 20 mg/kg of MDMA twice daily for 4 days resulted in significant depletion of serotonin in multiple brain areas as soon as 1 day after treatment (Boot et al., 2002) and for as long as 5 months after the treatment (Ricaurte et al., 1993). Li et al. (1989) also found that a 4-day regimen of 6 mg/kg of MDMA twice daily was toxic specifically to the serotonergic system in rats. Similarly, the present data demonstrated that s.c. administration of a slightly larger dose of MDMA (10 mg/kg) produced selective depletions of serotonin and 5-HIAA, but not dopamine, norepinephrine or epinephrine in most areas of the brain.

While most studies with rats have examined the neurotoxic effects of s.c. administration of MDMA, only a few studies have investigated the effects of intraperitoneal (i.p.) administration (Gurtman et al., 2002; Marston et al., 1999; Scanzello et al., 1993). Moreover, the dosing regimens for

i.p. MDMA administration have not been consistent across studies, which prohibits a direct comparison. For example, Gurtman et al. (2002) administered 5 mg/kg i.p. every hour for 4 h for two consecutive days and found that levels of serotonin and 5-HIAA, but not dopamine, were reduced for 10 weeks. In another study, Scanzello et al. (1993) administered 10 mg/kg of MDMA i.p. to rats every 2 h for a total dose of 40 mg/kg and obtained depletions of serotonin and 5-HIAA for periods up to 32 weeks in some brain areas. Although the present study only examined neurotransmitter levels within the first 2 weeks of a single MDMA treatment, the data clearly show that both i.p. and s.c. administration of MDMA can significantly reduce serotonin and 5-HIAA over that time period and that levels of these substances were reduced at the time of the first scopolamine challenges. In addition, the i.p. data give further credence to the notion that adrenergic transmission may also be affected in some brain areas after MDMA administration (Arrue et al., 2003; Mayerhofer et al., 2001). The effects on epinephrine were especially interesting because levels of this neurotransmitter have rarely been examined after MDMA.

Scopolamine and other muscarinic receptor antagonists have been widely reported to decrease response rate and increase errors in rats responding under a variety of complex operant tasks (Baron et al., 1998; Evenden, 2002; Shannon et al., 1990), including repeated-acquisition tasks (Cohn and Cory-Slechta, 1993; Howard and Pollard, 1983; Jutkiewicz et al., 2003). Similar to the present study, the data in a majority of these studies indicated that scopolamine has prominent effects on both response rate and errors, and that response rate was generally more sensitive than accuracy. In a study by Evenden (2002), for example, scopolamine only disrupted choice accuracy under a random reinforcement procedure at doses that markedly reduced response rates. Similarly, in two separate studies using a two-lever spatial alternation task with rats (Bymaster et al., 1993; Shannon et al., 1990), the percentage of correct responses was less sensitive to the effects of scopolamine than the overall response rate. In the study by Bymaster et al. (1993), this particular profile of effects was attributed specifically to antagonism at M2 receptors, as selective antagonism at M1 receptors by trihexylphenidyl and pirenzepine produced the opposite profile of effects (i.e., decreasing accuracy prior to decreasing response rate).

The error-increasing effects of scopolamine in these operant tasks are also consistent with much of the rodent literature involving various maze tasks where scopolamine-induced disruptions are frequently used as positive controls for cholinergic involvement (e.g., Daniel et al., 2003; Eckerman et al., 1980; Lichtman and Martin, 1996; Peele and Baron, 1988). For example, in one of the maze studies most closely related to the present study, Peele and Baron (1988) used a repeated-acquisition technique with a radial-arm maze to show that scopolamine, but not methylscopolamine, dose-dependently reduced arm-choice accuracy over the daily 14-trial sessions in which the rats acquired pellets from four of eight arms. In a study using a more traditional radial-arm maze task (Daniel et al., 2003), where subjects were required to obtain pellets from all eight arms without re-entering an arm, scopolamine 0.1 to 5.6 mg/kg decreased arm-choice accuracy and decreased the number of arms entered per minute (rate). As in many of the operant studies, rate was more potently disrupted than accuracy.

Among the most important and novel findings obtained in this study was the attenuation of scopolamine's rate-decreasing and error-increasing effects after each neurotoxic regimen of MDMA. Unlike the behavioral effects of MDMA, the effects of scopolamine could not easily be attributed to a decrease in sensitivity (i.e., functional tolerance) because it was only administered twice weekly throughout testing and the determinations that occurred between MDMA administrations were separated by at least 10 days (4 of MDMA and 6 following). These results were also particularly surprising because data from two other studies examining the effects of cholinergic blockade following MDMA-induced serotonin depletion were negative (Ricaurte et al., 1993; Robinson et al., 1993), and combined cholinergic and serotonergic blockade in rats has been shown to produce severe disruptions in complex brain function including disruptions in learning and memory (e.g., Nilsson et al., 1988; Riekkinen et al., 1991; Vanderwolf, 1987). However, methodological differences among these studies could help explain some of the different findings. First, some of the studies examining combined serotonin and cholinergic blockade used lesions, which are more permanent and less reversible than the effects of receptor antagonists. Lesions could also lead to the deterioration of neuronal pathways. Second, most of the studies that looked at antagonist-induced cholinergic blockade used single doses of the receptor antagonist and these were frequently very large doses. For example, Robinson et al. (1993) used a 50-mg/kg dose of atropine to examine cholinergic blockade after MDMA-induced serotonin depletion, whereas Vanderwolf (1987) used a 5-mg/kg dose of scopolamine to examine cholinergic blockade after depletion of serotonin with *p*-chlorophenylalanine (PCPA). Third, the design of the present study was unique in that scopolamine was administered to the same subjects before and after depletion of serotonin by MDMA and then tested the subjects on a learning task daily to ascertain behavioral changes. In two of the previous studies involving MDMA, for example, Ricaurte et al. (1993) did not administer scopolamine until 18 or 19 weeks after the neurotoxic treatment with MDMA and Robinson et al. (1993) did not begin training the subjects to perform the task until 2 days after the last injection of MDMA.

Finally, there are published data indicating that serotonin tonically inhibits the release of acetylcholine from brain areas such as the striatum (Fischer et al., 2000; Gillet et al., 1985; Jackson et al., 1988). Removal of this tonic inhibition by the depletion of serotonin could, therefore, increase the

release of acetylcholine and effectively compete with scopolamine for cholinergic receptors, thereby reducing the behavioral effects of scopolamine. Consistent with this interpretation, a recent study by Lehmann et al. (2002) found that cholinergic lesions induced nocturnal hyperlocomotion, reduced T-maze alternation, impaired reference-memory in the water maze and working memory in the radial maze, but combined serotonergic and cholinergic lesions actually attenuated the hyperlocomotion and working memory deficits compared to cholinergic lesions alone. Comparable to many of the aforementioned studies involving serotonergic lesions, Lehmann et al. (2002) also found that moderate serotonergic depletion alone generally had limited effect on complex brain function. Similarly, in the present study, recovery of responding occurred within 3 days of the last MDMA injection, but selective reductions of serotonin and 5-HIAA were evident in a variety of brain areas for a minimum of 14 days after a single neurotoxic regimen of MDMA. Possibly more important for learning and memory, the present data indicated that these reductions were correlated with significant alterations of cholinergic function.

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