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Extended Access to Nicotine Self-Administration Leads to Dependence: Circadian Measures, Withdrawal Measures, and Extinction Behavior in Rats

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ABSTRACT

The present study characterized nicotine intake, circadian patterns of food and water intake, precipitated somatic signs of withdrawal, and extinction of nicotine-seeking behavior in rats with 23-h access to intravenous self-administration (IVSA). Separate groups of animals were allowed access to nicotine IVSA (0.015, $n = 9$; 0.03, $n = 14$; 0.06, $n = 16$; mg/kg/0.1 ml infusion/s; fixed ratio 1) and trained to nosepoke for food and water 23 h/day for 40 consecutive days. Somatic signs of nicotine withdrawal were examined following saline or mecamylamine administration (1.5 mg/kg i.p.), and extinction of nicotine-seeking behavior was assessed. A dose-dependent decrease in lever responding and an increase in nicotine intake were observed, with the highest nicotine dose producing the lowest amount of lever responding and the highest amount of nicotine intake. Nicotine acutely reduced diurnal and nocturnal food intake, producing smaller and fewer meals, and an increased rate of eating. Differences in rate of nicotine intake between the light and dark phase decreased significantly, especially in rats receiving higher unit nicotine doses (0.03 and 0.06 mg/kg), along with long-term decreases in the circadian profile and amplitude of feeding. Mecamylamine precipitated robust withdrawal signs, the magnitude of which was positively correlated with the total amount of self-administered nicotine. Extinction of nicotine-seeking behavior was observed and was facilitated by removal of nicotine-associated cues. The results demonstrate that rats will self-administer nicotine to the point of producing dependence, as measured by somatic signs, resistance to extinction, and measures of food intake.

To more closely model tobacco use in humans, recent studies have examined extended access to nicotine intravenous self-administration (IVSA) in rats. For example, female rats display increased nicotine IVSA during the active phase of the light cycle during 3 weeks of continuous nicotine access (Cox et al., 1984). These rats also display a compensatory increase in nicotine IVSA when the dose is lowered (0.03 to 0.003 mg/kg) and a decrease in nicotine-seeking behavior when nicotine is replaced with saline. Moreover, male rats display nicotine IVSA in extended access models (6–23 h) using low nicotine doses (0.00375 mg/kg/injection), and the level of nicotine intake approximates that of human smokers (Valentine et al., 1997; Paterson and Markou, 2004; Kenny and Markou, 2006). The 23-h access model of nicotine IVSA seems to be sensitive to genetic differences, since nicotine intake is more quickly acquired and persistently maintained in Lewis versus Holtzman and Fisher strains of male rats (Brower et al., 2002). Furthermore, the 23-h model of nicotine IVSA is sensitive to passive nicotine administration. Nicotine intake decreased following implantation of a minipump that delivers doses of nicotine that are equal to, or higher than, peak levels associated with simulated nicotine intake (LeSage et al., 2002). In addition, nicotine intake in rats allowed 23-h access is attenuated in response to administration of a nicotinic receptor antagonist or in response to an

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ABBREVIATIONS: IVSA, intravenous self-administration; MESOR, midline estimating statistic of rhythm; ANOVA, analysis of variance.

increase in the ratio requirement for infusions of nicotine (Denoble and Mele, 2006). Studies using 23 h access to nicotine also demonstrate that increasing the unit dose of nicotine from 0.008 to 0.064 mg/kg/infusion resulted in an increase in nicotine intake (infusions displayed an inverted U-shaped dose-response curve) and that saline substitution resulted in extinction of nicotine-seeking behavior (LeSage et al., 2004; DeNoble and Mele, 2006). Furthermore, the latter study demonstrated that extinction of nicotine-seeking behavior in rats allowed 23-h access is reinstated by nicotine-associated cues, but not by priming injections of nicotine. Collectively, it seems that rats receiving higher doses of nicotine display higher levels of nicotine intake following extended access to this drug (Cox et al., 1984; Valentine et al., 1997; Denoble and Mele, 2006).

Studies examining extended (23-h) access to nicotine IVSA have provided valuable information regarding nicotine doses, patterns of intake across time, and time of day for maximal drug intake. However, the relationship between the levels of nicotine intake and the manifestation of the somatic withdrawal syndrome has not been examined in rats allowed 23-h access to nicotine IVSA.

Extinction of drug-seeking behavior can be interpreted as an indication of motivation to obtain the drug. Thus, the continued presence versus absence of nicotine-associated cues retards the extinction of nicotine-seeking behavior (Caggiula et al., 2001). Rats dependent on cocaine or heroin exhibit slower extinction of drug-seeking behavior (Shalev et al., 2002). Thus, rats that exhibit measures of nicotine dependence may be expected to exhibit slower rates of extinction compared with nondependent controls, and this hypothesis was tested in the present studies.

Rodent studies indicated that passive nicotine administration (i.e., experimenter-administered) alters the control of food intake and weight gain. For example, nicotine decreases feeding and body weight, and withdrawal from nicotine produces rebound overeating and an increase in weight gain, an effect that is mirrored in human subjects (Jo et al., 2002). However, studies examining the effects of nicotine on food intake have used noncontingent administration of nicotine via minipumps (Blaha et al., 1998; Miyata et al., 2001) or bolus administration of high-nicotine doses (Bellinger et al., 2003; Wellman et al., 2005) that might produce different effects than self-regulated intake of small unit doses of nicotine. Therefore, the present study also examined overall food intake, and microstructural changes in food intake, in rats receiving continuous access to voluntary nicotine IVSA.

The purpose of the present study was to test the hypothesis that rats allowed continuous access to nicotine over extended periods (i.e., 40 days) will display mecamylamine-precipitated somatic signs of nicotine withdrawal and that the degree of dependence will be a function of previous nicotine intake. A subhypothesis under test was that circadian measures of nicotine, food, and water intake also may serve as markers of dependence in rats given chronic 23-h access to nicotine.

Materials and Methods

Subjects

Male Wistar rats (n = 39; Charles River, Stone Ridge, NY) weighing 200 to 250 g at the beginning of the experiment were housed in groups of three per cage in a humidity- and temperature-controlled (22°C) vivarium on a 12-h light/dark cycle (lights on for 6:00 AM to 6:00 PM). Rats were handled daily during an initial 5-day acclimation period where they had ad libitum access to food and water. All procedures were conducted in strict adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Operant Chambers

Following the initial acclimation period, the rats were housed in operant IVSA chambers (MED Associates, St. Albans, VT) that were kept on a regular light/dark cycle (from 6:00 AM to 6:00 PM lights on) via a computer system that monitored real time. The house light was located inside the sound-attenuated chambers with continuous white noise. Each day at 10:00 AM, the rats were removed from the operant chambers and placed into their home cage (n = 2–3/cage) for 1 h so that the chambers could be cleaned and the water and food replenished. The exit port of the catheter fittings were connected to polyethylene tubing contained inside a protective metal spring that was suspended into the operant chamber from a liquid swivel attached to a balance arm. The nicotine was delivered via a syringe pump (Razel Scientific, St. Albans, VT) as described in Caine et al. (1993). Operant sessions were conducted using two retractable levers (i.e., active and inactive lever) that extended approximately 1 in. into the chamber.

Food and Water Training

During the first 5 days, the animals were allowed to perform nosepoke responses on a fixed ratio-1 schedule of reinforcement to obtain palatable chow pellets (45 mg of precision food pellets, Formula A/I; Research Diets, Lancaster, NH) from a pellet dispenser with a swing door mounted between two levers on the front wall of the chamber. A nosepoke response was also required in a separate hole positioned on the back of the chamber for administration of 0.1-ml aliquots of water into an adjacent metal dipper cup. By the fourth day of training, all of the rats had acquired stable levels of food and water responding. The animals were returned to their home cages (n = 2–3/cage) with ad libitum access to food and water for 2 days before catheter implantation surgery.

Intravenous Catheter Implantation

Rats were anesthetized with an isoflurane/oxygen vapor mixture (1.0–1.5%) and prepared with chronic indwelling intravenous catheters into the jugular vein as described in Caine et al. (1993). In brief, the catheters were implanted in the rat's jugular vein and the exit port was secured to the skull using cranioplastic cement. Animals were allowed to recover for 1 week. Catheters were flushed daily with 0.2 ml of sterile physiological saline containing heparin (30 USP units/ml) and the antibiotic Timentin (SmithKline Beecham Pharmaceuticals, Philadelphia, PA). If at any point during the experiment catheter leaks or abnormal shifts in IVSA behavior were observed, then rats received 0.1 ml of the ultrashort-acting barbiturate anesthetic Brevital sodium (1% methohexital sodium; Eli Lilly & Co., Indianapolis, IN) through the catheter. Animals with patent catheters exhibited prominent signs of anesthesia (pronounced loss of muscle tone) within 3 s of the intravenous injection. Data collected from animals with nonpatent catheters were excluded from the data analyses.

Lever Habituation and Re-Establishment of Food and Water Responding

Following recovery, the rats had an additional 5 days of food and water training to re-establish stable food and water intake before nicotine access. The exit port of the catheters were connected to the drug tether and swivel to habituate the animals to the drug lead even though drug was not yet available. The rats also were habituated to the levers by presenting them each day during the 5 days of re-establishment of food and water responding. Each response on the

lever that would become the "active" nicotine lever resulted in presentation of the drug cues (i.e., stimulus light and pump noise), whereas responses on the other "inactive" lever were recorded but had no scheduled consequences. Preliminary work before this project revealed that rats living in an operant chamber press a lever approximately 12 times throughout the course of a 23-h period. The majority of this low number of responses does occur in the active (night) phase of the rats' cycle. Therefore, this criterion was set as a point of habituation, and all rats that were included in the study responded less than 12 times before the introduction of nicotine.

Nicotine IVSA Sessions

On day 1 of nicotine access, responses on the drug-associated lever resulted in administration of various unit doses of nicotine (0.015, $n = 9$; 0.03, $n = 14$; or 0.06, $n = 16$ mg/kg/infusion/0.1 ml infusion). Each response on the active lever (that did not occur during the time-out period) resulted in the delivery of 0.015, 0.03, or 0.06 mg/kg nicotine base in a volume of 0.1 ml over a 1-s period. A 28-V white cue light was illuminated above the active lever at the onset of the 1-s infusion and was terminated after a 20-s time-out period, during which time-out responses were recorded but had no scheduled consequences. Separate groups of animals were used to examine IVSA of these doses of nicotine for 40 days. Two additional days of nicotine IVSA were included to assess withdrawal signs in the morning of day 41 and 42 of nicotine IVSA. The nicotine solutions were prepared daily based on the animals' weights from the previous day. Food and water were available throughout the entire 23-h nicotine IVSA sessions, including during the nicotine time-out periods.

Assessment of Nicotine Precipitated Withdrawal with Mecamylamine

Baseline and mecamylamine-induced signs of nicotine withdrawal were observed on days 41 and 42 of nicotine IVSA. Withdrawal signs were measured on day 41 (following saline administration) and day 42 (following mecamylamine administration) following 40 days of nicotine IVSA. In relation to the 23-h session, the somatic signs were technically assessed on days 40 and 41 because the signs were taken at 6:00 AM in the morning and the sessions began at 10:00 AM. The baseline measures are thought to reflect true basal values, since comparable baseline measures have been reported following mecamylamine administration in naive animals (O'Dell et al., 2006). Rats were removed from the IVSA boxes at the end of the dark phase (6:00 AM) of the light/dark cycle to observe signs following a period of high nicotine intake. Rats received saline on the first test day, and then mecamylamine (1.5 mg/kg i.p.) on the next day. The rats then were placed into a plastic opaque cylindrical container (30 × 29 cm), and 30 min later they were observed for 10 min for somatic signs of nicotine withdrawal according to the method developed by Malin et al. (1992). The signs recorded were blinks, body shakes, chews, cheek tremors, escape attempts, foot licks, gasps, writhes, genital licks, hops, head shakes, ptosis, scratches, teeth chattering, and yawns. Multiple successive counts of any sign required a distinct pause between episodes. Total number of somatic signs in the 10-min observation period was defined as the sum of the number of occurrences of all of the above-mentioned signs. The same observer scored all of the withdrawal signs and was blind to the animal's drug treatment.

The circadian pattern of nicotine intake was not compared with withdrawal signs because repeated tests using mecamylamine might have introduced a confound of repeated drug effects across dose conditions. Furthermore, acute administration of mecamylamine allowed us to examine a discrete time point that could be examined at the same time of day for all rats. Withdrawal signs were also not measured during extinction because it would have been impossible to control for individual differences in the rats' prior exposure to nicotine.

Extinction of Nicotine IVSA Behavior

After completing the nicotine IVSA phase of the experiment (42 days), nicotine was replaced with saline, and the animals responded in extinction for an additional 10 days. During the first 5 days of the extinction phase, responding on the active lever resulted in presentation of the drug-associated cues (i.e., infusions, pump noise, and cue light). During the next 5 days of extinction training, the cues were removed (i.e., turned off the pump and cue light) such that responses on the active lever had no scheduled consequences.

Drugs

The drugs used in these experiments were (−)-nicotine hydrogen tartrate salt and mecamylamine. Both drugs were purchased from Sigma/RBI (Natick, MA). The doses of mecamylamine refer to the salt, and the doses of nicotine refer to the free base form. All drugs were dissolved in 0.9% sterile saline, and mecamylamine was administered in a volume of 1 ml/kg. The drug doses were selected based on previous work in our laboratory (Watkins et al., 1999; Paterson and Markou, 2004) and that of others using extended access to nicotine IVSA (Valentine et al., 1997; Fu et al., 2001; Lesage et al., 2002, 2003).

Data Analysis

Nicotine IVSA, Baseline, and Mecamylamine Data. Repeated measures analyses of variance (ANOVAs) were performed with time as the within-subjects factor and dose as the between-subjects factor on various measures (nicotine responding, nicotine intake, and extinction responses) that were assessed in rats allowed 23-h access to nicotine for 40 days. One-way ANOVAs were used to examine dose-dependent effects of measures that were collapsed across time (e.g., dose × mean total responding). Post hoc comparisons were conducted using Fisher's protected least significant difference test. A Pearson's r correlational matrix analysis was performed on mean nicotine intake (mg/day) versus total score (i.e., counted signs) of mecamylamine-precipitated nicotine withdrawal. The probability for a type 1 error for all significance testing was set at 0.05.

Cosinor Analysis of Food, Water, and Nicotine Intake. Similar to a recent study from our laboratory (Chen et al., 2006), cosinor analysis was used for the analysis of the circadian regulation of food, water, and nicotine intake (Smolensky et al., 1976; Lentz, 1990). In brief, cosinor analysis is a form of time-series examination that models chronobiological rhythms as a cosine function with the following attributes: the midline estimating statistic of rhythm (MESOR; mean level around which the cosine function oscillates), amplitude [the distance from the MESOR to the extremes (peak or nadir) of the oscillation], acrophase (the time at which the cosine peak occurs relative to a time of interest, in this case the start of the session), and period (the time interval at which the cycle repeats; Smolensky et al., 1976; Lentz, 1990). The acrophase is the time at which circadian peak occurs. Figure 1 displays a schematic of the cosinor analysis measure. To examine changes in circadian regulation, a predefined period of 24 h was used according to the following equation:

$$y = \text{MESOR} + \text{amplitude} \times \cos\left(\frac{2\pi(\text{x} - \text{acrophase})}{24}\right)$$

Cosinor functions were fit individually to each rat's daily intakes, and the MESOR, amplitude, acrophase, and goodness of fit (r^2) were obtained from each and averaged across rats. Peaks were calculated as the MESOR + amplitude, and nadirs were calculated as the MESOR − amplitude. Because food and water intake occur in discrete episodes ("meals"), intake was cumulated into 3-h bins to facilitate modeling of the hypothesized underlying intake rhythm. Nicotine intake was cumulated identically for consistency. The first and last hours of the 23-h data collection period were not used for curve fitting because of potentially confounding influences of recent and

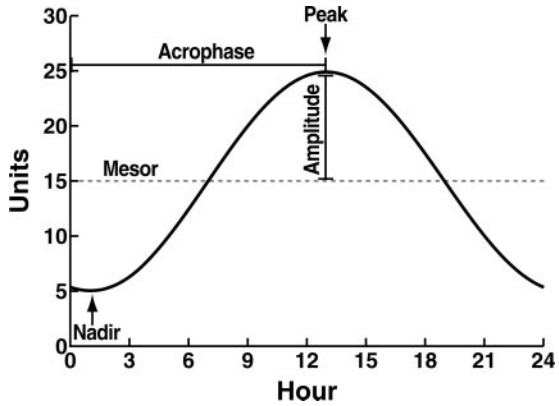

Fig. 1. For clarity, cosinor measures (e.g., MESOR, amplitude, and acrophase) of circadian regulation of drug and food intake are illustrated in this sample figure that depicts a cosinor function with MESOR = 15, amplitude = 10, peak = 25, nadir = 5, and acrophase = 13 h from the start of the session.

anticipated experimenter manipulation and transient inaccess to reinforcers. DataFit 8.0 computer software (Oakdale Engineering, Oakdale, PA) was used for curve fitting, and Instat 3.0 (GraphPad Software Inc., San Diego, CA) was used to test for significant differences in the central tendency or variability of the cosinor parameters. In all analyses, measures of ingestion were normalized per Kleiber's law as a power function of body weight (i.e., grams of food intake per [kilograms of body weight]$^{0.75}$) to account for increased metabolic needs of greater body mass (Sidhu, 1992). Because rats' weights changed by approximately 90 g over the course of the 40 days of nicotine IVSA, it is important to account for changes in body weight when interpreting changes in food intake. Kleiber's law is perhaps the best-validated bioenergetic law (also see Gillooly et al., 2001; Lindstedt and Schaeffer, 2002). In essence, it expresses how much more energy is required to sustain a larger body mass. Therefore, the correction applied in the present study clarifies that observed changes in intake are independent from potentially confounding metabolic mass-related changes in energy need.

One-way within-subjects ANOVAs with day as the repeated measures factor were performed on the data for all cosinor measures (amplitude, MESOR, acrophase, peak, nadir, and r^2). Where appropriate, Student-Newman-Keuls post hoc comparisons were conducted for within-subjects comparisons. Bonferroni-corrected t tests were used to examine whether the nadir of the primary group differed reliably from zero. Using Instat 3.0, Bartlett's method was used on the acrophase measure to test whether the variability (standard deviation) of acrophase changed across conditions. A significant increase in variability would indicate a desynchronization of the acrophase over time.

Meal Pattern Analysis of Food and Water Intake. For meal pattern analysis, a meal for rats was defined as a burst of responses for food or water that contained at least five food-directed responses, or 0.225 g, a value below lower bounds for food bout size estimated previously (Demaria-Pesce and Nicolaidis, 1998; Zorrilla et al., 2005). Meals were discriminated from one another by the threshold meal interval, or the maximum interval between ingestive responses that was considered to continue the ongoing meal. The threshold meal interval was estimated by determining the interevent interval(s) between feeding- and drinking-directed nosepokes that provides the most stable, joint estimates of meal size for food and total meal duration, thereby minimizing the negative consequences of misassigned events and time. This method was related to previous approaches in which transitions or stabilities in the slope of a function were identified through first-derivative analysis (Dado and Allen, 1993). This methodology explicitly considers drinking to be a part of meals, has recently been validated, and differs from conventional meal pattern analysis (Inoue et al., 2003; Zorrilla et al., 2005). A prior study using this apparatus and diet revealed that the threshold meal interval for Wistar rats was 5 and 10 min between food or water responses for nocturnal and diurnal intake, respectively (Zorrilla et al., 2005).

The estimated threshold meal interval was used to calculate descriptive statistics of average nocturnal and diurnal meal structure. Parameters included the 1) total quantity (food intake), 2) total duration of prandial intake, 3) meal frequency (the number of meals), 4) average meal size, 5) average meal duration, and 6) response rate of meals. Meal duration was calculated as the total time from the first to last response of a meal, and duration of eating within the meal was calculated as the duration of consecutive responses for food. Thus, transitions between eating and drinking were included in total meal duration but not in the duration of eating. Meal sizes for eating were calculated as the average number of food-directed responses during meals. Rates of eating were calculated by dividing each meal size by food duration. In the absence of experimental treatments, rats normally exhibit remarkable stability in these measures of meal patterning (calculated as two-way random effect intraclass correlation of absolute agreement) (Shrout and Fleiss, 1979) average ICC(3,4) = 0.77 across 3 weeks of testing (Zorrilla et al., 2005).

Results

Nicotine IVSA Behavior. Figs. 2 and 3 display responses and nicotine intake, respectively, in rats allowed extended access to nicotine IVSA for 40 days. The overall analysis of these measures revealed significant dose × time interaction effects for both nicotine responses ($F_{78,1404} = 4.2$; $p < 0.001$) and nicotine intake ($F_{78,1404} = 2.4$; $p < 0.001$). These effects seemed to be due to dose-dependent differences over time that developed following the initial access to nicotine. Specifically, on the first day of drug access, rats receiving the 0.03-mg/kg dose exhibited higher levels of responding and intake relative to all other groups, and this effect dropped dramatically by day 4 of nicotine access (Fisher's test; $p < 0.05$). All groups exhibited a main effect of time with decreases in nicotine responding (main effect of time: $F_{39,1404} = 2.8$; $p < 0.0001$) and intake (main effect of time: $F_{39,1404} =$

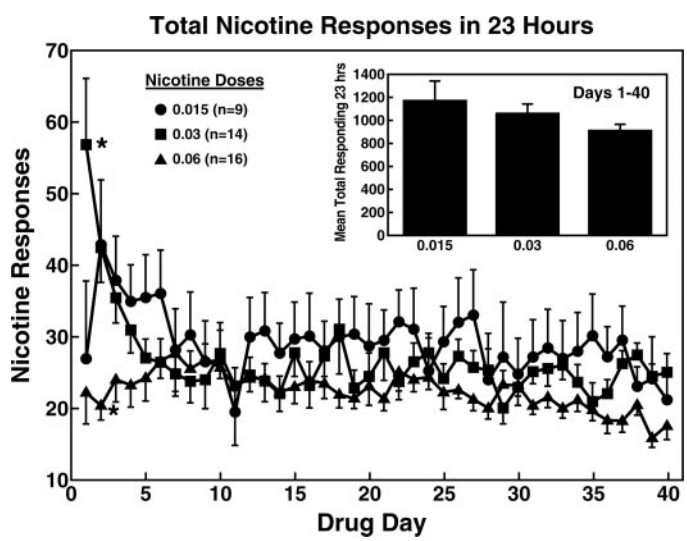

Fig. 2. Responding on the nicotine lever (mean ± S.E.M.) across days 1 to 40 in separate groups of rats allowed 23-h access to nicotine (0.015, 0.03, or 0.06 mg/kg/0.1 ml infusion) for 40 days. The inset represents the cumulative responses on the active lever from day 1 to day 40 of nicotine IVSA. There was no overall difference in responding across time in rats allowed access to different doses of nicotine. The asterisk reflects a significant difference relative to all other groups (Fisher's test; $p < 0.05$).

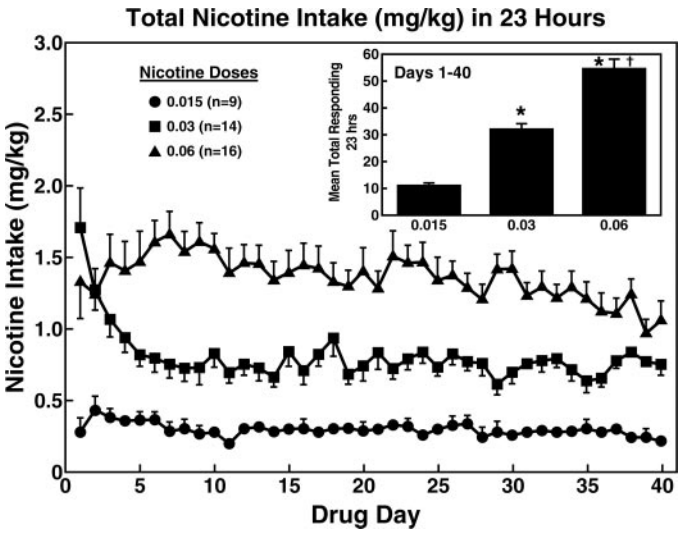

Fig. 3. Nicotine intake (mean ± S.E.M. in milligrams per kilogram) across days 1 to 40 in separate groups of rats allowed 23-h access to nicotine IVSA (0.015, 0.03, or 0.06 mg/kg/0.1 ml infusion) for 40 days. The inset reflects cumulative nicotine intake from day 1 to day 40 of nicotine IVSA. Although there was no difference in responding across time, rats allowed access to higher nicotine doses exhibited significantly more nicotine intake. Asterisks (*) reflect a significant difference relative to the 0.015 dose (Fisher's test; $p < 0.05$). The dagger reflects a significant difference relative to the 0.03 dose.

2.7; $p < 0.0001$) being observed in all groups. Although the patterns across time were similar across the measures of responding and intake, we did observe a highly significant main effect of dose on nicotine intake (Fig. 3, inset; $F_{2,36} = 45.8$; $p < 0.0001$). Rats receiving the 0.06-mg/kg nicotine dose exhibited higher levels of intake relative to both other groups, and rats receiving the 0.03-mg/kg nicotine dose exhibited higher intake relative to the 0.015-mg/kg dose (Fisher's test; $p < 0.05$).

Nicotine Intake in the First Hour of Access. Fig. 4 reflects the first hour of nicotine intake in rats allowed extended access to nicotine IVSA for 40 days. The overall analysis of the first and last 5 days of nicotine IVSA revealed a main effect of dose ($F_{2,36} = 8.12$; $p < 0.001$), with rats receiving the lowest nicotine dose exhibiting lower levels of nicotine intake across all days. There also was a main effect of time ($F_{9,18} = 3.1$; $p < 0.001$), with intake increasing in the first 5 days in rats receiving the highest doses and leveling off by day 5 of nicotine access. The first 5 days were analyzed because responding was stable after the first 5 days of nicotine IVSA.

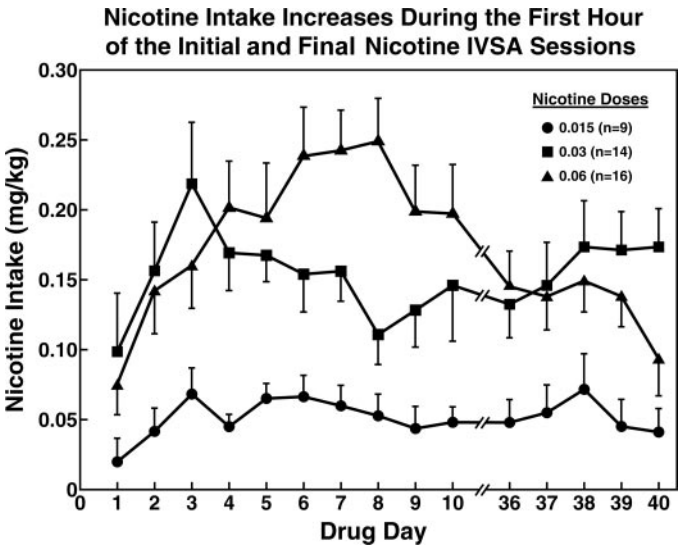

Fig. 4. Nicotine intake (mean ± S.E.M. in milligrams) in the first hour of each 23-h session across days 1 to 10 and days 36 to 40 of nicotine IVSA. Rats receiving the higher nicotine doses exhibited an increase in nicotine intake during the first hour of days 1 to 5 of nicotine IVSA. This increase in responding leveled off by day 5 and remained stable across the remaining nicotine IVSA sessions.

Circadian Pattern of Nicotine IVSA. Nicotine was self-administered in a circadian manner during the 21-h maintenance period, as reflected in good-to-excellent fits for the cosinor function (see r^2 values in Table 1). The highest rates of drug taking occurred approximately 13 h into the session, corresponding to the midpoint of the dark cycle (12:00 PM; see acrophase in Table 1). Higher unit drug doses led to increased average and maximum, but not minimum, rates of nicotine IVSA across the day, as reflected in main effects of dose for the MESOR and peak, but not nadir, respectively (Table 1; Fig. 5). Consequently, higher unit doses were associated with greater differences in nicotine intake between the light (nadir) and dark (peak) phase across the day (see amplitude in Table 1 and Fig. 5).

Following 5 weeks of extended access to nicotine IVSA (days 36–40), the circadian manner of drug intake changed significantly from the pattern observed during early acquisition (days 1–5), as reflected in main effects of time and time × dose interactions. First, nicotine IVSA became less circadian in nature, with 10 to 20% less of the variance in drug taking modeled by the cosinor function (see reduced r^2 values in Table 1). Second, mean and peak levels of drug intake decreased relative to the initial sessions (Fig. 5; Table 1; see MESOR and peak). In contrast, minimum levels of drug intake increased, especially in rats with access to higher unit doses of nicotine (Table 1, nadir). Accordingly, the difference in the rate of nicotine intake between the light phase and dark phase decreased significantly, especially in rats self-administering higher unit doses of nicotine (Fig. 5; Table 1; see amplitude). Thus, after 5 weeks of extended access to 0.03- or 0.06-mg/kg unit doses of nicotine, rates of nicotine IVSA became more consistent across the day (Fig. 5; Table 1; compare amplitude during days 1–5 with amplitude during days 36–40), as reflected in decreased peak and increased minimum (nadir) rates of intake. Time-course analyses indicated that changes in the circadian profile of nicotine intake across the day developed gradually, first becoming noticeable after approximately 2 to 3 weeks of extended nicotine IVSA access (Table 2), and continuing to change further from 3 to 5 weeks of access.

Importantly, increases in the consistency and nadir of responding for nicotine were behaviorally specific. The inactive lever responses during the "maintenance" phase of responding are presented in direct comparison with the active responses in Table 2. A comparison of these measures from the data presented in Table 2 reveals a consistent 2:1 ratio for the MESOR as well as the amplitude. The nicotine-maintained responding is both greater in magnitude (2:1) and different in time course from that on the inactive lever. Furthermore, unlike responding at the active, nicotine-associated lever, the nadir and amplitude of responding at the inactive lever were unchanged after 5 weeks in rats self-

TABLE 1

Mean ± S.E.M. estimates for selected measures of circadian drug-intake (milligrams per kilogram) rhythm in rats allowed 23-h access to nicotine IVSA

Amplitude, MESOR, peak, and nadir are expressed as micrograms of drug intake per kilogram of body weight per 3 h. Acrophase is expressed as hours following the onset of the test session, which was initiated 7 h before the onset of the dark cycle. Pearson correlations (r) reflect the relation of cumulative drug intake to the change in the circadian parameter from days 1 to 5 to days 36 to 40, calculated as a difference score (final − initial).

	Amplitude	MESOR	Acrophase	Peak	Nadir	Goodness of Fit
						r^2
0.015 Dose						
Days 1 to 5	1.8 ± 0.4	1.2 ± 0.2	13.2 ± 0.2	3.0 ± 0.6	−0.6 ± 0.2	0.86 ± 0.02
Days 36 to 40	1.3 ± 0.2	0.8 ± 0.1	13.0 ± 0.3	2.1 ± 0.3	−0.5 ± 0.1	0.71 ± 0.05
0.03 Dose						
Days 1 to 5	5.3 ± 0.5*	3.7 ± 0.3	12.1 ± 0.3	9.0 ± 0.8	−1.6 ± 0.2*	0.78 ± 0.02
Days 36 to 40	2.7 ± 0.5*†	2.3 ± 0.3	12.9 ± 0.5	5.1 ± 0.7	−0.4 ± 0.3†	0.63 ± 0.07
0.06 Dose						
Days 1 to 5	6.7 ± 0.8*	4.5 ± 0.6	13.0 ± 0.3	11.2 ± 1.4	−2.1 ± 0.3*	0.72 ± 0.04
Days 36 to 40	3.2 ± 0.4*†	3.6 ± 0.3	13.2 ± 0.4	6.8 ± 0.6	0.4 ± 0.4†	0.61 ± 0.05
Overall interaction	$F_{2,36} = 3.5$; $p < 0.04$	$F_{2,36} = 1.0$; p = N.S.	$F_{2,36} = 1.4$; p = N.S.	$F_{2,36} = 2.2$; p = N.S.	$F_{2,36} = 6.1$; $p < 0.005$	$F_{2,36} = 0.1$; p = N.S.
Main effect time	$F_{1,36} = 24.7$, $p < 0.0001$	$F_{1,36} = 11.8$, $p < 0.001$	$F_{1,36} = 1.4$, p = N.S.	$F_{1,36} = 21.5$, $p < 0.0001$	$F_{1,36} = 20.2$, $p < 0.0001$	$F_{1,36} = 11.5$, $p < 0.001$
Main effect dose	$F_{2,36} = 14.6$; $p < 0.0001$	$F_{2,36} = 20.3$; $p < 0.0001$	$F_{2,36} = 1.2$; p = N.S.	$F_{2,36} = 18.1$; $p < 0.0001$	$F_{2,36} = 0.8$; p = N.S.	$F_{2,36} = 2.4$; p = N.S.
Correlation of cumulative nicotine intake to changes in circadian drug-taking (r)						
Overall	−0.62‡‡‡	−0.33‡	0.00	−0.55‡‡‡	0.63‡‡‡	0.07
0.06 Dose	−0.62‡‡	−0.53‡	0.03	−0.62‡	0.48‡	0.17

* Significant difference from the lowest nicotine dose at the same time point.
† Significant difference from days 1 to 5 (Fisher's test; $p < 0.05$)
‡, ‡‡, ‡‡‡ Correlation that differs from 0 at $p < 0.06$, $p < 0.005$, and $p < 0.0002$, respectively.

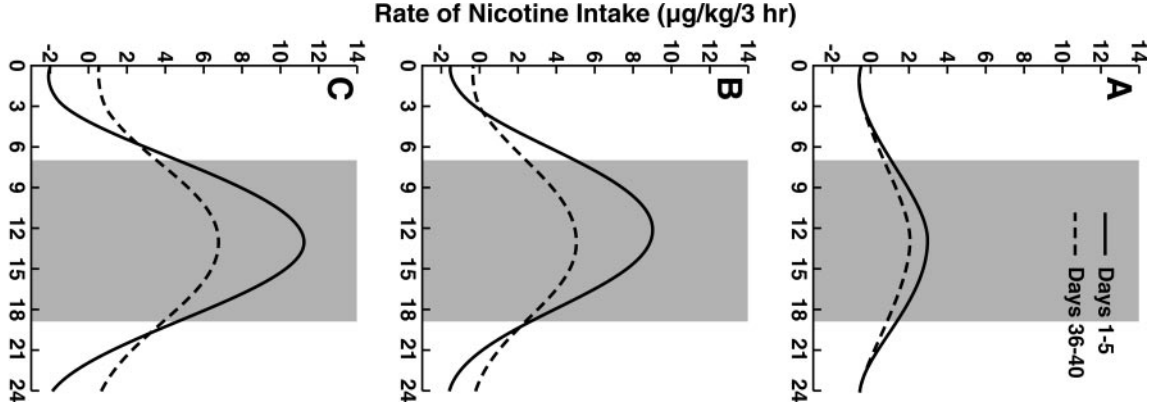

Fig. 5. Depicted here is the comparison of cosinor functions for nicotine intake on days 1 to 5 and days 36 to 40. A, 0.015-mg/kg dose. B, 0.03-mg/kg dose. C, 0.06-mg/kg dose.

administering the 0.06-mg/kg unit dose of nicotine (Table 2). Thus, rats differentially regulated responding at the active versus inactive levers across the day following extended nicotine access. Correlational analysis supported the interpretation that changes in how a rat maintained nicotine IVSA across the day were related to its cumulative history of nicotine intake. As shown in Table 1 and Fig. 5, rats that self-administered more nicotine across the 40 days of testing exhibited not only greater decreases in peak and mean (MESOR) rates of nicotine IVSA but also greater increases in minimum (nadir) rates of nicotine IVSA. Consequently, rats with a history of greater cumulative intake exhibited the largest reductions in circadian amplitude, reflecting an increased consistency of nicotine IVSA across the day relative to their initial pattern of drug-taking (Table 1, bottom). Correlations were of similar magnitude when analyses were limited to the 0.06-mg/kg dose, indicating that relations truly

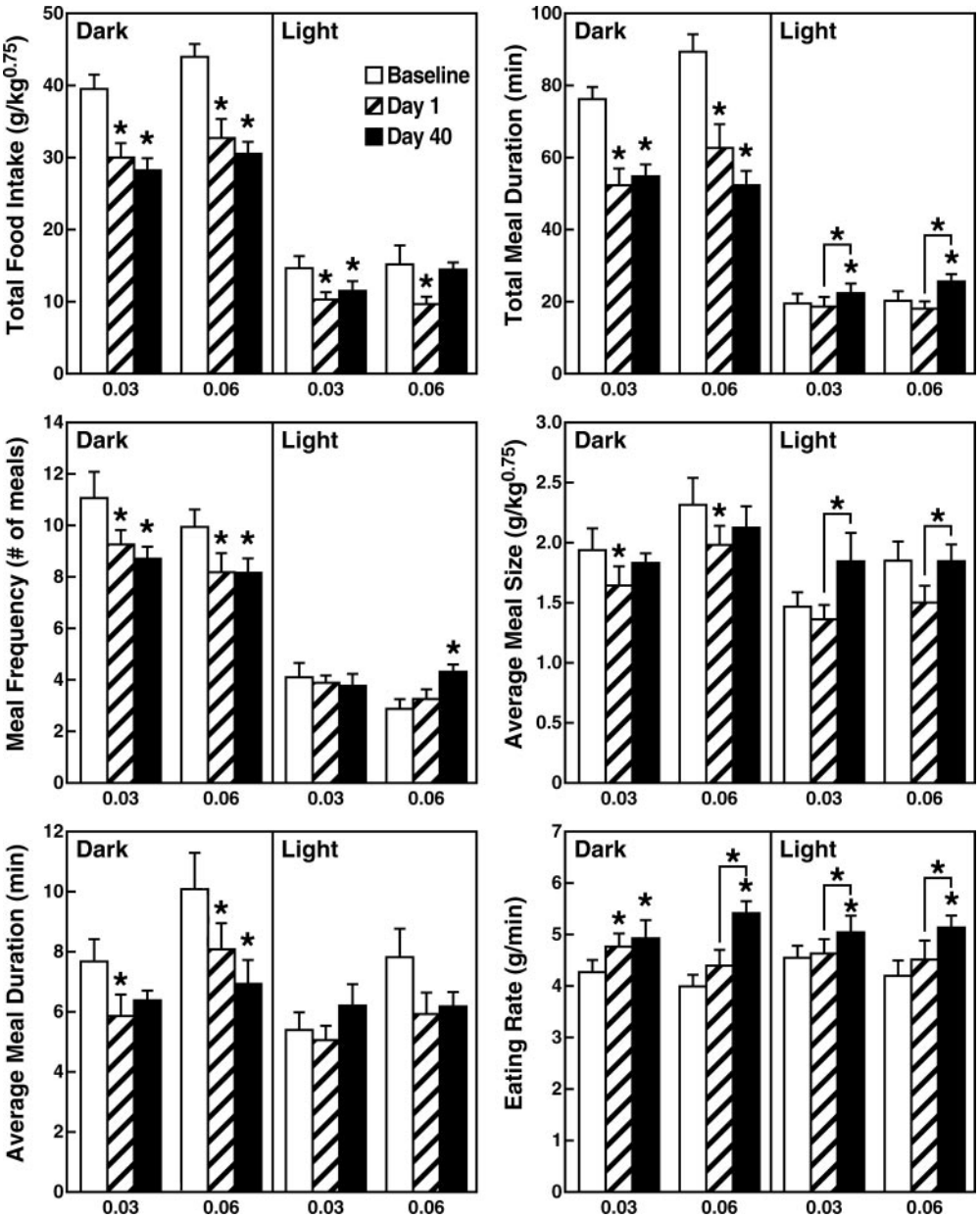

Fig. 6. Total food intake, total meal duration, meal frequency, average meal size, average meal duration, and eating rate during baseline (4 days before nicotine IVSA) and on day 1 and day 40 of nicotine IVSA. Asterisks (*) reflect a significant difference relative to baseline, or, if indicated, day 1 of nicotine IVSA (Newman-Keuls comparisons; $p < 0.05$).-

reflected individual differences in drug intake and not only differences in unit dose (Table 1, bottom).

Circadian Pattern of Food Intake. Food was consumed in a circadian manner during the 21-h observation period, with the majority of variance in intake modeled by a cosinor function (see r^2 values in Table 3). The highest rates of feeding were observed 10 to 12 h into the session, or 3 to 5 h into the dark cycle, and preceded the peak in nicotine intake (compare Figs. 5 and 7 and Tables 2 and 3, acrophase). On the initial day of access to intravenous nicotine IVSA, peak and mean rates of feeding dose-dependently decreased, as reflected in significant effects of time and time × dose (see Table 3, acute effect for MESOR and peak). Post hoc tests showed acute reductions in the peak and MESOR of feeding specifically in rats self-administering 0.03- and 0.06-mg/kg unit nicotine doses, changes also reflected as a blunting of the circadian feeding amplitude (Fig. 7; Table 3).

After 40 days of extended access to nicotine IVSA, the circadian pattern of feeding differed significantly from that observed when rats were drug-naive. Mean and peak levels of feeding were lower in rats across all unit doses (Fig. 7; Table 3; see repeated effect for MESOR and peak). Perhaps more interesting, pairwise comparisons indicated that food intake became less tightly modeled by a circadian rhythm in rats self-administering the highest unit nicotine dose (Table 3, decreased r^2). In addition, the amplitude of food intake was blunted at higher unit nicotine doses, accompanied by an increase in the nadir of food intake at the 0.06-mg/kg unit dose. Within-subjects pairwise comparisons did not indicate similar changes in the amplitude, nadir, or circadian quality of feeding in rats following 40 days of extended access to the 0.015-mg/kg unit dose of nicotine (Fig. 7; Table 3).

Time-course analyses of changes in the circadian pattern of feeding (averages of days 11–12, 14–15, and 20–21) showed that after the initial actions of nicotine (day 1), the diurnal rhythm of feeding actually returned to a more baseline-like

TABLE 2

Mean ± S.E.M. estimates for selected measures of the circadian rhythm of responding on the active and inactive lever in rats allowed 23-h access to the highest nicotine IVSA dose

Amplitude, MESOR, peak, and nadir are expressed as responses per 3 h. Acrophase is expressed as hours following the onset of the test session, which was initiated 7 h before the onset of the dark cycle.

0.06 Dose	Amplitude	MESOR	Acrophase	Peak	Nadir	Goodness of Fit (r^2)
Active						
Days 1 to 5	1.11 ± 0.14*	0.76 ± 0.09	13.1 ± 0.3	1.87 ± 0.23	−0.36 ± 0.06*	0.72 ± 0.04
Days 11 to 12	0.94 ± 0.10	0.81 ± 0.07	13.2 ± 0.4	1.75 ± 0.15	−0.12 ± 0.09	0.62 ± 0.04
Days 14 to 15	0.79 ± 0.09	0.74 ± 0.08	13.4 ± 0.7	1.53 ± 0.14	−0.05 ± 0.09	0.70 ± 0.05
Days 20 to 21	0.78 ± 0.10	0.67 ± 0.07	12.9 ± 0.3	1.45 ± 0.15	−0.10 ± 0.07	0.63 ± 0.05
Days 36 to 40	0.53 ± 0.06†	0.60 ± 0.06	13.2 ± 0.4	1.13 ± 0.12	0.07 ± 0.06*†	0.61 ± 0.05
Inactive						
Days 1 to 5	0.55 ± 0.11	0.40 ± 0.07	13.0 ± 0.4	0.95 ± 0.18	−0.15 ± 0.05	0.59 ± 0.05
Days 36 to 40	0.49 ± 0.12	0.30 ± 0.06	11.9 ± 1.1	0.80 ± 0.18	−0.19 ± 0.06	0.59 ± 0.05
Overall interaction	$F_{1,30} = 6.8$; $p < 0.01$	$F_{1,30} = 0.3$; $p =$ N.S.	$F_{1,30} = 1.0$; $p =$ N.S.	$F_{1,30} = 3.7$; $p =$ N.S.	$F_{1,30} = 13.4$; $p < 0.001$	$F_{1,30} = 1.1$; $p =$ N.S.
Main effect time	$F_{1,30} = 10.1$; $p < 0.003$	$F_{1,30} = 4.6$; $p < 0.04$	$F_{1,30} = 0.7$; $p =$ N.S.	$F_{1,30} = 8.6$; $p < 0.01$	$F_{1,30} = 9.5$; $p < 0.004$	$F_{1,30} = 1.2$; $p =$ N.S.
Main effect lever	$F_{1,30} = 6.7$; $p < 0.01$	$F_{1,30} = 14.6$; $p < 0.001$	$F_{1,30} = 1.0$; $p =$ N.S.	$F_{1,30} = 10.1$; $p < 0.003$	$F_{1,30} = 0.3$; $p =$ N.S.	$F_{1,30} = 1.1$; $p =$ N.S.

N.S., not significant.
* Significant difference from the inactive lever at that time point.
† Significant difference from days 1 to 5 (Fisher's test; $p < 0.05$).

pattern by days 11 to 12 of nicotine access. Subsequently, however, the rhythm of feeding changed from the second week of nicotine access (days 14–15) through the end of the third week of nicotine access (days 20–21) to a profile that resembled that observed on day 40. The chronic actions were reflected as a significant reduction, relative to baseline levels, in the amplitude, MESOR, and peak of feeding, as well as a progressive decrease in the degree to which feeding was modeled by a cosinor function. Changes in each of these measures followed a stepwise progression from days 11 to 12 to days 14 to 15 to days 20 to 21 (Table 3).

Meal Pattern Analysis. Meal pattern analyses also were performed on the diurnal and nocturnal intake profiles of rats receiving the two highest doses of nicotine (Fig. 6). Overall, rats exhibited higher levels of food intake during the nocturnal phase of their light cycle relative to their diurnal phase. Decreases in the total nocturnal quantity (Fig. 6, top left; $F_{2,54} = 50.9$; $p < 0.001$) and duration (Fig. 6, top right; $F_{2,54} = 44.4$; $p < 0.001$) of prandial food intake were observed. Nicotine self-administering rats took about two fewer meals per night (Fig. 6, middle left; $F_{2,54} = 6.64$; $p < 0.003$), going approximately 10 min longer between meals. When taken, meals were smaller (Fig. 6, middle right; $F_{2,54} = 2.97$; $p < 0.06$) and 2 to 3 min briefer (Fig. 6, bottom left; $F_{2,54} = 8.77$; $p < 0.001$) in feeding. The overall decrease in nocturnal food intake was accompanied by an increase in the eating rate (Fig. 6, bottom right; $F_{2,54} = 20.9$; $p < 0.001$). These findings indicate that although nicotine produced a decrease in food intake, the rate at which the meals were eaten was faster, consistent with the appetite-suppressant and stimulant effects of nicotine, respectively.

During the diurnal phase, the quantity of food intake initially decreased on the first day of nicotine IVSA access (Fig. 6, top left; $F_{2,54} = 8.29$; $p < 0.001$). However, the quantity and duration of diurnal feeding subsequently recovered and increased, respectively (Fig. 6, top left; $F_{2,54} = 5.69$; $p < 0.006$), accounted for by increases in meal size ($F_{2,54} = 4.22$; $p < 0.02$), and, in rats self-administering the 0.06-mg/kg dose, meal frequency (Fig. 6; time × dose: $F_{2,54} = 3.01$; $p < 0.05$). In general, increases in food intake were observed between day 1 and day 40 of nicotine IVSA, perhaps suggesting that rats came to compensate for low nocturnal food intake by eating more during the diurnal phase of their cycle. As was observed during the dark cycle, nicotine self-administering rats ultimately came to eat faster within meals during the light cycle (Fig. 6, bottom right; $F_{2,54} = 5.92$; $p < 0.005$).

Mecamylamine-Precipitated Withdrawal. Fig. 8 reflects the correlation of total nicotine exposure in the 40 days of nicotine IVSA with overt signs of mecamylamine-precipitated withdrawal in rats self-administering various unit doses of nicotine. The analysis of these data was performed on individual subject data, but the scatterplot is presented as means for clarity. Table 4 illustrates the individual basal and mecamylamine-precipitated withdrawal signs for the different nicotine IVSA groups. Mecamylamine produced an overall dose-dependent increase in total overt signs of precipitated withdrawal relative to baseline measures (dose × time; $F_{2,36} = 4.0$; $p < 0.03$) in rats allowed 40 days of 23-h nicotine access. An analysis of mecamylamine-precipitated withdrawal signs revealed that rats that had received the 0.06-mg/kg nicotine dose exhibited significantly more withdrawal

TABLE 3

Mean ± S.E.M. estimates for selected measures of circadian feeding rhythm in rats allowed 23-h access to nicotine IVSA

Amplitude, MESOR, peak, and nadir are expressed as grams food intake per kilogram 0.75 body weight to account for changes in metabolic need with changing body mass over time per Kleiber's law. Acrophase is expressed as hours following the onset of the test session, which was initiated 7 h before the onset of the dark cycle.

	Amplitude	MESOR	Acrophase	Peak	Nadir	Goodness of Fit
						r^2
0.015 Dose						
Baseline	25.6 ± 1.6	29.3 ± 1.0	11.7 ± 0.3	54.9 ± 1.8	2.8 ± 2.1	0.65 ± 0.05
Day 1	24.5 ± 2.3	28.5 ± 1.3	11.4 ± 0.2	53.0 ± 2.8	0.2 ± 2.8	0.67 ± 0.07
Day 40	22.1 ± 2.4	23.9 ± 1.1††	11.5 ± 0.3	46.1 ± 1.8†	5.1 ± 1.8	0.68 ± 0.04
0.03 Dose						
Baseline	24.1 ± 1.9	29.2 ± 0.7	10.5 ± 0.2	53.3 ± 2.3	5.2 ± 1.8	0.66 ± 0.04
Day 1	18.3 ± 1.9†	23.3 ± 1.2*†	9.7 ± 0.5	41.7 ± 2.5*†	5.0 ± 2.0	0.65 ± 0.06
Day 40	17.6 ± 2.0†	23.9 ± 1.1†	10.7 ± 0.4	41.7 ± 1.9†	6.5 ± 2.34	0.54 ± 0.06
0.06 Dose						
Baseline	27.1 ± 1.6	29.3 ± 1.3	12.5 ± 0.4	56.3 ± 2.5	2.2 ± 1.6	0.65 ± 0.05
Day 1	19.4 ± 2.3†	24.8 ± 1.3†	11.8 ± 0.6	44.2 ± 3.2†	5.5 ± 1.8	0.56 ± 0.04
Day 11–12	25.2 ± 1.6	26.8 ± 1.0†	11.9 ± 0.5	52.0 ± 1.8†	1.6 ± 2.1	0.60 ± 0.05
Day 14–15	22.9 ± 1.9†	25.4 ± 0.8†	11.5 ± 0.5	48.3 ± 1.9†	2.5 ± 2.2	0.59 ± 0.04
Day 20–21	21.7 ± 2.0†	22.6 ± 1.5†	12.1 ± 0.4	41.9 ± 3.1†	3.4 ± 1.9	0.54 ± 0.05†
Day 40	18.4 ± 2.2†	25.6 ± 0.9†	10.4 ± 0.8	44.0 ± 2.7†	7.2 ± 2.1†	0.49 ± 0.07†
Acute effect: Baseline versus Day 1						
Overall interaction	$F_{2,36} = 2.3$; p = N.S.	$F_{2,36} = 3.5$; $p < 0.04$	$F_{2,36} = 0.25$; p = N.S.	$F_{2,36} = 4.0$; $p < 0.03$	$F_{2,36} = 1.7$; p = N.S.	$F_{2,36} = 0.8$; p = N.S.
Main effect of time	$F_{1,36} = 16.1$; $p < 0.003$	$F_{1,36} = 24.4$; $p < 0.0001$	$F_{1,36} = 3.3$; p = N.S.	$F_{1,36} = 31.4$; $p < 0.0001$	$F_{1,36} = 0.01$; p = N.S.	$F_{1,36} = 0.5$; p = N.S.
Main effect of dose	$F_{2,36} = 1.1$; p = N.S.	$F_{2,36} = 1.4$; p = N.S.	$F_{2,36} = 10.8$; $p < 0.0002$	$F_{2,36} = 1.6$; p = N.S.	$F_{2,36} = 1.0$; p = N.S.	$F_{2,36} = 0.6$; p = N.S.
Repeated effect:baseline versus day 40						
Overall interaction	$F_{2,36} = 1.2$; p = N.S.	$F_{2,36} = 0.5$; p = N.S.	$F_{2,36} = 2.9$; p = N.S.	$F_{2,36} = 0.4$; p = N.S.	$F_{2,36} = 1.2$; p = N.S.	$F_{2,36} = 1.6$; p = N.S.
Main effect of time	$F_{1,36} = 21.7$; $p < 0.0001$	$F_{1,36} = 37.4$; $p < 0.0001$	$F_{1,36} = 2.2$; p = N.S.	$F_{1,36} = 46.9$; $p < 0.0001$	$F_{1,36} = 1.2$; p = N.S.	$F_{1,36} = 4.2$; $p < 0.05$
Main effect of dose	$F_{2,36} = 0.75$; p = N.S.	$F_{2,36} = 0.32$; p = N.S.	$F_{2,36} = 2.0$; p = N.S.	$F_{2,36} = 0.7$; p = N.S.	$F_{2,36} = 0.85$; p = N.S.	$F_{2,36} = 1.0$; p = N.S.

* Significant difference from the lowest nicotine dose at that time point.

† Significant difference from baseline (Fisher's test for day 40; $p < 0.05$ and Bonferroni-corrected Student's paired t test for follow-up time-course analysis of days 11 to 12, 14 to 15, and 20 to 21.

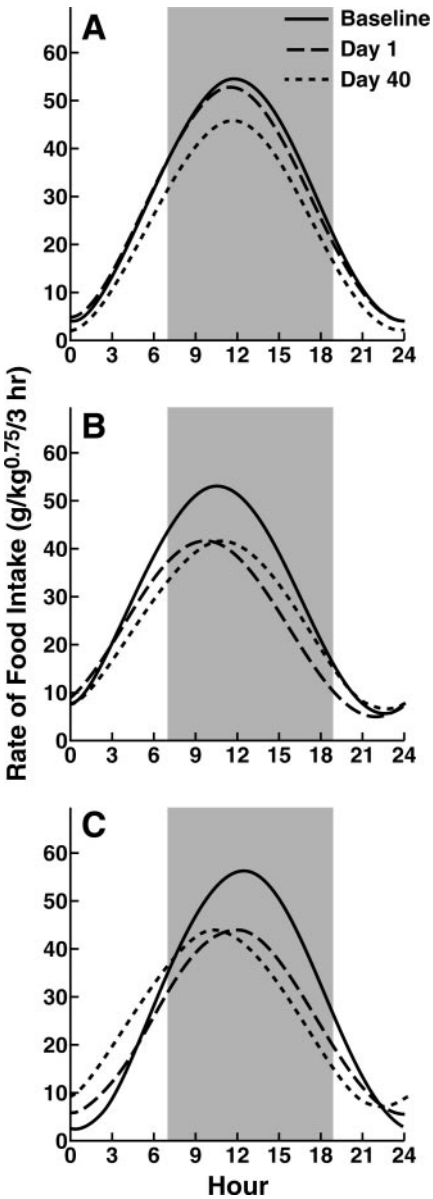

Fig. 7. Depicted here is the comparison of cosinor functions for food intake on days 1 to 5 and days 36 to 40. A, 0.015-mg/kg dose. B, 0.03-mg/kg dose. C, 0.06-mg/kg dose.

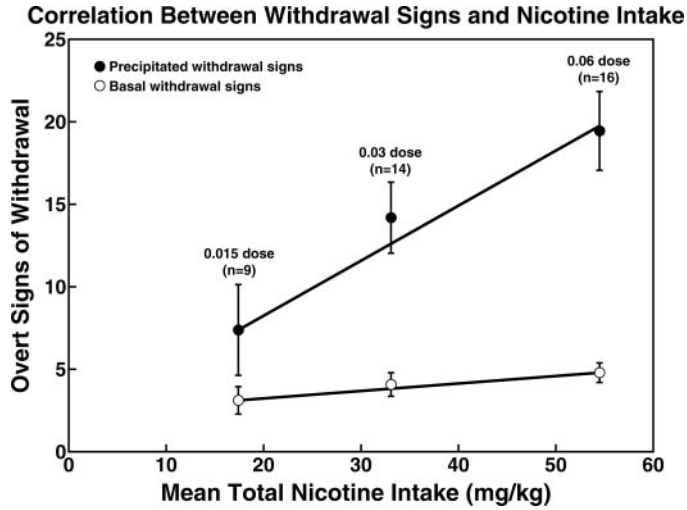

Fig. 8. Mecamylamine-precipitated nicotine withdrawal data are shown in correlation with mean total nicotine intake in rats that received 23-h nicotine IVSA sessions for 40 days. The analysis of these data was performed on individual subject data, but the scatter plot is presented as means for clarity. Total counted signs (mean) are compared with the total amount of nicotine that was self-administered. A positive linear correlation between total precipitated counted signs and total nicotine exposure was found ($r = 0.5$; $p < 0.02$).

signs relative to the 0.015-mg/kg nicotine dose. There was a slight trend for rats that had received the 0.03-mg/kg nicotine dose to exhibit more withdrawal signs relative to the lowest nicotine dose ($p < 0.08$). A correlational analysis revealed a significant positive correlation ($r = 0.5$; $p < 0.02$) between mean total nicotine exposure (i.e., total intake in the 40 days of IVSA) and total precipitated counted signs, indicating that increased nicotine exposure is associated with increased levels of precipitated withdrawal consistent with previous findings (Paterson and Markou, 2004).

Extinction of Nicotine-Seeking Behavior. Fig. 9 reflects responding during the last 5 days of IVSA and during the subsequent extinction phase where nicotine was replaced with saline and animals' responses resulted in the presentation of drug cues for 5 days and then for an additional 5 days where the drug cues were no longer presented. An overall analysis comparing the dose groups across the last 5 days of nicotine IVSA and the 10 extinction days revealed a dose-dependent reduction in responding on the nicotine lever across time (dose × time: $F_{20,360} = 2.5$; $p < 0.001$). An analysis comparing the average of the last 5 days of nicotine IVSA and the first day of extinction revealed a dose-dependent reduction in responding on the nicotine lever (dose × time; $F_{2,36} = 8.3$; $p < 0.001$), and this effect was significant at the 0.015- and 0.03-mg/kg nicotine dose (Fisher's test; $p < 0.05$). In contrast, a significant reduction in responding on the nicotine lever was delayed until the second day of extinction in animals receiving the 0.06-mg/kg dose. These animals also exhibited a significantly higher level of nicotine lever responding on day 10 of extinction. Extinction was facilitated by removal of the drug-associated cues in all groups (Fisher's test; $p < 0.05$).

Discussion

This study provides new information regarding various measures that can serve as markers of the development of dependence in rats allowed extended 23-h access to various doses of nicotine IVSA for 40 days. Rats allowed 23-h access to nicotine exhibited dose-dependent decreases in lever responding as the unit dose of nicotine was increased. The rate of nicotine intake became more regular and less circadian over time, especially in rats that self-administered the highest amount of nicotine, evident in relation both to unit dose and individual total intake. Nicotine IVSA initially decreased both nocturnal and diurnal feeding relative to prenicotine feeding levels, but with long-term nicotine IVSA of higher unit doses, diurnal feeding normalized in quantity and increased in duration. Mecamylamine precipitated robust somatic signs of nicotine withdrawal, and this effect was positively correlated with the amount of nicotine self-administered. Extinction of nicotine-seeking behavior was observed, and this effect was facilitated by the removal of

TABLE 4
Mean ± S.E.M. precipitated signs of withdrawal during baseline and following a challenge injection of mecamylamine in rats allowed 23-h access to nicotine IVSA

	Total	Blink	Gasp	Writhe	Teeth chatter	Yawn	Head shake	Ptosis
0.015 Dose								
Baseline	3.1 ± 0.8	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.3	2.1 ± 0.9	0	0.1 ± 0.1	0
Withdrawal	7.4 ± 2.8	2.3 ± 1.0	1.5 ± 0.7	3.0 ± 1.4	0.2 ± 0.15	0	0.2 ± 0.1	0.1 ± 0.1
0.03 Dose								
Baseline	4.1 ± 0.7	2.5 ± 0.5	0.8 ± 0.3	0.1 ± 0.1	0.7 ± 0.3	0	0	0
Withdrawal	14.2 ± 2.2	7.5 ± 2.0	1.9 ± 0.3	1.2 ± 0.5	1.5 ± 0.6	1.8 ± 0.5	0.3 ± 0.2	0.1 ± 0.1
0.06 Dose								
Baseline	4.8 ± 0.6	2.5 ± 0.5	0.8 ± 0.3	0.6 ± 0.6	0.4 ± 0.2	0.6 ± 0.2	0.4 ± 0.2	0
Withdrawal	19.3 ± 2.4	8.8 ± 1.8	2.1 ± 0.4	2.2 ± 0.6	2.9 ± 0.6	2.1 ± 0.8	0.8 ± 0.4	0.4 ± 0.1

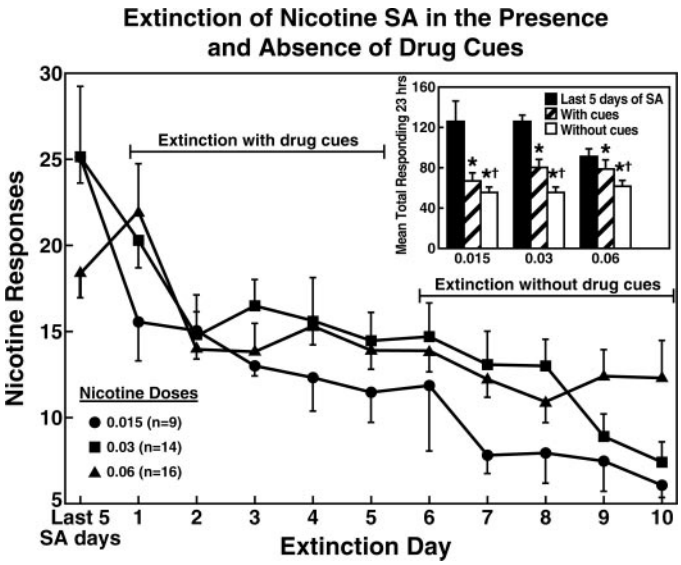

Fig. 9. Responding during the last 5 days of nicotine IVSA and during extinction where nicotine was replaced with saline for the first 5 days and then where the drug associated cues were removed. The inset reflects the average of responding over the last 5 days of nicotine IVSA and during each subsequent phase of extinction. Responding decreased by the first extinction day in rats receiving the two lowest doses and by the second extinction day in rats given access to the highest nicotine dose. Daggers (†) reflect a significant decrease from the last 5 days of nicotine IVSA (Fisher's test; $p < 0.05$). Responding was further decreased when the drug-associated cues were removed in all groups. Asterisks (*) reflect a significant difference relative to the average of the extinction condition where the drug cues were present (Fisher's test; $p < 0.05$).

drug-associated cues. In addition, extinction was slower in rats with higher nicotine intake.

Although rats exhibited less responding as the unit nicotine dose was increased, the change in responding was insufficient to compensate for changes in dose, and thus, nicotine intake increased as the unit dose of nicotine was increased. A similar pattern of behavior has been observed in previous studies using extended access to nicotine IVSA (Cox et al., 1984; Valentine et al., 1997; Denoble and Mele, 2006). Moreover, other laboratories using similar nicotine doses (0.00375–0.03 mg/kg/infusion) report an average daily nicotine intake (0.18–1.5 mg/kg/day; Donny et al., 1995; Rose and Corrigall, 1997; Paterson and Markou, 2004; Kenny and Markou, 2006) that is within the range observed in the present study (0.25–1.5 mg/kg/day), values that approximate the level of nicotine intake observed in human smokers (0.14–1.14 mg/kg/day; Benowitz and Jacob, 1984).

The circadian analysis of nicotine IVSA revealed that nicotine intake is the highest during the rats' night cycle, an effect that is consistent with previous reports (Cox et al., 1984; Valentine et al., 1997). Our findings extend previous work by demonstrating that, over time, nicotine intake becomes less circadian and more evenly distributed and consistent across the light and dark phases. The change toward greater stability was found to be most evident in rats that self-administered the most nicotine across the entire study, evident both as a function of unit nicotine dose and of individual levels of intake. Therefore, it seems that rats become motivated to maintain levels of nicotine intake within a circumscribed range across the light/dark phases. In confirmation of previous work (Paterson and Markou, 2004), rats that self-administered the most nicotine also exhibited the most mecamylamine-precipitated withdrawal signs, consistent with the hypothesis that increasing regularity of nicotine intake across light/dark phases is a marker of the development of nicotine dependence.

The acquisition of nicotine IVSA in the present study seemed to occur rapidly, because steady levels of nicotine intake were achieved within 5 days of nicotine IVSA. The acquisition of nicotine IVSA also occurred in the first hour of nicotine access, because rats exhibited an increase in intake in the first hour of the first five sessions. Previous acquisition curves for extended nicotine IVSA reflect an initial increase in total nicotine intake across the first 5 to 10 days (Valentine et al., 1997). However, the present study involved lever habituation for 5 days before nicotine access, and this procedure may account for the more rapid acquisition of stable nicotine self-administration in the present study.

Rats allowed extended access to other drugs of abuse, such as cocaine and heroin, exhibit an escalation of drug intake over time that is evident when examining the first hour of drug access across days (Ahmed and Koob, 1998; Ahmed et al., 2000; Chen et al., 2006). The present findings illustrate that rats allowed extended access to nicotine also exhibit escalation in the first hour by this definition, over time. However, unlike rats with extended access to heroin or cocaine (Ahmed and Koob, 1998; Ahmed et al., 2000), rats with 6- (Paterson and Markou, 2004), 12- (Kenny and Markou, 2006), or 23-h access to nicotine IVSA do not increase their total daily intake, and over 23 h they even decreased their peak rates of intake. The lack of an increase in total intake with nicotine is likely due to the fact that nicotine dependence is not characterized by increased intake over time (as it might be with cocaine or heroin) but by the ability to tightly regulate nicotine at a given level. The escalation that is seen in the first hour may reflect the initial learning at a given level of titration that occurs rapidly in the first 5 days of nicotine IVSA. Human smokers are readily able to titrate nicotine levels across the day, and they are readily able to

adjust puff rates based on changes in nicotine levels. Therefore, nicotine use may be based on maintaining a given level of nicotine that prevents or reverses aversive effects of nicotine withdrawal. The difference between nicotine versus cocaine and heroin may be due to toxic effects of nicotine that drive the user to delicately balance between positive and negative effects that are less pronounced with cocaine and heroin.

Nicotine IVSA initially (day 1) decreased the quantity and duration of feeding during both phases of the light/dark cycle. Acute anorexia was dose-related, not evident at the lowest 0.15-mg/kg unit dose, and reflected rats taking smaller and briefer meals, and, during the dark cycle, going longer between meals, resulting in a decreased meal frequency. The results differ from those previously observed following intermittent bolus nocturnal administration of nicotine (five daily i.p. injections at a 1.4–4 mg/kg/day dose for 1 week), which resulted in anorexia only during the dark phase and reportedly due only to decreases in meal size in rats maintained on low-fat diets (Bellinger et al., 2003; Wellman et al., 2005). Contrary to the present results, rats receiving noncontingent bolus nicotine doses exhibited increased meal frequency and shorter intermeal intervals (Bellinger et al., 2003). The current findings also differ from reported effects of continuous subcutaneous nicotine infusion (5–6 mg/kg/day), which also decreased food intake by reducing meal size but not meal frequency (Blaha et al., 1998; Miyata et al., 2001). Differences between studies may reflect differences in how meals were defined, with the current study using an empirical definition that recognized prandial drinking (Zorrilla et al., 2005). The actions of self-administered nicotine in the present study to prolong the intermeal interval are consistent with findings that intranasal nicotine enhances the satiety-promoting effects of a caloric premeal in human volunteers (Perkins et al., 2001), that smoking high-nicotine, but not low-nicotine, cigarettes delays gastric emptying in smokers (Gritz et al., 1988), and that chewing nicotine gum slows the mouth-to-cecum transit of a liquid meal in smokers (Scott et al., 1992).

Long-term IVSA of higher unit nicotine doses changed the microstructure of feeding differently from acute nicotine IVSA or from long-term administration of low unit doses. First, diurnal feeding increased in quantity and duration, changes that reflected larger and more frequent meals being taken during the light cycle. Second, the circadian quality of feeding was disrupted in rats that self-administered higher unit nicotine doses during the 40 days, evident in both a decreased fit of the cosinor function and a blunted difference between the extremes of the dark and light phases. Nicotine IVSA exerted two phases of action on the circadian regulation of feeding, an acute action from which the rhythm of feeding had fully normalized within 10 days, and a delayed action that began after ~2 weeks of access and progressed through 3 weeks of access to a profile that resembled that observed after almost 6 weeks of access. The findings did not reflect a general change in behavioral activity patterns, because responding at the inactive lever did not become less circadian or blunted in amplitude across light-dark phases. Similar changes in the biorhythm and microstructure of feeding result from chronic passive or self-administration of heroin in quantities sufficient to induce physical dependence, which has led to the resulting food intake patterns being proposed as markers of opioid dependence (Chen et al., 2006). Here, the relevant adaptations in feeding again were observed in rats that took nicotine in quantities sufficient to induce dependence (e.g., 0.06-mg/kg group), as reflected in increased somatic signs, and dose-relatedly less so in those that developed fewer or no somatic withdrawal signs. These results suggest that changes in food intake patterns also may serve as markers of the development of nicotine dependence in this model.

Mecamylamine precipitated robust somatic signs of withdrawal following the last nicotine IVSA session, and the magnitude of this effect was positively correlated with the total amount of nicotine that was self-administered. Dependence was observed in the present study in rats that received the 0.06-mg/kg dose, and these animals received 54.5 mg/kg total nicotine, as well as in rats self-administering the 0.03-mg/kg dose, which received 32.4 mg/kg total nicotine. These amounts of nicotine may reflect a minimum level of nicotine to which the rat must be exposed to produce somatic signs of nicotine dependence because rats receiving the 0.015-mg/kg dose did not exhibit mecamylamine-precipitated withdrawal signs, and they received only 15.6 mg of total nicotine (0.015 mg/kg $\times$ 25 presses each day for 40 days = 15 mg/kg of total nicotine). The number of withdrawal signs (19.3 $\pm$ 2.4) observed in rats receiving the highest nicotine dose is comparable with previous studies using passive (Malin et al., 1992; Hildebrand et al., 1999; Watkins et al., 1999; Markou and Paterson, 2001; Skjei and Markou, 2003; O'Dell et al., 2004) and active (Paterson and Markou, 2004) nicotine administration. These studies included groups of animals that received approximately 1 to 6.2 mg/kg/day nicotine via osmotic minipumps for 7 days or longer (i.e., approximately 7.0–50 mg/kg total nicotine for 7 days) and animals self-administering 0.88 $\pm$ 0.06 mg/kg/day nicotine (Paterson and Markou, 2004).

The present study illustrated that extinction of nicotine-seeking behavior was observed following the replacement of nicotine with saline, consistent with previous reports using extended access to nicotine IVSA in female (Cox et al., 1984) and male rats (Denoble and Mele, 2006). The rate of extinction was dose-dependent, because rats receiving the higher doses of nicotine exhibited more lever pressing during extinction relative to rats receiving lower doses of nicotine. Furthermore, rats receiving the highest dose of nicotine exhibited the greatest degree of dependence, as measured by mecamylamine-precipitated signs of withdrawal. Thus, higher levels of responding during extinction may also serve as a marker of dependence. The decrease in responding following cue removal may be confounded by the passage of time, therefore, previous nicotine exposure may affect both response-nicotine and cue-nicotine associative strength but not necessarily both.

Previous studies have found that escalation of heroin or cocaine intake is associated with increased incentive motivational effects of drug-associated cues (Ahmed et al., 2000; Paterson and Markou, 2003). Nonetheless, an early study also found that rats that previously self-administered the highest unit dose of heroin exhibited the greatest rates of responding for a previously heroin-associated cue in the absence of physical dependence (Davis and Smith, 1976). Furthermore, in accordance with previous work (Cox et al., 1984; Denoble and Mele, 2006), we found that extinction rates were

further decreased when responding on the nicotine-associated lever did not result in presentation of the nicotine-associated stimulus (light and pump noise). This finding is consistent with the greater levels of responding observed throughout extinction in rats that continue to be presented with previously nicotine-associated cues versus cue-absent controls (Caggiula et al., 2001) and with the growing literature illustrating the importance of drug-associated cues in the maintenance of nicotine IVSA behavior (Chaudhri et al., 2006). For example, nicotine IVSA is more robust in the presence of cues that predict nicotine availability (Caggiula et al., 2001; Cohen et al., 2005), and these cues can serve as powerful secondary reinforcers that reinstate extinguished nicotine-seeking behavior (LeSage et al., 2004; De Vries et al., 2005; Paterson et al., 2005).

In summary, the present study revealed that key changes in drug and food intake serve as markers of the development of nicotine dependence in rats given 23-h access to nicotine. Specifically, there was increased regularity of nicotine intake across the light and dark phases that replaced the early pattern of nicotine intake primarily during the dark cycle. Such a change in the pattern of nicotine intake may serve as an early indicator of the development of nicotine dependence. During the early days of nicotine exposure, there was a decrease in food intake reflecting the anorexogenic effects of nicotine seen also in humans (Jo et al., 2002). These anorexogenic effects were reflected in reductions in both meal size and meal frequency and are consistent with prior clinical reports that nicotine augments satiety (Bray, 2000). However, long-term decreases in the circadian profile and amplitude of feeding, especially increased meal taking during the diurnal phase of the cycle, were uniquely seen in the group of subjects that self-administered the most drug and that exhibited the most somatic signs of nicotine dependence upon administration of the nicotinic acetylcholine receptor antagonist mecamylamine. Finally, the present results showing somatic signs of nicotine withdrawal and resistance to extinction in the high-dose group provide strong support for the hypothesis that unlimited access to nicotine leads to drug dependence.

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