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Sex differences and repeated intravenous nicotine: behavioral sensitization and dopamine receptors

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Abstract

The present study examined the sex-dependent expression of behavioral sensitization as well as changes of dopamine (DA) transporters and D_1, D_2, and D_3 receptors following repeated intravenous nicotine administration. Male and female Sprague–Dawley rats were implanted with indwelling jugular catheters, equipped with subcutaneous intravenous injection ports. Rats were habituated to activity chambers for 3 days and were subsequently administered 15-s bolus injections of intravenous nicotine (50 μg/kg/ml) 1/day for 21 days. Animals were placed in activity chambers for 60 min immediately after the 1st and 21st nicotine injection. Observational time sampling was also performed. Brains were subsequently removed and frozen for autoradiographic DA transporter/DA receptor analysis on the afternoon females were in proestrus. With one exception, no robust sex differences were observed for locomotor activity or any rearing measures either during baseline or after initial nicotine injection. Females exhibited markedly more behavioral sensitization of locomotor activity, rearing, duration of rearing, and incidence of observed rearing. There were no sex differences in the number of D_1 or D_2 receptors. Females exhibited an increased number of DA transporters and decreased D_3 receptors in the NAcc, relative to males. Multiple regression analyses suggest that D_3 receptors and DA transporters in various striatal and NAcc subregions differentially predicted nicotine-induced behaviors for males and females. Collectively, these findings demonstrate that repeated intravenous nicotine produces sex differences in the expression of behavioral sensitization, and suggest that nicotine-induced changes of DA transporters and D_3 receptors are partly responsible for increased behavioral sensitization in female rats.

Keywords: Sex differences; Nicotine; Sensitization; Dopamine transporters; Dopamine receptors

1. Introduction

Tobacco use is the leading preventable cause of death in the United States (Jaffe, 1990; USDHHS, 2004). Women tobacco users in particular represent a public health concern as the incidence of smoking-related lung cancer surpassed breast cancer as the leading cause of cancer for women in the United States (Grunberg et al., 1991). Indeed, epidemiological studies indicate that the rise in tobacco-related health problems in women reflect the increased use and dependence on tobacco (Waldron, 1991; Kandel et al., 1998). Together, these findings indicate that women and men exhibit differential cigarette smoking behavior, and furthermore, suggest that investigation of biologically based sex differences in response to nicotine will be fruitful.

The behavioral sensitization procedure is a widely used research protocol that models the neuropharmacological changes in the human central nervous system following repeated exposure to psychostimulant drugs of abuse, such as nicotine (Kalivas, 1995). Behavioral sensitization describes the augmentation of a behavioral response following repeated administration of psychostimulant drugs (Downs and Eddy, 1932; Emmett-Olglesby, 1995; Kalivas and Weber, 1988; Post, 1980; Post and Contel, 1983; Zahniser and Peris, 1992). Although a number of neurobiological mechanisms have been invoked to explain behavioral sensitization (Kalivas, 1995), it is clear that the mesocorticolimbic dopamine (DA) pathway is one critical neural substrate. This pathway originates in the ventral tegmental area (VTA) and terminates in the nucleus accumbens (NAcc; Evenden and

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Ryan, 1988; Wise and Rompre, 1989). Studies suggest that neurons in the VTA are integral for the initiation of behavioral sensitization, whereas those in the NAcc are important for the expression of behavioral sensitization (Kalivas, 1995; Kalivas and Stewart, 1991).

The development and expression of nicotine-induced behavioral sensitization is dependent on the dose of nicotine, dosing regimen, and sex of the animal (Booze et al., 1999a,b; Domino, 2001; Miller et al., 2001; Stolerman et al., 1995). Like cocaine, nicotine is believed to produce behavioral sensitization by activating the mesocorticolimbic DA pathway. Thus, acute nicotine administration increases the concentration of DA in microdialysate from rat ventral striatum and NAcc (Benwell and Balfour, 1992; Pontieri et al., 1996), and produces c-*fos* activation in the anterior cingulated cortex, striatum, and NAcc (Pagliusi et al., 1996; Pich et al., 1997). Moreover, nicotine increases neuronal activity in the cingulate cortex, frontal cortex, and NAcc in tobacco smokers, as determined by magnetic resonance imaging (Stein et al., 1998).

Most of the basic sciences research effort that has examined the effects of repeated nicotine administration has almost exclusively used male subjects. The few studies that have compared the effects of nicotine in male and female rats do suggest that the response to nicotine differs depending on the sex of the rat. For example, chronic administration of nicotine induced increased locomotor activity in females, relative to male rats (Kanyt et al., 1999). Nicotine affected bodyweight and eating behavior in female rats (Grunberg et al., 1986, 1987; Levin et al., 1987), and produced increased body weight following the cessation of nicotine administration in female, relative to male rats (Grunberg et al., 1986, 1987). Taken together, the literature on nicotine-induced sex differences, albeit small, suggests that females exhibit increased sensitivity to nicotine on a number of measures (Battig, 1981; Rosecrans, 1971, 1972).

Recent research suggests that repeated intravenous nicotine administration induced sex-dependent behavioral sensitization in rats (Booze et al., 1999a). In these studies, male and female rats were implanted with indwelling jugular catheters equipped with intravenous injection ports, and administered repeated, intravenous nicotine (50 μg/kg/ml) once a day for 14 days. The results suggest that repeated intravenous administration of nicotine induced behavioral sensitization in male and female rats, and furthermore, produced a sex difference in the expression of behavioral sensitization. Specifically, female rats exhibited increased sensitivity to repeated nicotine, relative to males. The nicotine-induced sex difference was observed with both automated (i.e., interruption of photobeams) and observational time sampling data (i.e., rearing). Repeated nicotine administration did not interfere with intact female vaginal cytology, or produce persistent vaginal estrus, estrus acyclicity, or changes in body weight. Furthermore, the pharmacokinetic analysis of nicotine revealed indistinguishable peak arterial nicotine concentrations in male and female rats. This research is in accord with previous research demonstrating nicotine-induced sex differences in rats (Battig, 1981; Grunberg et al., 1986, 1987; Kanyt et al., 1999; Levin et al., 1987; Rosecrans, 1971, 1972), and was the first demonstration that intravenous nicotine administration produced a sex difference in the expression of behavioral sensitization.

Characterizing the sex differences in response to repeated nicotine administration is necessary to develop an animal model that will be useful in testing novel therapeutic approaches toward smoking cessation in humans. For example, it is necessary to understand if male and female rats' differences in behavior are related to differential expression of DA transporters and D_3 receptors following repeated nicotine administration. If so, then these differences might impart the need for different therapeutic approaches to smoking cessation for men and women tobacco users. Two groups of rats were used to determine if there are sex differences in the expression of behavioral sensitization and DA transporter/D_3 protein following repeated intravenous nicotine injection. Based on previous findings using the intravenous route of nicotine administration (Booze et al., 1999a), it was hypothesized that females would exhibit more behavioral sensitization relative to males. It was also hypothesized that the receptor and transporter changes would be greater in females than in males. Finally, it was hypothesized that there would be sex differences in the relationship between the expression of behavioral sensitization and the proteins associated with repeated administration of intravenous nicotine.

The purpose of the present study was to replicate and extend our previous findings by showing that (1) behavioral sensitization following intravenous administration of nicotine is robust, (2) the expression of nicotine-induced behavioral sensitization is greater in females than males, and (3) the distribution of DA transporters and DA receptors in the striatum and NAcc predicts the behavioral sensitized response, and perhaps the sex difference in the expression of nicotine-induced behavioral sensitization.

2. Method

2.1. Animals

Adult male and female, Sprague–Dawley rats (70 days old) were obtained from Harlan Laboratories, (Indianapolis, IN). Prior to delivery, all rats were surgically implanted with an Intracath intravenous catheter (22 ga, Becton/Dickinson General Medical, Grand Prarie, TX), which was used as a subcutaneous, dorsally implanted port for chronic intravenous injections. The subcutaneous implantable access port was developed and described by Mactutus et al. (1994). Upon arrival at the animal care facilities, rats were placed in quarantine for 7 days, and then transferred to the colony. Animals were pair housed throughout the experiment and the catheters were flushed daily with 0.2 ml of heparinized

(2.5%) saline. Rodent food (Pro-Lab Rat, Mouse Hamster Chow #3000) and water were provided ad libitum. The colony was maintained at 21 ± 2 °C, 50 ± 10% relative humidity, and a 12L:12D cycle with lights on at 0700 h (EST). The Institutional Animal Care and Use Committee (IACUC) of the University of South Carolina approved the animal protocol for this research.

2.2. Experimental design and procedures: locomotor activity and behavioral sensitization

Animals were habituated to the locomotor activity chambers for two 60-min sessions, 1/day. A baseline measure of activity was obtained on Day 3. For the third (baseline) activity test session, rats were injected with saline immediately prior to placement in the activity chambers for a 60-min session.

The activity monitors, similar to those previously described in Wallace et al. (1996), were 16-cm diameter, round open-field chambers (Flex-Field, San Diego Instruments, San Diego, CA) that detected free movement of animals by infrared photocell interruptions. This equipment used an infrared photocell grid (32 emitter/detector pairs) to measure total locomotor activity. The activity monitors measured behavior, termed centrally and peripherally directed behavior that occurred in the center of the enclosure and activity that occurred outside of that central region, respectively. Total activity, as well as entry into the centermost compartment, rearing, centrally directed and peripherally directed locomotor activity, was measured by assessing the number and type of photocell interruptions within a 60-min period. Photocell interruptions were collected in 10-min intervals. In addition to the automated monitoring, an observational time sampling procedure was employed. An observer, unaware of the treatment condition of the animal, observed and recorded the animal's behavior, using a well-established protocol (Fray et al., 1980). Each rat was observed for 10 s at six time periods (1, 5, 10, 15, 30, and 60 min). During each time sampling period, behavior was recorded as present/absent (see Table 1 for the behaviors recorded during the sampling period). The animals' locomotor activity response to nicotine was assessed on only two occasions: immediately after the first (Day 1) and last (Day 21) nicotine injections. This latter procedure is important to preclude the repeated pairing of nicotine injection and the testing environment that otherwise confounds the neural expression of sensitization with learning via classical conditioning (Anagnostaras and Robinson, 1996; Bevins and Palmatier, 2003). Testing occurred between 1500 and 1700 h under dim light conditions, in the absence of direct overhead lighting (< 10 lx). All animals were sacrificed according to procedures used in Booze et al. (1999a). Briefly, the proestrus status of all female rats was monitored. Each male was matched to a female, and the male and female matched set was sacrificed when the female rat was in proestrus. Both male and female rats received the daily intravenous nicotine injection after Day 21 until proestrus was observed.

Table 1
Descriptions of behaviors measured in the observational time sampling procedure

Behavior	Description
Still	Animal's body is completely still
Locomotion	Animal has moved all four legs from one location to another
Rearing	Animal has raised up on two hind legs
Head-up sniff	Animal's head is raised while sniffing
Head-down sniff	Animal's head is lowered while sniffing
Head bobbing	Rhythmic moving of the animal's head up and down
Grooming	Animal grooms or licks body
Pivoting	Animal moves front of body while hind legs remain stationary
Scanning	Animal visually searches while moving head to the side
Circling	Animal rotates around one hind foot
Scratching	Animal scratches anywhere on body
Lying down	Animal lies down, but is not asleep

2.3. Experimental design and procedures: autoradiographic analysis of DAT, D_1, D_2, and D_3 receptors

2.3.1. Animals and brain dissection

Animals were transcardially perfused with 50 ml of 0.9% NaCl (37 °C) and 150 ml 0.9% NaCl following injection of sodium pentobarbital (60 mg/kg ip). The brains were rapidly dissected and immediately frozen. Frozen brains were placed in a cryostat and sliced into sagittal sections at a thickness of 20 μm. Slices were systematically sampled throughout the striatum starting at bregma +1.60 mm to the anterior commissure at bregma −0.40 mm (Paxinos and Watson, 1998). The sections were thaw-mounted onto Super Frost Plus Microscope slides (Fisher Scientific, Pittsbugh, PA) and stored in −80 °C. Slides were removed from −80 °C storage to a −20 °C environment 1 h prior to assay. Sections were thawed on a hotplate for 2–3 min (BT) and circled using a PAP pen to retain incubation buffer over the entire section.

2.3.2. DA transporter autoradiography

Sections for DA transporter autoradiography were preincubated in 137 mM NaCl, 2.7 mM KCl, 10.14 mM Na_2HPO_4, and 1.76 mM KH_2PO_4 for 30′ at RT (22–24 °C; pH 7.4). Sections were incubated to equilibrium in a wet incubation chamber with 20 pM [^{125}I] RTI-121 (NEN Life Science Products, Boston, MA) at RT for a period of 60′. The cocaine analog RTI-121 has been shown to bind with high affinity to DA transporters but not to serotonin or norepinephrine uptake sites. Nonspecific binding was defined to be [^{125}I] RTI-121 labeling observed in the presence of 200 μM Nomifensine—a selective DA uptake inhibitor interacting with the DA transporter at a site different from that of cocaine.

After incubation, sections were rinsed in ice-cold buffer (0–4 °C; pH 2 × 20′) followed by a 10-s rinse in cold distilled water to remove buffer salts. Further slides were dried under a stream of cold air for about 20′, in a cold room. The dried slides were placed against a ^{125}I-sensitive film (Kodak Scientific Imaging Film Biomax MR 24 × 30 cm, single emulsion film) and a set of [^{125}I] microscale standards (Amersham Life Scientific, Piscataway, NJ) for approximately 48 h in 0–4 °C.

2.3.3. D_1 autoradiography

Sections for D_1 DA receptor autoradiography were preincubated in Tris buffer containing 50 mM Tris–HCl, 120 mM NaCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$ for 30 min at RT (22–24 °C; pH 7.4). Sections were incubated to equilibrium in a wet incubation chamber at RT for a period of 60 min with 0.1 nM [^{125}I]-(+)-SCH 23982 (NEN Life Science Products) and 1 uM Ketanserin (to block binding to 5-hydroxytryptamine, 5-hydroxytryptomine, and alpha-2 adrenergic receptors). Nonspecific binding was defined to be [^{125}I]-(+)-SCH 23982 labeling and 1 uM Ketanserin (Research Biochemicals International, Natick, MA) observed in the presence of 1 μM SCH 23390 HCl (Research Biochemicals International). After incubation, sections were rinsed in ice-cold buffer (0–4 °C; pH 2 × 10 min) followed by a 10-s rinse in ice-cold distilled water to remove the buffer salts from the sections. Further slides were dried under a stream of cold air for about 20 min in a cold room. The dried slides were placed against a ^{125}I-sensitive film (Kodak Scientific Imaging Film Biomax MR 24 × 30 cm, single emulsion film) and a set of [^{125}I] microscale standards (Amersham Life Scientific) for approximately 48 h in 0–4 °C. The film was exposed for 24 h.

2.3.4. D_2 autoradiography

Sections for DA D_2 receptor autoradiography were preincubated in Tris buffer containing 50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$ at RT (22–24 °C; pH 7.4) for 5 min up to 30 min. Sections were incubated in fresh buffer including 1 mM ascorbic acid containing 0.15 nM [^{125}I] Iodosulpride (Amersham Life Scientific) for total binding and placed in a wet incubation chamber for 60 min at 22–24 °C (pH 7.4). Nonspecific binding was defined to be [^{125}I] Iodosulpride labeling in the presence of 1 μM (−) Sulpiride (Research Biochemicals International). Slides were rinsed in ice-cold (0–4 °C) buffer 2 × 5 min followed by a 10-s rinse in ice-cold distilled water to remove excess salts. The dried slides were placed against a ^{125}I-sensitive film (Kodak Scientific Imaging Film Biomax MR 24 × 30 cm, single emulsion film) and a set of [^{125}I] microscale standards (Amersham Life Scientific) for approximately 48 h in 0–4 °C. The film was exposed for 48 h.

2.3.5. D_3 autoradiography

Sections for dopamine D_3 receptor autoradiography were preincubated in 50 mM Tris–HCl, 100 mM NaCl at 30 °C for 30 min (pH 7.4). Further sections were incubated in 50 mM Tris–HCl containing 40 mM NaCl, 100 uM Gpp (=5 min-Guanylylimidodiphosphate, Sigma, St. Louis, MO), 0.5 uM DTG [=1,3-Di (2-tolyl)-guanidine, Research Biochemicals International] with 0.05 nM [^{125}I]-7-OH-PIPAT (NEN Life Science Products cat # NEX 307) for 60 min at RT. For nonspecific binding, 10 μM of the unlabeled ligand 7-OH-DPAT (Research Biochemicals International) was added to the incubation buffer. Following incubation, all sections were washed (and agitated) in 50 mM of ice-cold Tris–HCl and 40 mM of NaCl buffer (pH 7.4) for 3 h. The buffer was changed every hour. Sections were quickly dipped in ice-cold distilled water to remove excess salts from the sections. The dried slides were placed against a ^{125}I - sensitive film (Kodak Scientific Imaging Film Biomax MR 24 × 30 cm, single emulsion film) and a set of [^{125}I] microscale standards (Amersham Life Scientific) for approximately 48 h in 0–4 °C. The film was exposed for 72 h.

The autoradiographic films were developed using the Kodak D-19 developer for 2 min in 20 °C and Kodak rapid fixer for 5 min in 20 °C followed by 20 min rinse in running water under safelight darkroom condition. Computer-assisted densitometry methods (MCID System, Imaging Research) were used to obtain specific binding images (general autoradiography methods according to Booze et al., 1989; Booze and Wallace, 1995).

2.4. Drug treatment

The nicotine treatment was always administered as a bolus injection delivered in a volume of 1 ml/kg body weight (15 s), and was followed by flushing (15 s) with 0.2 ml heparinized (2.5%) saline (i.e., the approximate volume of the catheter). The dose of nicotine bitartrate (50 μg/kg/1 day) is calculated on the weight of the base and dissolved in saline for an injection volume of 1 ml/kg. The intravenous dosing regimen used in the present experiment has been shown to produce plasma nicotine levels comparable to levels demonstrated in tobacco smokers (Booze et al., 1999a; Benowitz et al., 1982).

2.5. Data analysis

The behavioral data were analyzed using analysis of variance (ANOVA) techniques (BMDP statistical Software, 1990; Winer, 1971). A mixed factorial ANOVA, with sex as a between-subjects factor and day as a within-subjects factor, was conducted for the automated locomotor, rearing duration, and entry data. Subsequent planned contrasts were conducted to determine sex differences in the automated behavioral data. Chi-square tests were used to compare rearing incidence between males and females on the data derived from the observational time sampling procedure. The observational data is nominal and between subjects in nature and thus was analyzed using the chi-square test. In addition, a Pearson *r* correlation was conducted to assess the

relationship between the automated and observed rearing data for sensitized animals (i.e., Day 21 data).

The autoradiography data were also analyzed using ANOVA techniques (BMDP statistical Software, 1990; Winer, 1971). A mixed factorial ANOVA, with sex as a between-subjects factor and brain region as a within-subjects factor, was conducted for each receptor ligand and the DA transporter data. Subsequent planned contrasts were conducted to determine sex differences in the autoradiography data.

As previous research shows that repeated intravenous cocaine produced a dose-dependent increase in striatal D_3 receptor density, and a dose-dependent decrease of NAcc D_3 receptor density (Wallace et al., 1996), a second level analysis was conducted using sex, protein (DA transporter and D_3), and region (ventral, medial, dorsal and lateral Striatum; core and shell of the NAcc). The goal of this latter analysis was to determine whether there was an interaction between the D_3 receptor density changes and those of the DA transporter. Finally, potential interrelationships between striatal and NAcc DA transporter/D_3 receptor number and two measures of "sensitized" behavioral activity were assessed using multiple regression analysis. The purpose of this last set of analyses was to determine whether any of the various changes in brain proteins of the dopmaniergic system were significantly predictive of the observed "sensitization" of behavior.

3. Results

3.1. Locomotor activity and behavioral sensitization

3.1.1. Acute effects of nicotine

Female and male locomotor activity, duration rearing, and entry into the centermost region of the compartment, for Days 0, 1, and 21, are illustrated in Figs. 1, 2, and 3,

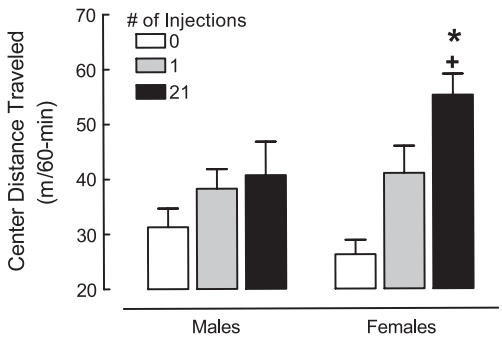

Fig. 1. Mean (± S.E.M.) central directed locomotor activity during a 60-min session, as a function of sex, for the baseline condition following acute intravenous saline, following an acute intravenous nicotine (50 μg/kg/ml) injection, and following the 21st injection of nicotine. All animals were habituated to the test environment for two sessions prior to baseline measurement. * Females, Days 1 and 21, $P<.05$; $^{+}$ Females compared to males on Day 21, $P<.05$. $n=16$ rats/group.

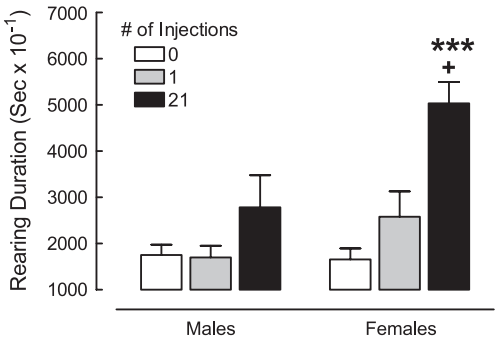

Fig. 2. Mean (± S.E.M.) duration spent rearing during a 60-min session, as a function of sex, for the baseline condition following acute intravenous saline, following an acute intravenous nicotine (50 μg/kg/ml) injection, and following the 21st injection of nicotine. All animals were habituated to the test environment for two sessions prior to baseline measurement. *** Females, Days 1 and 21, $P<.0001$; $^{+}$ Females compared to males on Day 21, $P<.05$. $n=16$ rats/group.

respectively. Planned contrasts revealed that there were no significant main effects of sex on rat's habituated baseline locomotor [$F(1,30)=1.3$, $P=.26$], rearing behavior [$F(1,30)<1.0$], or entries into the centermost compartment [$F(1,30)=1.2$, $P=.27$]. After the initial intravenous injection of nicotine, there was no sex difference in centrally directed locomotor activity [$F(1,30)<1.0$] or rearing [$F(1,30)=1.93$, $P=.17$], but there was a significant sex difference of entry to cross into the center zone [$F(1,30)=7.0$, $P<.05$]. Thus, females exhibited more entries into the centermost portion of the chamber, relative to males. The initial intravenous injection of nicotine did not induce hypoactivity of centrally directed locomotor activity, rearing, or entry to cross into the center zone in either the male or female rats.

3.1.2. Repeated effects of nicotine

ANOVA revealed significant main effects of sex for duration rearing [$F(1,30)=4.8$, $P<.05$] and entry [$F(2,60)=17.5$, $P<.0001$]. Significant main effects of day for locomotor activity [$F(2,60)=16.7$, $P<.0001$], duration rearing [$F(2,60)=20.7$, $P<.0001$], and entry [$F(2,60)=11.6$, $P<.001$] indicate that robust sensitization was produced with the present protocol. Moreover, significant Sex × Day interactions were revealed in the locomotor activity [$F(2,60)=4.3$, $P<.05$], duration rearing [$F(2,60)=5.3$, $P<.01$], and entry [$F(2,60)=5.2$, $P<.01$] measures demonstrating that females were markedly more sensitive than males to the effects of repeated nicotine (see Figs. 1, 2, and 3, respectively). Planned contrasts further suggested that females exhibited more locomotor activity [$F(1,30)=4.1$, $P<.05$], increased duration rearing [$F(1,30)=7.3$, $P<.05$], and more entries into the centermost chamber [$F(1,30)=20.7$, $P<.0001$] on Day 21, relative to male rats. Male rats did not exhibit nicotine-induced sensitization for the measures of locomotor activity, duration rearing, or entry into the center compartment.

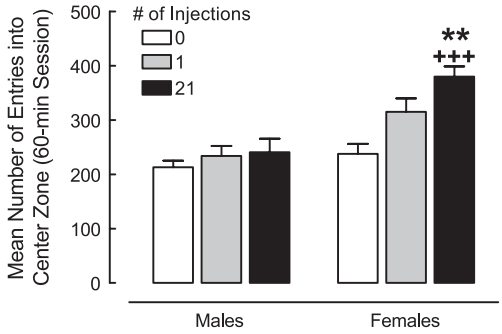

Fig. 3. Mean (± S.E.M.) number of entries into the centermost region of the locomotor activity chamber during a 60-min session, as a function of sex, for the baseline condition following acute intravenous saline, following an acute intravenous nicotine (50 μg/kg/ml) injection, and following the 21st injection of nicotine. All animals were habituated to the test environment for two sessions prior to baseline measurement. ** Females, Days 1 and 21, $P<.001$; $^{+++}$ Females compared to males on Day 21, $P<.0001$. $n=16$ rats/group.

3.2. Observational time sampling of behavior

A modified version of the observational time sampling method, first described by Frey et al. (1980), was used in the present experiments to examine the behaviors that rats exhibit following acute or repeated intravenous nicotine administration.

Fig. 4 illustrates one of the behaviors, rearing incidence, which further reflects the behavioral sensitization response to acute (Day 1) or repeated (Day 21) intravenous nicotine in male and female rats. There were no significant alterations in rearing behavior as a function of gender in the animals' acute response to intravenous nicotine [$\chi^2(1)=1.8$, $P=.19$]. However, the sensitized rearing response on Day 21, relative to Day 1 [$\chi^2(1)=8.1$, $P\leq.005$], displayed a pronounced sex difference in this effect [male vs. female on Day 21; $\chi^2(1)=22.8$, $P\leq.0001$]. Thus, females exhibited a 230% increase in rearing [Day 1 vs. Day 21; $\chi^2(1)=30.1$, $P\leq.0001$], whereas males showed a smaller, albeit significant [Day 1 vs. Day 21; $\chi^2(1)=4.4$, $P\leq.04$], 70% increase in rearing on Day 21 relative to Day 1. It is interesting that the female's acute response to nicotine is similar to the sensitized rearing response exhibited by males on Day 21. This observation further indicates the profound nicotine-induced sex difference in response to a low intravenous dose of nicotine.

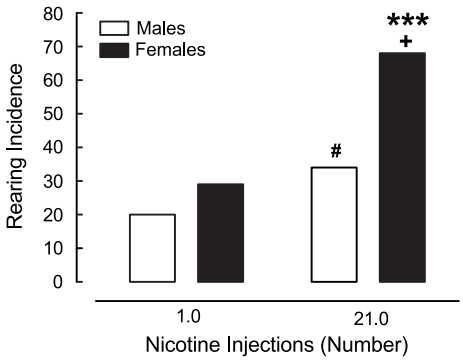

Fig. 4. Total number of rearing responses to acute and repeated intravenous nicotine dosing (50 μg/kg 1/day for 1 or 21 days) is illustrated for the observational time sampling of behavior as a function of sex. Behavioral sensitization was observed after the 21st nicotine injection in male and female rats, and there was significantly greater increase in the nicotine-treated animals. No measure of variance is indicated on the graph because the incidence (nominal) data were analyzed using the χ^2 statistic. *** Females, Days 1 and 21, $P<.0001$; $^{\#}$Males, Days 1 and 21, $P<.05$; $^{+}$Females compared to males on Day 21, $P<.05$. $n=16$ rats/group.

3.3. Relationship between automated and observed rearing behaviors

The current study demonstrated that repeated intravenous nicotine administration induced behavioral sensitization in male and female rats as measured by both rearing duration (automated) and rearing incidence (observed) measures. However, the relationship between the automated measure and the observational time sampling measure of rearing behavior has not been examined. Understanding the relationship between these two measures is beneficial when determining the utility of an animal model that may be used to further elucidate the sex differences in response to repeated nicotine administration. Thus, a Pearson r correlation was conducted on the automated and observed rearing data from Day 21. The analysis revealed that the automated and the observational time sampling measures of rearing behavior are highly correlated ($r=.74$, $P<.01$; $n=32$). The relationship between the automated and observational rearing measures is illustrated by the scatterplot in Fig. 5.

3.4. D_1 and D_2 receptor autoradiography

The means (± S.E.M.) for the D_1 and D_2 receptor data are presented in Table 2. ANOVA conducted for the D_1 data

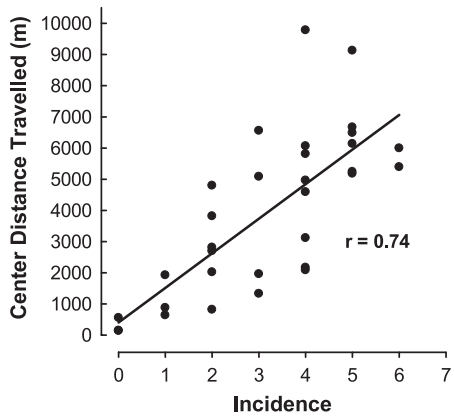

Fig. 5. Pearson r correlation of the automated and observational rearing measures is positively correlated. The correlational analysis is collapsed across sex. $n=32$.

Table 2
D_1 and D_2 receptor number means (± S.E.M.) for region and sex

	D_1		D_2	
	Female	Male	Female	Male
Dorsal striatum	275.7 (34.6)	272.7 (19.0)	84.4 (3.8)	87.0 (3.4)
Lateral striatum	241.4 (28.9)	237.1 (14.8)	74.0 (3.1)	75.1 (3.2)
Ventral striatum	305.2 (37.7)	286.9 (20.1)	98.0 (4.3)	99.2 (5.1)
Medial striatum	278.2 (32.0)	264.3 (16.0)	72.2 (2.9)	74.9 (2.3)
NAcc core	214.5 (23.9)	222.5 (13.3)	60.7 (2.8)	74.9 (2.6)
NAcc shell	196.7 (18.5)	211.9 (17.7)	65.9 (3.0)	60.3 (2.9)

revealed a significant main effect of region [$F(5,105)=25.9$, $P<.0001$], but neither of sex nor the interaction of Sex × Region. The ANOVA conducted for the D_2 data indicated a main effect for region [$F(5,150)=82.8$, $P<.0001$] and a significant Region × Sex interaction [$F(5,150)=5.1$, $P<.001$], primarily attributable to the opposite sex differences observed within the NAcc core and shell. The main effect for sex was not significant.

3.5. DA transporter and D_3 receptor autoradiography

DA transporter and D_3 receptor number is illustrated for female and male rats in the top and bottom panels in Fig. 6, respectively. ANOVA revealed significant main effects for protein [$F(1,30)=597.6$, $P<.0001$] and region [$F(5,26)=34.3$, $P<.0001$], and significant Protein × Sex [$F(1,30)=4.2$, $P<.05$] and Protein × Region [$F(5,26)=61.7$, $P<.0001$] interactions. Simple main effects analyses indicate that the NAcc core [$F(1,30)=8.4$, $P<.01$] and NAcc shell [$F(1,30)=10.1$, $P<.01$] were the regions primarily responsible for the Protein × Sex interaction. The data show that males exhibit higher numbers of D_3 receptors than females. Interestingly, the opposite finding was observed for DA transporter number; females exhibited higher numbers of DA transporters relative to male subjects (see Fig. 6). These findings suggest that a sex difference in the expression of DA transporters and D_3 receptors in the NAcc was found following repeated administration of intravenous nicotine.

3.6. Relationship between D_3 receptors and DA transporter in striatum and NAcc and locomotor activity

Multiple regression analyses were conducted to examine the relationship between brain proteins and behaviors in male and female rats. In the first analysis, stepwise multiple regression was used to suggest the regions of brain DA protein measures that would predict the sensitized entry data into the center of the open field: Striatal (medial and lateral), NAcc (shell) D_3 density, and NAcc (shell) DA transporter density were factors selected. Two separate correlational analyses, each using the same regions/receptor types as factors, but confined to either male or female subjects, were then conducted. The relationship between protein and entry behavior for female and male rats is illustrated in panel A of Fig. 7. The correlational analyses demonstrated significant relationships between entry behavior and striatal/NAcc D_3 and DA transporter density for both females ($r=.79$, $P<.05$) and males ($r=.77$, $P<.05$). These results suggest robust relationships between entry behavior and striatal/NAcc D_3 and DA transporter density for both male and female rats repeatedly pretreated with intravenous nicotine. Fig. 7, panel A, suggests that the slope of regression lines are similar, i.e., similar r values, but that the intercepts of these lines are different. An ANOVA of regression coefficients was conducted to determine if there was a significant difference in the slopes/intercepts of the male and female regression lines. The analysis revealed a significant main effect of group [$F(5,22)=4.3$, $P<.01$] indicating that the regression line that fits the female data is located higher on the graph than the regression line plotted for the male subjects.

In the second analysis, stepwise multiple regression was used to suggest the regions of brain DA protein measures that would predict the sensitized duration of rearing data: striatal (dorsal and medial) D_3 and DA transporter (dorsal) density factors were selected. Two separate correlations, each using the same regions/receptor types as factors, but

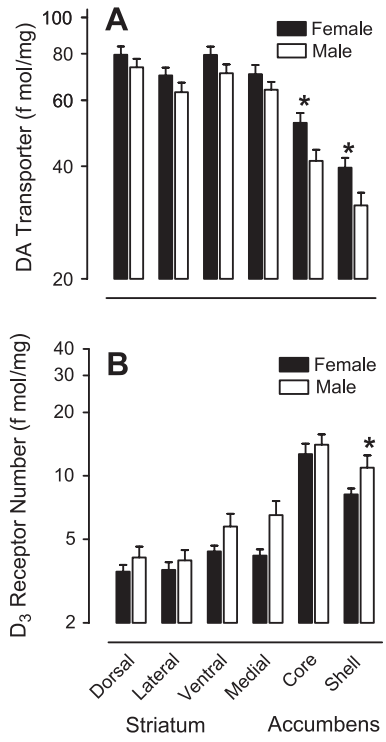

Fig. 6. (A) Mean (± S.E.M.) number of DA transporters in the striatum and NAcc for male and female rats on Day 21. Female rats displayed more DA transporters in the core and shell regions of the NAcc, relative to males. No sex differences were detected in the striatum. * Females compared to males, $P<.01$. $n=16$ rats/group. (B) Mean (± S.E.M.) number of D_3 receptors in the striatum and NAcc for male and female rats on Day 21. Female rats displayed less D_3 receptors in the NAcc, relative to males. No sex differences were detected in the striatum. * Females compared to males, $P<.01$. $n=16$ rats/group.

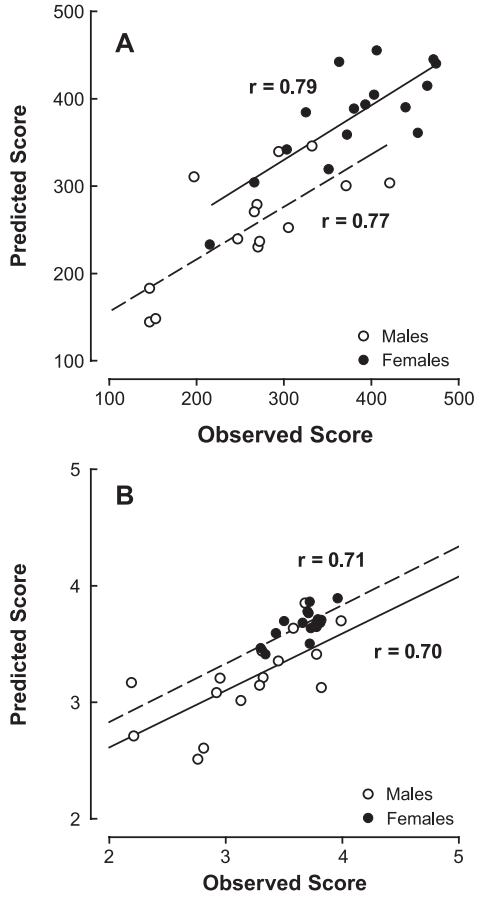

Fig. 7. Multiple regression analysis of entry and rearing behavior and D_3/DAT density. Data are expressed as observed entry (A) or rearing (B) score (*x* axis) vs. predicted entry score (*y* axis) as a function of sex. Predicted entry and rearing scores were obtained from multivariate linear regression analysis of behavior and autoradiography data. Predicted values represent the entry scores based on each animal's number of entries into the centermost portion of the locomotor activity chamber, and rearing duration on Day 21. A significant relationship was found between the number of entries and D_3 receptors in the striatum (medial and lateral) and NAcc (shell), and DA transporters in the NAcc (shell) for both male and female rats. A significant relationship was also found between duration rearing and striatal (dorsal and medial) D_3 and DA transporter (dorsal) proteins. $n = 16$ rats/group.

confined to either male or female subjects, were conducted. The relationship between protein and duration rearing for female and male rats are illustrated in panel B of Fig. 7. The correlational analyses demonstrated significant relationships between duration rearing and striatal D_3 and DA transporter density for both females ($r = .71$, $P < .05$) and males ($r = .70$, $P < .05$). These results suggest robust relationships between duration rearing and striatal D_3 and DA transporter density for both male and female rats repeatedly pretreated with intravenous nicotine. Fig. 7, panel B, suggests that the *r* values are similar, but that the intercepts of these lines are different. An ANOVA of regression coefficients revealed a significant main effect of group [$F(4,24) = 7.4$, $P < .001$] indicating that the regression line that fits the female data is located higher on the graph than the regression line plotted for the male subjects.

4. Discussion

The present experiment demonstrated that repeated IV nicotine administration (50 μg/kg, 21 days × 1/day) produced sex differences in the expression of sensitized behavior across four measures of activity. Thus, female rats exhibited more locomotor and rearing behaviors after 21 days of nicotine administration, relative to males. Autoradiography data suggest that there were no sex differences in the expression of D_1 or D_2 receptors following repeated nicotine administration. Interestingly, female rats exhibited more DA transporters in the core and shell region of the NAcc and fewer D_3 receptors in the shell region of the NAcc, compared to males. Moreover, exploratory multiple regression analyses indicate that various sensitized behaviors are differentially predicted by regional DA transporter and D_3 receptor differences between male and female rats.

A caveat to our use of the term behavioral sensitization should be added as the present experiment did not include saline control groups that would otherwise permit statements about the absolute levels of behavioral sensitization observed. Using similar procedures, but with the study of intravenous cocaine, saline-injected males displayed a linear increase in centrally directed locomotor activity across 14 days of injection with repeated testing (Wallace et al., 1996). Although that particular effect is most likely attributable to context conditioning due to the repeated pairing of saline injection and the test environment, the present lack of any statistically detectable increase in activity in injected males after 21 days of intravenous nicotine injection is particularly striking. Furthermore, with similar procedures in the study of the effects of intravenous cocaine in females, saline-injected animals displayed no significant increases in activity after 14 days of injection with repeated testing, even with the benefit of context conditioning (Booze et al., 1999b). Again, the contrast to the present study is particularly striking with the females displaying marked within subject increases and highly significant differences from similarly injected males. Having now achieved a set of parameters that provides a robust sex difference in relative behavioral sensitization, future research will incorporate saline-injected controls to further characterize sex differences in the development and expression of behavioral sensitization.

The present experiments demonstrate that the nicotine-induced sex difference in the expression of behavioral sensitization is robust, and that this intravenous injection procedure may serve as a useful animal model to further examine nicotine-induced sex differences in rats. Differences in the procedures of an initial experiment, which first reported intravenous nicotine-induced sex differences in the expression of behavioral sensitization (Booze et al., 1999a), compared to the procedures in the present experiment, further demonstrate the reliability of this finding. For example, the sex-dependent response to repeated intravenous nicotine administration was observed when automated locomotor activity was measured in square or round activity

chambers, and if intravenous nicotine administration occurred over a 14- or 21-day period (present experiment). Furthermore, the modified time sampling observation, which was also used in the Booze et al. (1999a) study, revealed that rearing behavior became sensitized to repeated intravenous nicotine administration in the present study despite these slight procedural differences. Taken together, this evidence suggests that the repeated intravenous nicotine administration procedure reliably produces a sex difference in the expression of behavioral sensitization across varying testing situations.

The sex-dependent expression of behavioral sensitization for the locomotor activity measures of distance traveled and duration rearing, and the observational measure of rearing incidence cannot be explained by baseline sex differences in locomotor activity (Beatty, 1979; Burke and Broadhurst, 1966; Cronan et al., 1985; Rodier, 1971; van Haaren and Meyer, 1991; Wang, 1923) because both female and male rats exhibited similar activity following 3 days of habituation to the locomotor activity chambers. Notably, however, the current study reports one incidence of an acute, nicotine-induced sex difference. Thus, females exhibited more entries into the center portion of the compartment following the 1st and 21st nicotine injections, compared to males. It can be concluded that the acute injection of nicotine produced increased entry behavior in females, relative to males, because there was no sex difference in the number of entries into the center zone during baseline. This finding is in accord with previous research which suggests that nicotine stimulates more locomotor activity in female compared to male rats (Battig, 1981; Kanyt et al., 1999). The sex-dependent expression of behavioral sensitization is not likely due to differential nicotine pharmacokinetics between male and female rats. Booze et al. (1999a) investigated nicotine pharmacokinetics in female and male rats following 14 days of repeated administration of the intravenous nicotine dose 50 μg/kg/infusion used in the present experiments and failed to find any evidence of a sex difference in arterial plasma nicotine pharmacokinetics. Importantly, the present experiment replicated and extended the sex-dependent expression of nicotine-induced behavioral sensitization in multiple measures of locomotor activity (Booze et al., 1999a), thus adding to the growing number of studies demonstrating sex differences in response to nicotine treatment (Battig, 1981; Booze et al., 1999a; Grunberg et al., 1986, 1987; Kanyt et al., 1999; Levin et al., 1987; Rosecrans, 1971, 1972).

Our laboratory has examined the expression of behavioral sensitization following repeated administration of intravenous cocaine (Wallace et al., 1996) and nicotine (Booze et al., 1999a; present experiment). Across these experiments, circular and square open-field arenas have been used to measure changes in locomotor activity (Wallace et al., 1996; present study, and Booze et al., 1999a, respectively). Although we have not systematically compared the expression of psychostimulant-induced behavioral sensitization when circular vs. square chambers are used, it appears that the former style of open-field arena is more sensitive to an array of locomotor behaviors. Future studies will directly compare circular and square activity chambers to determine if there are parametric differences in the expression of psychostimulant-induced behavioral sensitization.

One critical consideration with regard to establishing an animal model that can be utilized to investigate the neurobiological mechanisms of sex differences is the route of drug administration. The intravenous route of administration in a rat model (see Booze et al., 1999a; Mactutus et al., 1994; Wallace et al., 1996) removes the process of drug absorption and provides near instantaneous distribution of nicotine through the vasculature, as well as 100% bioavailability of nicotine to the arterial side of the circulation. This particular method of intravenous drug injection was used because it closely mimics the pharmacokinetics of drug action observed for stimulant drugs commonly abused by humans via smoking or intravenous injection (Benowitz, 1990a, 1990b; Henningfield et al., 1993; Russell and Feyerabend, 1978). The various exposure routes, e.g., subcutaneous, peroral, and tablet/minipump, exhibit substantially different nicotine kinetic profiles in comparison to the intravenous route of administration used in the current experiments. The intravenous route of administration produces a characteristic rapid peak concentration of nicotine followed by precipitous clearance, whereas the subcutaneous, peroral, and tablet/minipump routes of administration produce moderate and sustained plasma levels of nicotine due to the absorptive and protracted distribution processes. The most advantageous route of administration for animal models of nicotine abuse would be one that closely mimics the pharmacokinetics observed in humans. For example, cigarette smoking delivers nicotine in doses that produce rapid increases in plasma nicotine concentration. The levels of nicotine in the arterial circulation may be 10-fold greater than levels in the venous blood (Benowitz, 1990a, 1990b; Henningfield and Keenan, 1993).

The distinction between arterial and venous sampling is crucial because arterial levels reflect the peak levels delivered to the brain following intravenous injection or cigarette smoking. Nicotine delivered through cigarette smoking has a distribution half-life of 10–20 min in plasma, followed by an elimination half-life of 2–3 h (Benowitz et al., 1991, 1982). Nicotine administered by other delivery systems also has an elimination half-life of 2 h and can mimic the average plasma nicotine levels, but cannot achieve the rapid and large increase in arterial nicotine levels associated with cigarette smoking. Previous research examined the pharmacokinetics of intravenous nicotine (50 μg/kg) administration and demonstrated peak arterial nicotine levels that are in the clinically relevant range (Booze et al., 1999a). Moreover, these values are consistent with previously established values in male rats with methods that have used the quantification of ^{14}C-labeled nicotine (Adir et al., 1976). Given these findings, the intravenous dosing method is preferable to other routes of administration in determining the neurobiological mecha-

nisms responsible for the sex-dependent expression of behavioral sensitization.

The present research also investigated the density of D_1, D_2, and D_3 receptors and DA transporters in the striatum and NAcc following repeated intravenous administration of nicotine. Interestingly, a sex difference in the expression of D_3 receptors and DA transporters was observed in the NAcc. Thus, females exhibited fewer D_3 receptors in the NAcc (shell), and more DA transporters (core and shell), relative to males. There was no sex difference in the expression of D_1 or D_2 receptors in either the striatum or the NAcc. A caveat of the current study, in not including saline-injected groups, is that it cannot be determined if the repeated intravenous nicotine-induced changes in the expression of DA receptors or DA transporters were above or below baseline levels. Future studies will determine whether the sex difference in the expression of D_3 receptors/DA transporters are due to differential changes from up- or down-regulation of these proteins in male and female rats, respectively.

Recent research suggests that normal locomotor activity in rats is the result of the activity between two opposing DA receptor subtypes: D_1/D_2 receptors that promote locomotor activity and D_3 receptors that inhibit locomotor activity. According to this suggestion, behavioral sensitization is a consequence of changes in D_1/D_2 and D_3 receptor activity as a response to repeated administration of psychostimulant drugs. Thus, "sensitized behavior" might reflect an increase in D_1/D_2-mediated excitation, or may instead be the result of a decrease in D_3-regulated inhibition of activity (Chiang et al., 2003; Le Foll et al., 2003; Menalled et al., 1999; Richtand et al., 2003; Sautel et al., 1995; Waters et al., 1994). This suggestion is supported by neurophysiological studies which indicate that in the VTA, stimulation of D_3 receptors reduces neuronal firing rate (Lejeune and Millan, 1995), and that activation of D_3 receptors in the NAcc results in an attenuation of DA release (Gilbert and Cooper, 1995). These findings suggest that the increased behavioral sensitization exhibited in females relative to males in the present experiment may be due to the differential expression of D_3 receptors. Thus, if D_3 receptors inhibit locomotor activity, then female rats may have exhibited increased expression of behavioral sensitization compared to male rats because females had fewer D_3 receptors in the NAcc. Interestingly, female rats also exhibited a nicotine-induced increase in DA transporters in the NAcc, relative to males. Previous findings report that cocaine regulates DA transporter function by increasing DA transporter number on the presynaptic membrane via DA transporter trafficking in cells expressing human DA transporters (Little et al., 2002). Currently, it is not known whether nicotine can stimulate DA transporter trafficking as previously shown with cocaine. Further experiments are needed to determine if repeated intravenous nicotine administration produces differential regulation of D_3 receptors and DA transporters in male and female rats.

The behavioral experiments from the present study demonstrated that repeated intravenous nicotine administration produced a sex difference in the expression of behavioral sensitization. The results of the correlational analyses, which determined the relationship between DA transporter/D_3 protein and behavior, suggest that although both sexes are expressing a similar protein/behavior relationship, there is a sex difference in the absolute number of proteins and behavioral counts/observations observed. This sex difference was observed for both the entry and duration rearing behaviors, indicating that the sex difference is present regardless of type of sensitized behavior examined. Collectively, the correlational findings further show that the nicotine-induced behavioral changes observed for male and female rats are more pronounced in the female sex.

The present experiments demonstrate that the sex difference in the expression of nicotine-induced behavioral sensitization is reliable, and may be mediated by the differential expression of D_3 receptors and DA transporters in male and female rats. Changes in D_3 receptors and DA transporters, however, are not the only factors that play a role in psychostimulant-induced sensitization. One important issue is how gonadal hormones regulate D_3 receptors, and thus, nicotine-induced behavioral sensitization in male and female rats. Previous work shows that ovariectomized rats that were administered an estradiol implant and repeatedly injected with cocaine exhibited a decrease in D_2 and D_3 functioning and an increase in cocaine-induced rearing (Febo et al., 2003). Work from our laboratory shows that castration increased nicotine-induced activity relative to intact males, and ovairectomy decreased activity relative to intact females (Booze et al., 1999a). These studies clearly show that gonadal hormones modulate psychostimulant effects on animal behavior. Future work that will elucidate the role of gonadal hormones on the development and expression of nicotine-induced behavioral sensitization in both male and female rats is warranted.

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