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Lack of persistent changes in the dopaminergic system of rats withdrawn from methamphetamine self-administration

Roman Stefanski [a,b], Sun-Hee Lee [a,c], Sevil Yasar [a,d], Jean L. Cadet [e], Steven R. Goldberg [a,*]

[a] *Preclinical Pharmacology Section, National Institutes of Health, National Institute on Drug Abuse, Intramural Research Program, 5500 Nathan Shock Drive, Baltimore, MD 21224, USA*

[b] *Department of Pharmacology, Institute of Psychiatry and Neurology, Al. Sobieskiego 1/9, 02-957 Warsaw, Poland*

[c] *Department of Toxicology, National Institute of Toxicological Research, Korea Food and Drug Administration, 5, Nokbun-dong, Eunpyung-gu, Seoul, 122-704, South Korea*

[d] *Johns Hopkins School of Medicine, Division of Geriatric Medicine and Gerontology, Johns Hopkins Bayview Medical Center, 5505 Johns Hopkins Circle, Baltimore, MD 21224, USA*

[e] *Molecular Neuropsychiatry Section, National Institutes of Health, National Institute on Drug Abuse, Intramural Research Program, 5500 Nathan Shock Drive, Baltimore, MD 21224, USA*

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Abstract

A continuing challenge for studies in the neurobiology of drug abuse is to identify and characterize long-lived neuroadaptations that can trigger craving and relapse. We previously reported that rats that had actively self-administered methamphetamine for 5 weeks and were then withdrawn from methamphetamine for 24 h showed marked decreases in somatodendritic dopamine D_2 autoreceptor levels in the ventral tegmental area and median and dorsal part of the substantia nigra zona compacta with a corresponding down-regulation of dopamine D_1 receptors in the shell of the nucleus accumbens. The purpose of the present study was to determine whether neuroadaptive changes in dopamine receptors or transporters in the brains of rats withdrawn for 24 h from chronic methamphetamine self-administration are persistent changes that can be demonstrated long after withdrawal. A "yoked" procedure was used in which rats were tested simultaneously in groups of three, with only one rat actively self-administering methamphetamine while the other two received yoked injections of either methamphetamine or saline. In vitro quantitative autoradiography was used to determine densities of dopamine uptake sites and dopamine D_1 and D_2 receptors in different brain regions following 7- and 30-day periods of withdrawal from chronic methamphetamine self-administration. No changes in dopamine transporter and dopamine receptor numbers were detected in any brain region examined in rats self-administering methamphetamine compared with littermates receiving yoked infusions of either methamphetamine or saline. Thus, neuroadaptive changes in densities of dopamine receptors or transporters in certain brain areas may contribute to the reinforcing effects of methamphetamine during the acquisition and maintenance phases of self-administration, but do not appear to contribute to the long-lasting neuroadaptive effects of chronic methamphetamine self-administration, which may trigger craving and relapse.

Keywords: Methamphetamine; Drug self-administration; Neuroadaptation; Dopamine; (Rat)

1. Introduction

Neuropharmacological studies have established the mesolimbic dopamine system as a major neural substrate of the reinforcement processes involved in chronic psychostimulant self-administration (Hoebel et al., 1983; Carboni et al., 1989; Koob and Le Moal, 1997; Nestler and Aghajanian, 1997). However, a continuing challenge for studies in the neurobiology of addiction is to identify and characterize long-lived neuroadaptations that can trigger craving and relapse months, or even years, after drug use has stopped. The recent escalation of methamphetamine abuse has focused awareness of the need to elucidate methamphetamine-induced neuroadaptations and relate them to behavioral and motivational components of addiction, such as reinforcement, craving and relapse. Recently, we reported that in rats withdrawn from chronic methamphetamine self-administration for 24 h there were significant decreases in somatodendritic dopamine D_2 autoreceptor levels in the ventral tegmental area and medial and dorsal part of the substantia nigra zona compacta with a

* Corresponding author. Tel.: +1-410-550-1522; fax: +1-410-550-1648.
E-mail address: sgoldber@intra.nida.nih.gov (S.R. Goldberg).

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corresponding down-regulation of dopamine D_1 receptors in the shell of the nucleus accumbens (Stefanski et al., 1999). Since the decreases in levels of dopamine D_1 and D_2 receptors which occurred in rats self-administering methamphetamine did not occur in littermates that received either yoked injections of methamphetamine or saline, these changes likely reflected motivational states that were only present when methamphetamine injection was contingent on active drug self-administration.

In the present study, we wished to determine whether neuroadaptive changes in densities of brain dopamine receptors or transporters of rats withdrawn for 24 h from chronic methamphetamine self-administration are persistent changes that can be demonstrated long after withdrawal. In vitro quantitative autoradiography was used to determine densities of dopamine uptake sites and dopamine D_1 and D_2 receptors in different brain regions following a 7- and a 30-day withdrawal from chronic methamphetamine self-administration. To distinguish the neurobiological correlates of motivational symptoms associated with actively self-administered methamphetamine from those of methamphetamine administered passively at the same dose and frequency, we again utilized a ''yoked'' procedure. Rats were run simultaneously in groups of three, with only one rat actively self-administering methamphetamine while the other two received yoked injections of either methamphetamine or saline.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (Charles River, Wilmington, MA, USA) weighing approximately 300 g at the start of the experiment were individually housed in a temperature- and humidity-controlled environment under a 12-h light/dark cycle (lights on at 7:00 a.m.). In contrast to many previous investigations, rats had no operant history and food and water were available ad libitum in the home cage. Rats were trained and tested between 12:00 and 5:00 p.m. Animals used in this study were maintained in facilities fully accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC) and all experimentation was conducted in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, NIH, and the Guide for Care and Use of Laboratory Animals, National Research Council Academy Press, Washington, DC, 1996.

2.2. Surgery

Under Equithesin (pentobarbital 9.72 mg/ml, chloral hydrate 44.4 mg/ml, propylene glycol 44%) anesthesia (3 ml/kg, i.p.), all rats were implanted with a chronic silastic catheter in the external jugular vein, as described previously (Stefanski et al., 1999). A nylon bolt was fixed to the skull with dental cement and was anchored by stainless steel screws, which served as a tether to protect the catheter. At least 10 days was allowed for recovery before the start of the experiments. Catheters were flushed each day with a 0.1-ml saline solution containing heparin (1.25 U/ml) and gentamicin (0.16 mg/kg). Catheter patency was tested periodically, or whenever an animal displayed behavior outside baseline parameters, with the ultrashort-acting barbiturate anesthetic methohexital (10 mg/kg, i.v.) for loss of consciousness within 5 s. In addition, the patency of all catheters was verified at the end of the experiment.

2.3. Apparatus

Self-administration sessions were conducted in 20 standard operant chambers (Coulbourn Instruments, Allentown, PA, USA) equipped with two, nose-poke operanda. Nose-poke responses in one of the holes (defined as ''active'') resulted in drug delivery to the animal when schedule requirements were met, whereas nose pokes in the other hole (defined as ''inactive'') were recorded but not reinforced. Each nose poke produced a brief feedback tone. The position of the ''active'' and ''inactive'' holes remained unchanged throughout the study. A house light was on during drug availability but was turned off during the entire infusion and time-out periods. The injector system contained a fluid swivel (Alice King Chatham, Hawthorne, CA, USA) mounted on top of each chamber. One end of the swivel was connected via polyethylene tubing encased in a protective stainless steel spring tether to the animal's catheter while the other end of the swivel was connected via polyethylene tubing to the infusion pump (Harvard Apparatus, Model 22, South Natick, MA, USA). The operant chambers were enclosed in ventilated, sound-attenuating cubicles and controlled by an IBM compatible computer using the MED Associates (East Fairfield, VT, USA) MED-PC software package.

2.4. Yoked self-administration procedure: contingent vs. noncontingent methamphetamine administration

After complete recovery from surgery, self-administrations sessions were started, as described previously (Munzar et al., 1999; Stefanski et al., 1999). Rats were tested simultaneously in groups of three, with two rats serving as yoked controls that received an injection of either 0.1 mg/kg methamphetamine or saline which was not contingent on responding each time a response-contingent injection of 0.1 mg/kg methamphetamine was self-administered by the third paired rat (see Stefanski et al., for further details). Unlike self-administering rats, nose-poke responses by the yoked rats were recorded but had no programmed consequences. Sessions were conducted Monday to Friday and were 2 h in duration. At the beginning of each session, a noncontingent priming injection was automatically delivered, which has been shown to elicit drug-seeking behavior in rats with drug

self-administration experience (e.g., De Wit and Stewart, 1981) and reports of "wanting" or "craving" in experienced human drug users (Jaffe et al., 1989). Once responding was initiated, the number of responses required to produce each injection was gradually increased over a 2-week period to a final value of five (a 5-response fixed-ratio schedule of drug injection; FR 5). Following each injection there was a 30-s time-out period during which responding was recorded but had no programmed consequences. After 5 weeks, rats self-administering methamphetamine and their littermates receiving yoked injections of methamphetamine or saline were sacrificed 7 and 30 days after the last session and densities of dopamine uptake sites and dopamine D_1 and D_2 receptors were measured in different brain regions.

2.5. Quantitative autoradiography

The brains were quickly removed and frozen by immersion in isopentane (-35 °C) for 30 s and stored at -70 °C until sections of 20 μm were cut on cryostat (-20 °C) and thaw-mounted on gelatin-chromate coated slides. The slides were desiccated and stored at -70 °C until used for autoradiography.

2.6. Dopamine transporter assay

Sections were equilibrated at room temperature and incubated for 60 min at 25 °C with 0.07 nM [^{125}I]-3β-[4-(trimethylstannyl]phenyl]-tropane-2β-carboxylic acid isopropyl ester ([^{125}I]RTI-121) (2200 Ci/mmol; DuPont NEN, Boston, MA, USA) in a binding buffer consisting of 137 mM NaCl, 2.7 mM KCl, 10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4 and 10 mM NaI. After incubation, sections were washed twice (for 20 min each time) in ice-cold buffer, dipped in deionized ice-cold water and then dried with a stream of cool dry air. Nonspecific binding was defined by the addition of 10 μM 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl] piperazine dihydrochloride (GBR-12909) (Research Biochemicals Int., Natick, MA, USA). The dry labeled slides and plastic standards (^{125}I-labeled microscales, Amersham, Arlington Heights, IL, USA) were apposed to radiosensitive films (Hyperfilm-βmax, Amersham) in light-tight cassettes for 2 days at 4 °C.

2.7. Dopamine D_1 receptor assay

Sections were equilibrated at room temperature and preincubated in 50 mM Tris–HCl buffer (pH 7.4) containing 120 mM NaC1, 5 mM KC1, 2 mM $CaC1_2$ and 1 mM $MgC1_2$ at 25 °C for 15 min to remove endogenous catecholamines. Sections were then incubated for 60 min at 25 °C in buffer containing 4 nM *N*-methyl-3*H*-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride ([^{3}H]SCH 23390) (81.4 Ci/mmol; DuPont NEN) and 1 μM mianserin (Research Biochemicals Int.) to block serotonin 5-HT_2 receptors. After incubation, sections were washed twice (for 5 min each time) in ice-cold buffer, dipped in deionized ice-cold water and dried with a stream of cool dry air. Nonspecific binding was defined by the addition of 10 μM unlabeled *R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride (*R*(+)SCH 23390) (Research Biochemicals Int.). The dry labeled slides and plastic tritium standards (^{3}H-labeled microscales, Amersham) were apposed to tritium-sensitive films (Hyperfilm-^{3}H, Amersham) in light-tight cassettes for 7 days at room temperature.

2.8. Dopamine D_2 receptor assay

Sections were equilibrated at room temperature and preincubated in 50 mM Tris–HCl buffer (pH 7.4) containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ at 25 °C for 15 min to remove endogenous catecholamines. Sections were then incubated for 30 min at 25 °C in buffer containing 0.1 nM [^{125}I]Iodosulpride (2000 Ci/mmol; Amersham) and 5 nM *S*(+)-(4*aR*,10*bR*)-3,4,4*a*,10*b*-Tetrahydro-4-propyl-2*H*,5*H*-[1]benzopyrano-[4,3-*b*]-1,4-oxazin-9-ol hydrochloride (PD 128,907) (Research Biochemicals Int.) to block dopamine D_3 receptors. After incubation, sections were washed twice (for 5 min each time) in ice-cold buffer, dipped in deionized ice-cold water and dried with a stream of cool dry air. Nonspecific binding was defined by the addition of 1 μM unlabeled domperidone (Research Biochemicals Int.). The dry labeled slides and plastic standards (^{125}I-labeled microscales, Amersham) were apposed to radiosensitive films (Hyperfilm-βmax, Amersham) in light-tight cassettes for 4 days at 4 °C.

2.9. Densitometry

Films were developed in Kodak GBX developer and fixative according to manufacturer's instructions and autoradiograms were analyzed using a Macintosh computer-based image analysis system (National Institutes of Health, Image 1.58 software program). Quantitation of brain images was performed using standard curves generated from the ^{125}I- and ^{3}H-labeled microscales (Amersham). Anatomical regions were defined according to Paxinos and Watson (1986).

2.10. Immunohistochemistry

Slides were brought to room temperature and fixed in 4% paraformaldehyde (in 0.1 M phosphate buffer) for 10 min. Sections were then washed four times for 15 min each time in 50 mM Tris-buffered saline (TBS; pH 7.6) before being incubated in 1% H_2O_2 and 100% methanol for 10 min. After washing with TBS four times (5 min each time), the slide-mounted sections were incubated in 3% normal goat serum and 0.2% Triton X-100 in TBS for 1 h, and then exposed to a rabbit polyclonal antibody (diluted 1:10,000 in TBS containing 1% normal goat serum) specific for tyrosine

hydroxylase (Eugene Tech International, Ridgefield Park, NJ, USA) for 18 h at room temperature. After incubation with the primary antibody, sections were washed for three times (5 min each time) in 50 mM TBS before being incubated with biotinylated goat antirabbit secondary immunoglobulin G (IgG) which was diluted 1:200 in TBS (Vector Laboratories, Burlingame, CA, USA) for 1.5 h at room temperature. Following four washes in TBS (15 min each time), the sections were incubated with ABC reagent (Vector Laboratories) for 2 h. Tyrosine-hydroxylase-immunopositive neurons were visualized by 3,3′ -diaminobenzidine, nickel and H_2O_2 using a commercially available kit (DAB substrate kit, Vector Laboratories). Negative controls were run using normal goat serum instead of the primary antibody. Tyrosine-hydroxylase-immunoreactive neurons in the substantia nigra zona compacta and ventral tegmental area were counted manually on four slices from each animal ($n=6$ rats/group) using a Zeiss microscope at a magnification of 400 × with a superimposed grid. The person counting cells was blind to animal treatment. Cell size and relative density of the tyrosine-hydroxylase-positive neurons in the substantia nigra zona compacta and ventral

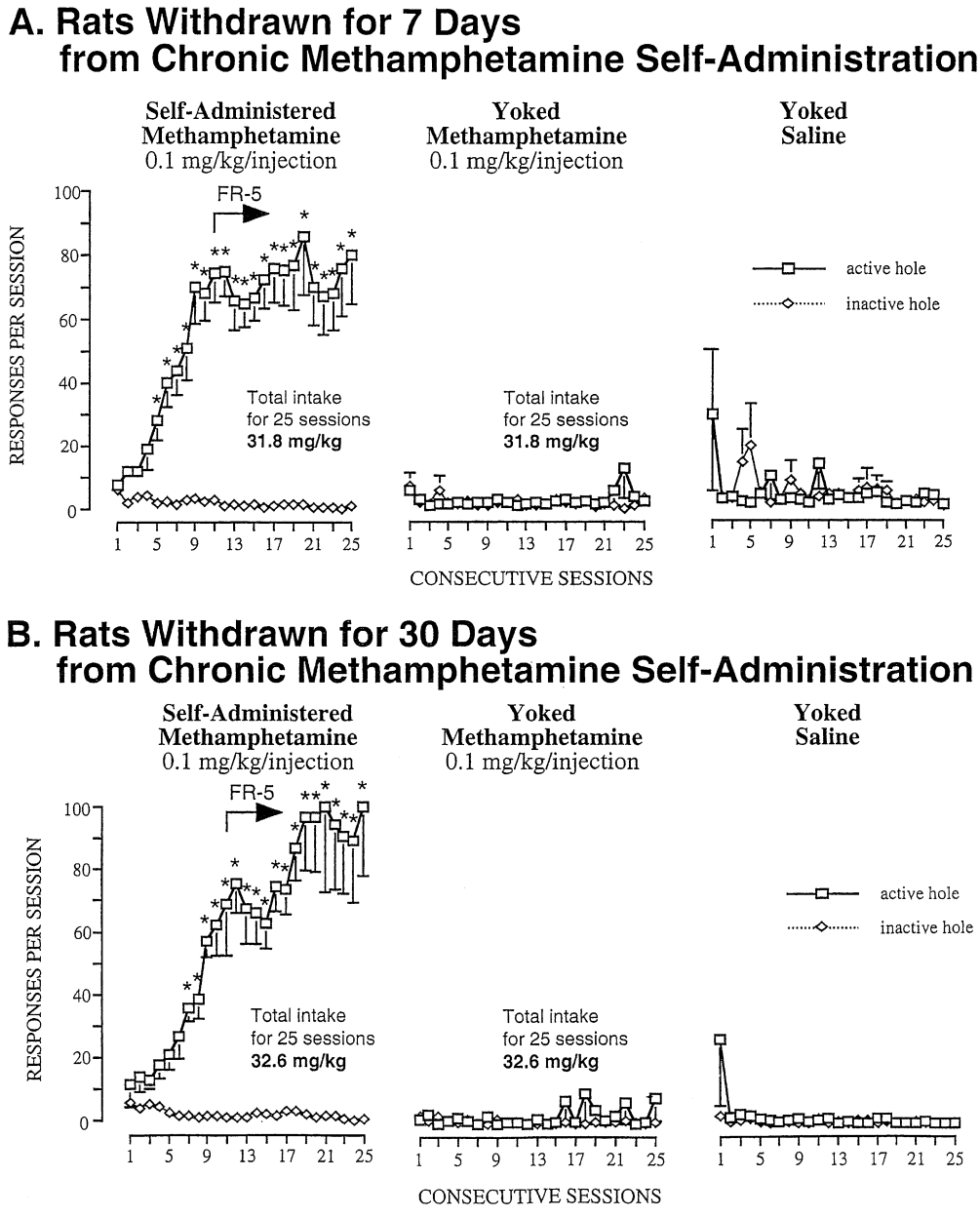

Fig. 1. The mean number (± S.E.M.) of responses in the active and inactive holes for rats that were allowed to acquire self-administration of methamphetamine at a dose of 0.1 mg/kg/injection ($n=6$) and their littermates that received yoked infusions of methamphetamine ($n=6$) or saline ($n=6$) during each of the daily 2-h sessions. The arrow indicates the period when methamphetamine self-administration was maintained under the final FR-5 schedule of reinforcement. Asterisks (*) denote significant differences ($P<0.01$) between active and inactive nose-pokes.

tegmental area were assessed on 10 tyrosine-hydroxylase-positive cells per each animal ($n=6$ rats/group) using the microscope at a magnification of 630 × and a Macintosh computer-based image analysis system (Image 1.58, NIH). Only tyrosine-hydroxylase-positive neurons with a complete nucleus in the section were assessed and outlined with the screen cursor driven by a hand-held mouse. This allowed for the measurement of neuron size and relative density of tyrosine-hydroxylase-staining. Relative density of tyrosine-hydrozylase-immunoreactive fibres was also measured in four representative areas of the striatum and two representative areas of the nucleus accumbens (500 × 500 μm) per each rat using the microscope at a magnification of × 400 and Image 1.58, NIH. All sections were processed at the same time under the same conditions.

2.11. Drugs

S(+)-Methamphetamine hydrochloride was obtained from Research Biochemicals Int. and dissolved in sterile physiological saline. Injection speed was adjusted daily according to the weight of each rat in order to provide an injection of 0.1 mg/kg in a volume of 250 μl/kg over a 2-s period.

2.12. Statistical analysis

Self-administration, data are presented as group means, and bars show the standard errors of the means. Data were analyzed using multifactorial analysis of variance (ANOVA) for repeated measures and post-hoc Student's *t*-test comparisons were performed to locate differences between group means. For quantitative autoradiography, data are presented as mean ± S.E.M. percentage of change compared to the yoked methamphetamine group. The error bars represent mean ± S.E.M. of individual values expressed as a percent of the corresponding mean value for the yoked-methamphetamine group. Since our autoradiography data do not appear to originate from normally distributed populations with equal variances and sample sizes are small ($n=6$) the significance of the difference between means was determined with a Mann–Whitney *U*-test. For immunohistochemistry, the values for number or size of tyrosine-hydroxylase-positive neurons in the substantia nigra zona compacta and ventral tegmental area are presented as the group mean, and bars show the standard error of the mean. The significance of the difference between means was determined with a Student's *t*-test.

3. Results

3.1. Yoked self-administration procedure: contingent vs. noncontingent methamphetamine administration

The number of active-hole responses required to produce each injection of methamphetamine was increased over days, reaching a final value of 5 (fixed-ratio 5 schedule of drug injection; FR 5) by the 11th session of training. Fig. 1A shows results for rats withdrawn for 7 days from chronic methamphetamine self-administration and their littermates

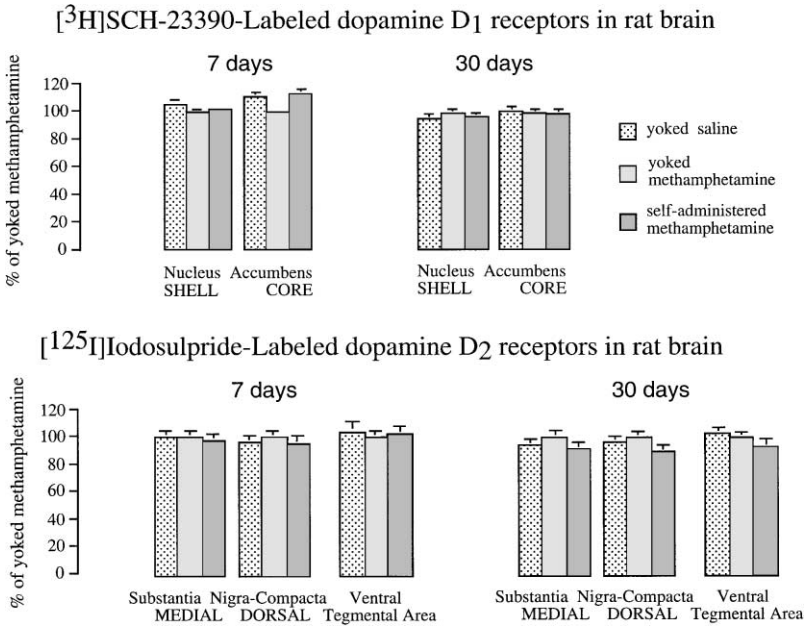

Fig. 2. Effects of withdrawal from chronic methamphetamine self-administration for 7 or 30 days on dopamine D1 receptors and D2 receptors in various brain regions. Data are presented as mean (± S.E.M.) percentage of change compared to the yoked-methamphetamine group. Sample sizes are as follows: yoked-saline group ($n=6$), yoked-methamphetamine group ($n=6$), methamphetamine self-administration group ($n=6$). The significance of the difference between means was determined with a Mann–Whitney *U*-test, $*P<0.05$ and $**P<0.01$. The self-administration group was compared with the yoked-methamphetamine group and the latter with the yoked-saline group.

receiving yoked injections of methamphetamine or saline. In the methamphetamine self-administration group of rats (Fig. 1A, left panel), a two-factor ANOVA for repeated measures revealed significant differences between active and inactive hole responding [$F(1,299) = 752.35$, $P < 0.001$] over the 25 sessions [$F(24,299) = 5.09$, $P < 0.001$]. In addition there was an overall significant interaction between nose-poke responding and session [$F(24,299) = 6.01$, $P < 0.001$]. Post-hoc analysis revealed that a significant preference for the active hole occurred during sessions 5–25 ($P < 0.01$). In contrast, with the yoked-methamphetamine group of rats (Fig. 1A, middle panel), a two-factor ANOVA for repeated measures indicated no significant effect of session [$F(24,299) = 0.88$, n.s.]. Also, there was no significant difference between active and inactive hole responding [$F(1,299) = 1.36$, n.s.] and the interaction between nose-poke responding and sessions failed to reach significance [$F(24,299) = 0.99$, n.s.]. With the yoked-saline group of rats (Fig. 1A, right panel), although a two-factor ANOVA for repeated measures indicated a significant effect of session [$F(24,299) = 1.85$, $P < 0.05$], there was no significant difference between active and inactive hole responding [$F(1,299) = 0.43$, n.s.] and the interaction between nose-poke responding and sessions failed to reach significance [$F(24,299) = 0.44$, n.s.].

Fig. 1B shows similar results for rats withdrawn for 30 days from chronic methamphetamine self-administration and their littermates receiving yoked injections of methamphetamine or saline. Again, the number of responses required to produce each injection was increased over days, reaching a final value of 5 (FR-5) by the 11th session. In the methamphetamine self-administration group of rats (Fig. 1B, left panel), there were significant differences between active and inactive hole responding [$F(1,299) = 465.75$, $P < 0.001$] over the 25 sessions [$F(24,299) = 4.9$, $P < 0.001$]. In addition, there was an overall significant interaction between nose-poke responding and session [$F(24,299) = 5.54$, $P < 0.001$] and a significant preference for the active hole occurred during sessions 7–25 ($P < 0.01$). With the yoked-methamphetamine group of rats (Fig. 1B, middle panel), there was a significant effect between active and inactive hole responding [$F(1,299) = 5.28$, $P < 0.05$], but there was no significant effect of session [$F(24,299) = 0.71$, n.s.] and no significant interaction between nose-poke responding and sessions [$F(24,299) = 0.73$, n.s.]. With the yoked-saline group of rats (Fig. 1B, right panel), there was a significant effect of session [$F(24,299) = 1.78$, $P < 0.05$], but there was no significant difference between active and inactive hole responding [$F(1,299) = 2.72$, n.s.] and the interaction between nose-poke responding and sessions [$F(24,299) = 1.26$, n.s.] failed to reach significance.

3.2. Neuroadaptations to methamphetamine self-administration: contingent vs. noncontingent infusions of drug

Fig. 2 shows the effects of withdrawal from chronic methamphetamine self-administration for 7 or 30 days on dopamine D_1 receptors in the nucleus accumbens shell and core and dopamine D_2 receptors in the medial and dorsal substantia nigra compacta and the ventral tegmental area. We previously reported (Stefanski et al., 1999) that 24 h after withdrawal from chronic methamphetamine self-administration there were significant decreases in numbers of dopamine D_1 receptors in the nucleus accumbens shell and in the medial and dorsal substantia nigra compacta and the ventral tegmental area in rats self-administering methamphetamine compared with littermates receiving yoked infusions of either methamphetamine or saline. In contrast, no changes in dopamine D_1 or D_2 receptor numbers were found in the present study in any of these brain regions, or in any of the other brain regions examined, 7 days after withdrawal (Fig. 2 and Table 1) or 30 days after withdrawal (Fig. 2 and Table 2) in rats self-administering methamphetamine compared with littermates receiving yoked infusions of either methamphetamine or saline There were also no changes in numbers of dopamine transporters in any of the brain regions examined in rats self-administering metham-

Table 1
Effects of withdrawal from chronic methamphetamine self-administration for 7 days on dopamine transporters, dopamine D_1 receptors and dopamine D_2 receptors in various brain regions

	Self-administered methamphetamine	Yoked methamphetamine	Yoked saline
[^{125}I]RTI-121-labeled dopamine transporters in rat brain			
NAcc shell	100.8 ± 2.05	100 ± 1.21	100.9 ± 1.47
NAcc core	102.7 ± 6.99	100 ± 4.07	101.3 ± 3.07
Ant. caudate	99.4 ± 5.94	100 ± 3.37	101.2 ± 2.98
Middle caudate	100.0 ± 2.64	100 ± 2.64	110.5 ± 3.24
Post. caudate	110.6 ± 4.19	100 ± 3.07	118.1 ± 4.27
SNCom. medial	96.7 ± 5.06	100 ± 4.52	98.4 ± 4.13
SNCom. dorsal	94.2 ± 4.39	100 ± 4.05	96.3 ± 4.25
VTA	97.6 ± 5.43	100 ± 3.61	99.6 ± 3.26
[^{3}H]SCH-23390-labeled dopamine D_1 receptors in rat brain			
NAcc shell	102.5 ± 2.01	100 ± 2.42	106.2 ± 2.97
NAcc core	113.7 ± 3.36	100 ± 1.71	111.2 ± 3.65
Ant. caudate	97.9 ± 3.62	100 ± 3.13	102.6 ± 2.43
Middle caudate	101.9 ± 2.32	100 ± 2.98	99.4 ± 2.57
Post. caudate	96.3 ± 2.67	100 ± 3.83	93.4 ± 3.77
SN reticulata	98.4 ± 5.86	100 ± 6.47	96.3 ± 3.97
[^{125}I]Iodosulpride-labeled dopamine D_2 receptors in rat brain			
NAcc shell	102.5 ± 3.43	100 ± 4.04	103.1 ± 3.12
NAcc core	96.2 ± 2.65	100 ± 3.65	99.8 ± 5.13
Ant. caudate	97.3 ± 4.17	100 ± 4.35	99.1 ± 4.71
Middle caudate	97.9 ± 4.54	100 ± 3.86	98.8 ± 3.16
Post. caudate	104.3 ± 5.04	100 ± 4.92	97.3 ± 3.81
SNCom. medial	97.9 ± 4.95	100 ± 4.71	99.8 ± 5.44
SNCom. dorsal	96.1 ± 5.04	100 ± 5.01	97.4 ± 3.33
VTA	102.4 ± 5.55	100 ± 5.14	104.1 ± 7.08

Data are presented as mean (± S.E.M.) percentage of change compared to the yoked-methamphetamine group. Sample sizes are as follows: yoked-saline group ($n = 6$), yoked-methamphetamine group ($n = 6$), methamphetamine self-administration group ($n = 6$). The self-administration group was compared with the yoked-methamphetamine group and the latter with the yoked-saline group.

Table 2
Effects of withdrawal from chronic methamphetamine self-administration for 30 days on dopamine transporters, dopamine D_1 receptors and dopamine D_2 receptors in various brain regions

	Self-administered methamphetamine	Yoked methamphetamine	Yoked saline
[^{125}I]RTI-121-labeled dopamine transporters in rat brain			
NAcc shell	102.7 ± 1.44	100 ± 1.77	102.9 ± 1.41
NAcc core	105.1 ± 3.01	100 ± 4.03	109.3 ± 4.45
Ant. caudate	104.5 ± 1.27	100 ± 5.07	110.5 ± 4.32
Middle caudate	108.1 ± 3.13	100 ± 4.49	107.2 ± 4.19
Post. caudate	101.6 ± 3.04	100 ± 5.55	108.4 ± 3.97
SNCom. medial	86.7 ± 4.44	100 ± 6.32	97.7 ± 5.07
SNCom. dorsal	86.6 ± 3.18	100 ± 6.94	98.6 ± 4.99
VTA	90.8 ± 3.46	100 ± 5.82	97.9 ± 5.48
[^{3}H]SCH-23390-labeled dopamine D_1 receptors in rat brain			
NAcc shell	97.4 ± 2.51	100 ± 3.12	96.2 ± 3.09
NAcc core	99.6 ± 3.56	100 ± 2.95	101.6 ± 3.26
Ant. caudate	98.5 ± 2.18	100 ± 1.57	98.4 ± 3.31
Middle caudate	96.3 ± 1.57	100 ± 1.71	100.3 ± 2.04
Post. caudate	98.6 ± 2.61	100 ± 3.92	105.3 ± 4.54
SN reticulata	93.3 ± 1.49	100 ± 5.47	103.3 ± 3.91
[^{125}I]Iodosulpride-labeled dopamine D_2 receptors in rat brain			
NAcc shell	97.4 ± 4.88	100 ± 3.11	102.7 ± 3.74
NAcc core	91.9 ± 4.99	100 ± 1.96	96.6 ± 2.66
Ant. caudate	92.3 ± 6.41	100 ± 4.96	99.7 ± 4.44
Middle caudate	99.4 ± 3.61	100 ± 2.21	92.3 ± 4.31
Post. caudate	99.5 ± 4.43	100 ± 3.32	106.5 ± 3.37
SNCom. medial	91.8 ± 4.05	100 ± 4.91	94.8 ± 3.96
SNCom. dorsal	89.7 ± 2.41	100 ± 4.21	96.5 ± 4.29
VTA	92.9 ± 5.66	100 ± 3.39	102.9 ± 4.33

Data are presented as mean (± S.E.M.) percentage of change compared to the yoked-methamphetamine group. Sample sizes are as follows: yoked-saline group ($n=6$), yoked-methamphetamine group ($n=6$), methamphetamine self-administration group ($n=6$). The self-administration group was compared with the yoked-methamphetamine group and the latter with the yoked-saline group.

phetamine compared with littermates receiving yoked infusions of either methamphetamine or saline either 7 days (Table 1) or 30 days (Table 2) after withdrawal from chronic methamphetamine self-administration.

Seven days after withdrawal from chronic methamphetamine self-administration, the mean [^{125}I] RTI-121 binding in the shell and core of the nucleus accumbens, anterior, middle and posterior caudate, medial and dorsal part of the substantia nigra zona compacta and ventral tegmental area regions of the yoked-methamphetamine group were 1.92 ± 0.09, 2.99 ± 0.14, 3.72 ± 0.12, 3.48 ± 0.13, 2.42 ± 0.14, 1.78 ± 0.13, 1.28 ± 0.11 and 1.99 ± 0.14 fmol/mg tissue, respectively. The mean [^{3}H]SCH-23390 binding in the shell and core of the nucleus accumbens, anterior, middle and posterior caudate and substantia nigra zona reticulata regions of the yoked-methamphetamine group were 248.8 ± 7.6, 282.6 ± 5.8, 284.8 ± 9.3, 276.4 ± 8.4, 192.6 ± 7.9 and 163.8 ± 12.6 fmol/mg tissue, respectively. The mean [^{125}I] Iodosulpride binding in the shell and core of the nucleus accumbens, anterior, middle and posterior caudate, medial and dorsal part of the substantia nigra zona compacta and ventral tegmental area regions of the yoked-methamphetamine group were 2.4 ± 0.14, 3.09 ± 0.11, 3.82 ± 0.13, 3.54 ± 0.12, 2.72 ± 0.11, 1.26 ± 0.14, 0.88 ± 0.12 and 1.22 ± 0.13 fmol/mg tissue, respectively.

Thirty days after withdrawal from chronic methamphetamine self-administration, the mean [^{125}I] RTI-121 binding in the shell and core of the nucleus accumbens, anterior, middle and posterior caudate, medial and dorsal part of the substantia nigra zona compacta and ventral tegmental area regions of the yoked-methamphetamine group were 2.01 ± 0.08, 3.1 ± 0.11, 3.89 ± 0.13, 3.55 ± 0.12, 2.62 ± 0.12, 1.92 ± 0.12, 1.13 ± 0.14 and 2.03 ± 0.13 fmol/mg tissue, respectively. The mean [^{3}H]SCH-23390 binding in

Table 3
Effects of contingent vs. noncontingent infusions of methamphetamine on the number and size of tyrosine hydroxylase-immunoreactive cells in the substantia nigra zona compacta and ventral tegmental area

	Rats withdrawn from chronic methamphetamine self-administration for 7 days		Rats withdrawn from chronic methamphetamine self-administration for 30 days	
	Substantia nigra zona compacta	Ventral tegmental area	Substantia nigra zona compacta	Ventral tegmental area
Cell number				
Self-administered methamphetamine	106.22 ± 8.24	82.67 ± 6.89	105.65 ± 4.87	83.23 ± 4.56
Yoked methamphetamine	104.34 ± 6.44	79.34 ± 7.32	106.34 ± 4.98	86.23 ± 6.43
Yoked saline	106.35 ± 5.23	84.23 ± 6.44	104.44 ± 6.34	82.56 ± 5.34
Cell size (μm^2)				
Self-administered methamphetamine	192.45 ± 5.98	169.45 ± 5.46	189.45 ± 6.78	169.45 ± 6.89
Yoked methamphetamine	189.56 ± 6.12	173.78 ± 6.23	193.54 ± 5.67	172.56 ± 5.67
Yoked saline	193.56 ± 4.88	172.94 ± 5.21	190.56 ± 6.45	170.23 ± 6.34

For cell number, the values represent means ± S.E.M. for 24 slices from six rats per each group. For cell size, the values represent mean ± S.E.M. for 60 neurons from six rats per each group. The significance of the difference between means was determined with a Student's *t*-test. The self-administration group was compared with the yoked-methamphetamine group and the latter with the yoked-saline group.

the shell and core of the nucleus accumbens, anterior, middle and posterior caudate and substantia nigra zona reticulata regions of the yoked-methamphetamine group were 263.4 ± 9.4, 294.2 ± 8.7, 289.2 ± 5.4, 272.4 ± 4.9, 183.2 ± 8.9 and 171.3 ± 11.4 fmol/mg tissue, respectively. The mean [^{125}I] Iodosulpride binding in the shell and core of the nucleus accumbens, anterior, middle and posterior caudate, medial and dorsal part of the substantia nigra zona compacta and ventral tegmental area regions of the yoked-methamphetamine group were 2.54 ± 0.07, 3.22 ± 0.06, 3.95 ± 0.14, 3.66 ± 0.06, 2.88 ± 0.08, 1.33 ± 0.12, 0.94 ± 0.11 and 1.18 ± 0.12 fmol/mg tissue, respectively.

3.3. Tyrosine hydroxylase immunohistochemistry in the ventral tegmental area and substantia nigra zona compacta

There were no changes in cell number or size of tyrosine-hydroxylase-immunoreactive dopamine neurons detected in the ventral tegmental area or substantia nigra zona compacta of rats withdrawn from chronic methamphetamine self-administration for either 7 or 30 days compared with littermates receiving yoked infusions of methamphetamine or saline (Table 3). Relative densities of accumbens and striatal tyrosine-hydroxylase-immunoreactive fibres were not significantly different in the three groups of rats (data not shown).

4. Discussion

The present experiment focused on neuroadaptive changes in the dopaminergic system of rats that persist after 7–30 days of withdrawal from chronic daily exposure to self-administered methamphetamine. We previously reported that rats, which had actively self-administered methamphetamine for 5 weeks and were then withdrawn from methamphetamine for 24 h (1 day), showed marked decreases in somatodendritic dopamine D_2 autoreceptors levels in the ventral tegmental area and medial and dorsal part of the substantia nigra zona compacta with a corresponding down-regulation of dopamine D_1 receptors in the shell of the nucleus accumbens (Stefanski et al., 1999). In the present experiments, we found that these changes were no longer evident after either a 7- or a 30-day withdrawal period, indicating the transient nature of this neural adaptation. Moreover, densities of dopamine uptake sites and dopamine D_1 and D_2 receptors in these rats were not significantly different from those observed in littermates that received yoked injections of either methamphetamine or saline.

The present results are consistent with previous reports that alterations in dopamine neurons produced by repeated, response-independent drug administration were relatively transient and clearly not involved in the maintenance and expression of persistent behavioral and cognitive aspects of the addiction process (Kamata and Rebec, 1984; White and Wang, 1984; Henry et al., 1989; Ackerman and White, 1990; Gao et al., 1998). Indeed, it has been reported that 4 days after the last of a series of amphetamine injections, impulse-regulating somatodendritic autoreceptors on ventral tegmental area dopamine neurons are subsensitive to the dopamine D_2 agonist quinpirole (Wolf et al., 1993). According to White and Wang (1984), this subsensitivity of dopamine D_2 autoreceptors persists up to 8 days after withdrawal from repeated amphetamine administration, although the degree of subsensitivity was less than that measured 24 h (1 day) after withdrawal from treatment. In the study by Wolf et al. (1993), somatodendritic dopamine D_2 autoreceptor subsensitivity was no longer evident after a 10- to 14-day withdrawal period, indicating the transient nature of these neural adaptations. In addition to dopamine autoreceptor subsensitivity, neuroadaptations that have been identified as transient in ventral tegmental area dopamine neurons include decreases in the level of the inhibitory G proteins which couple to dopamine D_2 autoreceptors (Self and Nestler, 1995), increases in dopamine release (Kalivas and Duffy, 1993), increased levels of tyrosine hydroxylase (Sorg et al., 1993), enhanced sensitivity of AMPA receptors (Zhang et al., 1997) and increased expression of Glu_1 and $NMDA_1$ glutamate receptor subunits (Fitzgerald et al., 1996).

In contrast to transient adaptations in the ventral tegmental area, more prolonged effects that persist for up to 1 month in the nucleus accumbens include a supersensitivity to dopamine D_1-mediated responses (Henry and White, 1991; Wolf et al., 1994), increased levels of the cyclic AMP synthesizing enzyme adenylate cyclase and the cyclic AMP-dependent protein kinase A (Terwilliger et al., 1991; Self et al., 1995), and decreased levels of inhibitory G_i proteins that inhibit cyclic AMP formation (Terwilliger et al., 1991; Striplin and Kalivas, 1993; Self et al., 1995). In addition, in vitro intracellular recordings from nucleus accumbens neurons in slices prepared from rats pretreated with methamphetamine revealed highly sensitized dopamine D_1 receptor mediated hyperpolarizations (Higashi et al., 1989). Although dopamine D_1 receptor supersensitivity may contribute to the sensitized locomotor stimulant effects of drugs, it is unlikely that enhanced dopamine D_1 responses trigger relapse to drug-seeking behavior, since dopamine D_1 agonists appear to suppress, rather than induce, relapse to cocaine-seeking behavior following a noncontingent priming injection of cocaine in self-administration experienced rats (Self et al., 1996). While a robust enhancement of amphetamine-stimulated dopamine release in the nucleus accumbens has been reported when rats were tested 15–21 days after withdrawal from an escalating amphetamine dose regimen (Robinson et al., 1988), the response to amphetamine was diminished rather than enhanced when rats were tested 2–6 days after the last injection of amphetamine (Segal and Kuczenski, 1992). These observations further suggest that enhanced, amphetamine-stimulated, dopamine release can be detected only when the drug has been discontinued for an extended period of time and support the hypothesis that persistent drug craving during withdrawal

from amphetamines may result, in part, from more persistent alterations within the mesostriatal dopamine system. The mechanism for these alternations does not appear to involve adaptations in the levels of dopamine receptors or transporters, since we failed to find any changes in dopamine uptake sites and dopamine D_1 and D_2 receptor binding in mesolimbic and nigrostriatal dopaminergic systems of rats withdrawn from chronic methamphetamine self-administration for 7 or 30 days in the present experiments.

To exclude the possibility that neuroadaptations in the dopaminergic system may represent a loss of dopaminergic neurons in the cell body and terminal regions as a result of methamphetamine-induced neurotoxicity, we investigated the effects of 5-week exposure to methamphetamine on dopamine transporters and tyrosine-hydroxylase-immunoreactive neurons in the ventral tegmental area and substantia nigra zona compacta. Densities of dopamine transporters in the cell body and terminal regions were not significantly different in rats self-administering methamphetamine compared with littermates receiving yoked infusions of either methamphetamine or saline. No changes in cell number and size of tyrosine-hydroxylase-immunoreactive dopamine neurons in the substantia nigra zona compacta and ventral tegmental area, as well as in relative density of accumbens and striatal tyrosine-hydroxylase-immunoreactive fibres, were observed in the three groups of rats. Thus, limiting daily methamphetamine intake through the use of relatively short sessions, timeout periods, and a requirement of multiple responses to produce an injection, likely prevented development of neurotoxicity or more general neurobiological changes which might have masked potential neuroadaptations.

In conclusion, different stages of withdrawal from chronic methamphetamine self-administration involve different neurobiological adaptations. The present results indicate that the downregulation of somatodendritic dopamine D_2 autoreceptors as well as dopamine D_1 receptors in the shell of the nucleus accumbens, which we previously reported in rats withdrawn from chronic methamphetamine self-administration for 24 h (Stefanski et al., 1999), were no longer evident after a 7-day or 30-day withdrawal period. This suggests that neuroadaptive changes in densities of dopamine receptors or transporters in certain brain areas may contribute to the reinforcing effects of methamphetamine during the acquisition and maintenance phases of self-administration, but do not appear to contribute to long-lasting neuroadaptive effects of chronic methamphetamine self-administration which may trigger craving and relapse. It is possible that use of a higher injection dose of methamphetamine on the descending limb of the dose–response curve (e.g., 0.3 mg/kg/injection; see Stefanski et al., 1999), which results in about a twofold greater intake of methamphetamine over 5 weeks (25 days) of methamphetamine self-administration (unpublished findings; Stefanski et al.), would have produced longer lasting changes in densities of dopamine receptors or transporters. It also may be important to focus on neurotransmitter systems other than dopaminergic ones to further identify and characterize long-lasting neuroadaptations in specific brain regions that underlie the particularly long-lived aspects of addiction, such as drug craving and relapse. Finally, there are also possible neuroadaptive responses in the dopaminergic system that may not involve altered dopamine receptors or transporters, such as altered dopaminergic signalling. The answers may be found not only in the same neurochemical systems and neurocircuitry responsible for the reward process associated with chronic drug use, but also in the multiple neuroadaptive mechanisms that establish long-term memories of drug rewards.

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