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## Differential effects of withdrawal from intermittent and continuous nicotine exposure on reward deficit and somatic aspects of nicotine withdrawal and expression of $\alpha 4\beta 2^*$ nAChRs in Wistar male rats

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### Abstract

**Background**—Chronic nicotine exposure produces neuroadaptations in brain reward systems and  $\alpha 4\beta 2$  nicotinic acetylcholine receptors (nAChRs) in the corticolimbic brain areas. We previously demonstrated opposite effects of nicotine exposure delivered by self-administration or pumps on brain reward thresholds that can be attributed to the different temporal pattern and contingency of nicotine exposure. We investigated the effects of these two factors on reward thresholds and somatic signs during nicotine withdrawal, and on nAChRs binding in corticolimbic brain areas.

**Methods**—The intracranial self-stimulation procedure was used to assess reward thresholds in rats prepared with pumps delivering various doses of nicotine continuously or intermittently. Separate group of rats were randomly exposed to nicotine via pumps (non-contingent) or nicotine self-administration (contingent) to determine [<sup>125</sup>I]-epibatidine binding at  $\alpha 4\beta 2^*$  nAChRs.

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#### Authors contributions

SS, MJM and AM were responsible for the study concept and design. SS, XJ contributed to the acquisition of animal data. MJM, TDMCB, MPT contributed to the acquisition of molecular data. SS, XJ, MJM completed data analysis and interpretation of findings. SS drafted the manuscript. SS, AM and MJM provided critical revision of the manuscript for important intellectual content. All authors critically reviewed the content and approved the final version for publication.

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There are no actual or potential financial conflicts of interest.

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**Results**—Withdrawal from continuous non-contingent nicotine exposure led to significant elevations in thresholds and increases in somatic signs in rats, while there was no significant effect of withdrawal from intermittent non-contingent nicotine exposure at the same doses.  $\alpha 4\beta 2^*$  nAChRs were upregulated during withdrawal from continuous non-contingent nicotine exposure.  $\alpha 4\beta 2^*$  nAChRs were upregulated in the ventral tegmental area and prelimbic cortex during withdrawal from non-contingent intermittent exposure and in the nucleus accumbens during withdrawal from contingent intermittent nicotine exposure to the same dose.

**Conclusions**—During non-contingent nicotine exposure, the temporal pattern of nicotine delivery differentially affected thresholds and somatic signs of withdrawal. Upregulation of  $\alpha 4\beta 2^*$  nAChRs was brain site-specific and depended on both temporal pattern and contingency of nicotine exposure.

### Keywords

$\alpha 4\beta 2^*$  nicotinic acetylcholine receptors; reward thresholds; somatic signs; intracranial self-stimulation; cotinine

## 1. Introduction

Chronic exposure to nicotine or other drugs of abuse produces changes in brain reward circuits resulting in the development of dependence (Koob and Volkow, 2010, Markou, 2008). It is well accepted that nicotine dependence is maintained by both the positive reinforcing and reward enhancing effects of nicotine, as well as the motivational effects of reward deficits associated with nicotine withdrawal (D'Souza and Markou, 2011). Extensive animal research in our laboratory demonstrated that both contingent exposure to nicotine self-administration (Kenny and Markou, 2006) and non-contingent acute (Harrison et al., 2002, Lindblom et al., 2005) or chronic (Cryan et al., 2003, Paterson et al., 2007, Skjei and Markou, 2003) nicotine administration via osmotic minipumps lowered brain reward thresholds reflecting reward enhancing effects of nicotine. By contrast, cessation of chronic subcutaneous non-contingent nicotine exposure resulted in elevations in brain reward thresholds reflecting a negative affective state, as well as somatic signs of withdrawal (Epping-Jordan et al., 1998, Semenova and Markou, 2003, Skjei and Markou, 2003). Similarly, the termination of extended 6 h access to nicotine self-administration produced somatic signs of nicotine withdrawal indicating the development of dependence on nicotine (Paterson and Markou, 2004). However, withdrawal from contingent 6 h exposure to nicotine self-administration resulted in an enhancement of brain reward function for at least 36 days after cessation of nicotine exposure (Kenny and Markou, 2006). This finding was unexpected and in contrast to the effects of nicotine withdrawal after non-contingent exposure via minipumps (Epping-Jordan et al., 1998, Semenova and Markou, 2003, Skjei and Markou, 2003). The temporal pattern and contingency of nicotine exposure may contribute to differential effects on brain reward function.

Differential adaptation in nicotinic acetylcholine receptor (nAChR) function may also be involved in the opposite effects of contingent vs non-contingent nicotine exposure on brain reward function. To date, 12 neuronal subunits of nAChRs have been identified with nine  $\alpha$ -type ( $\alpha 2$ - $\alpha 10$ ) and three  $\beta$ -type ( $\beta 2$ - $\beta 4$ ) receptors that form structurally and functionally

distinct hetero- and homo-pentameric receptors (Changeux, 2010, Collins et al., 2009). Nicotine dependence is believed to be mediated primarily via  $\alpha 4\beta 2^*$  (the asterisk indicates involvement of other subunits) nAChRs among others (Picciotto and Kenny, 2013). Chronic cigarette smoking in humans leads to nAChR upregulation of high affinity  $\alpha 4\beta 2^*$  nicotine binding sites in the brain (Benwell et al., 1988, Breese et al., 1997, Cosgrove et al., 2009, Gentry and Lukas, 2002, Perry et al., 1999, Staley et al., 2006). Studies in rodents showed an upregulation of high affinity nicotine binding sites after chronic non-contingent nicotine exposure via external or internal osmotic minipumps, repeated nicotine injections or intravenous infusions (Collins et al., 1990, Fasoli et al., 2016, Marks et al., 1983, Rowell and Li, 1997, Sanderson et al., 1993, Schwartz and Kellar, 1983, Ulrich et al., 1997). Similarly, exposure to chronic contingent nicotine administration also results in increased nAChR expression in rodents (Donny et al., 2000, Metaxas et al., 2010, Parker et al., 2004). The nicotine-induced upregulation of nAChR may contribute to increased sensitivity to nicotine (Dani and Heinemann, 1996, Wonnacott, 1990) and the long-lasting elevations in brain reward function after cessation of nicotine self-administration (Kenny and Markou, 2006).

In the present study we compared the effects of nicotine dose and temporal pattern (intermittent *vs* continuous) of non-contingent nicotine exposure via osmotic minipumps on brain reward function and somatic signs during nicotine withdrawal in rats. We used intermittent nicotine exposure because the delivery method using continuous nicotine infusion to induce dependence does not closely mimic the intermittent pattern of nicotine intake of human smokers (Brynildsen et al., 2016). That is, a chronic smoker uses tobacco every 20 to 30 min with intermittent inhalation of mainstream smoke, with an extended period of withdrawal during sleep (Ghosheh et al., 2001). The rats were exposed to nicotine at doses equal to both contingently self-administered (Kenny and Markou, 2006, Paterson et al., 2008) and non-contingently administered via minipumps (Skjei and Markou, 2003). Further, we compared nAChR expression in corticolimbic brain areas in rats self-administering nicotine intravenously for 12 h/day (i.e., contingent intermittent exposure), and rats treated non-contingently with nicotine via minipumps (continuously or intermittently). Finally, nicotine and cotinine levels in plasma were compared after contingent intermittent, non-contingent intermittent and non-contingent continuous nicotine exposures for 21–22 days.

## 2. Materials and Methods

### 2.1. Subjects

Male Wistar rats (Charles River, Raleigh, NC) weighing 320–360 g at the beginning of the experiments) were housed in groups of two in a humidity- and temperature-controlled vivarium on a reverse 12 h light/dark cycle (lights off at 7 am). Rats had *ad libitum* access to food and water until behavioral training commenced. Training and testing occurred during the dark cycle. All experiments were in accordance with the guidelines of the American Association for the Accreditation of Laboratory Animal Care and the National Research Council's Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

## 2.2. Drugs

(–)Nicotine hydrogen tartrate (Sigma, St. Louis, MO) was dissolved in sterile 0.9% saline solution and infused through 28-day subcutaneous osmotic minipumps (1.2, 3.16 or 6.32 mg/kg nicotine base per 12h or 24h for 20 days). For the 12 h nicotine exposure, nicotine concentrations were doubled to match the nicotine dose delivered during 24 h exposure. For intravenous self-administration, nicotine was dissolved in sterile saline (pH adjusted to ~7).

## 2.3. Intracranial self-stimulation (ICSS) apparatus, surgery, and procedure

The ICSS apparatus, surgery, and procedure were identical to those described previously (Harrison et al., 2001, Semenova and Markou, 2003). Briefly, training and testing occurred in 16 sound-attenuated (San Diego Instruments, San Diego, CA) Plexiglas chambers (Med Associates, St. Albans, VT) that contained a metal wheel manipulandum. Brain stimulation was delivered by constant current stimulators (San Diego Instruments, San Diego, CA). Under isoflurane/oxygen vapor mixture (1–1.5% isoflurane) anesthesia, subjects were prepared with bipolar stainless steel electrodes (Plastics One, Roanoke, VA) in the posterior lateral hypothalamus (anterior/posterior, –0.5 mm from bregma; lateral, ±1.7 mm; dorsal/ventral, –8.3 mm from dura) (Pellegrino et al., 1986) with the incisor bar elevated 5.0 mm above the interaural line. Subjects were trained to respond for electrical stimulation under a discrete-trial current-threshold intracranial self-stimulation procedure, modified from the original procedure developed by Kornetsky and colleagues (Kornetsky et al., 1979). Each test session lasted 30–40 min and provided two dependent variables for behavioral assessment: reward threshold and response latency (Harrison et al., 2001, Markou and Koob, 1992, Semenova and Markou, 2003).

## 2.4. Intravenous self-administration and food responding, chambers, surgery and procedure

Methodological details of intravenous self-administration (IVSA) apparatus, catheter construction, surgery and acquisition of nicotine- and food-maintained responding have been described elsewhere (Paterson and Markou, 2004). Training and testing occurred in 24 sound-attenuated (San Diego Instruments, San Diego, CA) Plexiglas chambers (Med Associates, St. Albans, VT). Briefly, rats were food-restricted to 20 g of chow per day and trained to respond for food pellets, progressing from a fixed-ratio 1 time-out 1 s (FR1 TO1) to a FR5 TO 20 s schedule of reinforcement, with sessions lasting approximately 30 min. After the completion of food training, rats were prepared with intravenous catheters inserted into the right jugular vein under isoflurane/oxygen vapor mixture (1–2% isoflurane) anesthesia and were allowed to self-administer nicotine (0.03 mg/kg/infusion, base). Responding on the active lever (previously paired with delivery of a food pellet) resulted in the delivery of the nicotine solution in a volume of 0.1 ml over a 1 s period, and the presentation of a cue light above the active lever that remained lit for 20 s, during which time responses on the active lever had no consequences (i.e., time-out period). Responding on the inactive lever (introduced during the first self-administration session) had no consequences. Rats received 20 g rat chow per day, at least 1 h after termination of testing. Animals were allowed to self-administer nicotine for 12 h/day, 7 days/week during the dark cycle. Control rats were trained to respond for food, and then allowed to self-administer

saline 12 h/day, 7 days/week. Rats were allowed to self-administer saline or nicotine for a total of 21–22 sessions.

### 2.5. Rating of somatic signs of nicotine withdrawal

Somatic signs of nicotine withdrawal were counted under white light conditions in cylindrical Plexiglas chambers (diameter 15 cm) with sawdust bedding on the floor. Each subject was observed for 10 min by an observer blind to the subjects' treatments. The standard checklist used was adapted from an opiate withdrawal signs checklist (Epping-Jordan et al., 1998, Malin et al., 1992). The following signs were recorded: blinks, body and head shakes, chews, cheek tremors, teeth chattering, escape attempts, foot licks, genital licks, gasps, writhes, scratches, ptosis, and piloerection. Multiple successive counts of any sign required a distinct pause between episodes. Ptosis was counted as 1 for appearance or 0 for non-appearance during the 10 min period of observation.

### 2.6. Modified minipump assembly

The modified osmotic minipumps were made in our laboratory (Figure 1) as an improved modification of previously described gating device (Azar et al., 2004). The gating device consisted of a stainless steel bilateral guide cannula (C235G-2.0-SPCL, Plastics One, Roanoke, VA, USA) connected with a standard minipump via silastic tubing (Baxter Scientific, McGraw Park, IL, USA). The bilateral cannula consisted of the input cannula defined as the cannula connected to the osmotic minipump, and the output cannula defined as the cannula cut flush with the mesh that delivered drug subcutaneously. Silastic tubing was attached to the input cannula bent at a right angle and encased in dental cement anchored with a 2.2 cm<sup>2</sup> durable plastic mesh (CMP-0500-C, Small Parts, Miami Lakes, FL, USA). The gating device was connected to the osmotic minipump by securing one side of a piece of tygon tubing to the end of the input cannula, and the other end to the minipump flow lead. Once the modified minipump was implanted, gating the device to the "ON" (drug delivery) or "OFF" (no drug delivery) position was achieved by securing a single piece of tygon tubing from the top of the input cannula to the top of output cannula. To discontinue subcutaneous drug delivery, the tygon tubing was removed from the top of the input and output cannula. A small metal cap was positioned on the top of cannula assembly to protect the tygon tubing when gating was positioned ON.

### 2.7. Osmotic minipump implantation and removal

Fourteen days after ICSS electrode implantation, osmotic minipump surgery was conducted. Rats were anesthetized with an isoflurane/oxygen vapor mixture (1–2%). Standard minipumps (model 2ML4, Alzet Osmotic Pumps, Cupertino, CA) were inserted subcutaneously (back of the animal parallel to the spine) with the flow moderator directed posteriorly. For the modified minipump implantation, an incision 4 cm in length was made perpendicular to the median plane of the body. Then, subcutaneous space was made anterior to the incision to allow space for implantation of the gating device with the corresponding minipump. Once the minipump and the gating device were inserted, a small incision was made above the top of the gating device to allow for the protrusion of the gating device through the skin. After modified or standard minipump implantation, the wound was closed with 9 mm stainless steel wound clips (Becton Dickinson Primary Care Diagnostics, Sparks,

MD), and triple-antibiotic ointment was applied to the incision area. On day 21, the minipumps were surgically removed using the aforementioned procedure. The concentration of nicotine was adjusted to compensate for differences in the rats' body weights at the time of minipump implantation.

## 2.8. Nicotinic acetylcholine receptor binding analyses

Brains were rapidly removed 12 h after the last nicotine exposure and dissected into coronal sections using a rat brain matrix. The brain regions were identified according to the rat brain atlas (Paxinos and Watson, 1998) and dissected on an ice-cooled metal plate using a round tissue punch (1.5 mm in diameter). The bilateral brain sites dissected were the prelimbic cortex (PLC) as part of the prefrontal cortex, the ventral tegmental area (VTA), the nucleus accumbens (Acb), the amygdala (AMY), the hypothalamus (HYP), and the hippocampus (HIP), the habenula (HB) and the interpedicular nucleus (IPN). Brain punches were promptly transferred to tubes that were immediately frozen in cooled isopentane and subsequently placed on dry ice before being stored at  $-80^{\circ}\text{C}$ .

[ $^{125}\text{I}$ ]-epibatidine binding was measured as described previously (Whiteaker et al., 2000). The tissue was homogenized in hypotonic buffer and centrifuged at  $20,000 \times g$  to obtain the particulate fraction. The particulate fraction was washed by resuspension in fresh buffer followed by recentrifugation a total of three times. The washed particulate fraction was frozen. On the day of assay the washed pellets were resuspended in the overlying buffer and centrifuged at  $20,000 \times g$  for 10 min. The supernatant was discarded and the pellet was resuspended in ice-cold solution. Resuspension volume varied among brain regions and was adjusted such that less than 10% of the [ $^{125}\text{I}$ ]-epibatidine was bound to the protein at the highest ligand concentration. Samples were incubated in 96-well polystyrene plates for 2 h at room temperature in a final incubation volume of  $30 \mu\text{l}$  with either buffer (total) or buffer containing cytosine (50 nM) or cytosine (100  $\mu\text{M}$ ), along with 200 pM [ $^{125}\text{I}$ ]-epibatidine. Buffer composition was (NaCl 144 mM; KCl, 1.5 mM;  $\text{CaCl}_2$ , 2.0 mM;  $\text{MgSO}_4$ , 1.0 mM, HEPES, 25 mM; pH = 7.5). The 100  $\mu\text{M}$  cytosine defines non-specific binding whilst the total and 50 nM cytosine enables determination of cytosine-sensitive and cytosine-resistant binding sites. After incubation, samples were diluted with 200  $\mu\text{l}$  of ice-cold wash buffer and filtered under vacuum (0.2 atm) onto glass fiber filters treated with 0.5% polyethylenimine (top filter, MFS Type B; bottom filter, Type A/E, Pall Bioscience). An Inotech Cell Harvester (Inotech Biosystems International, Rockville, MD) collected the samples, which were subsequently washed six times with ice-cold buffer. Filters were transferred to a 96-well scintillation plate and counted using a Wallac TriLux 1450 MicroBeta scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA) at 30% efficiency after the addition of 150  $\mu\text{l}$  of Optiphase Supramix scintillation mixture (PerkinElmer Life and Analytical Sciences, Waltham, MA). Protein was measured using the Lowry method (Lowry et al., 1951) with bovine serum albumin standard.

Cytosine-sensitive [ $^{125}\text{I}$ ]-epibatidine binding sites correspond to  $\alpha 4\beta 2^*$  nAChRs (Whiteaker, Jimenez, 2000), and were calculated after the subtraction of cytosine-resistant from total [ $^{125}\text{I}$ ]-epibatidine binding.  $\alpha 7$  nAChR binding, which would assess  $\alpha 7$  nAChR binding, was not examined because  $\alpha$ -bungarotoxin binding returns to baseline almost immediately



after chronic treatment (Marks et al., 1985) and because prior work has shown that  $\alpha 7$  nAChRs are not involved in nicotine withdrawal/reward (Fowler et al., 2008).

## 2.9. Nicotine and cotinine concentration analyses

On days 21–22 of the experiment, blood samples (300  $\mu$ L) were collected from the tip of the tail after exposure to nicotine for 12 h. The trunk blood samples were collected from the site where animal was decapitated at the 12 h nicotine withdrawal time point (i.e., 12 h after the last nicotine exposure). Nicotine and cotinine levels in the blood were measured (Apredica, Watertown, MA) in both nicotine- and saline-exposed rats to ensure blinding and verification of assay sensitivity. Briefly, plasma samples were analyzed with liquid chromatography/mass spectrometry technique using an Agilent 6410 mass spectrometer coupled with an Agilent 1200 HPLC and a CTC PAL chilled autosampler, all controlled by MassHunter software (Agilent). After separation on a Polar silica HILIC (Sepax) HPLC column using an acetonitrile-water gradient system, peaks were analyzed by mass spectrometry (MS) using ESI ionization in multiple reaction monitoring (MRM) mode. The lower limit of quantitation for nicotine and cotinine was 27 nM in plasma.

## 2.10. Experimental design

Experimental design is presented in Figure 2.

### 2.10.1. Experiment 1. The effects of temporal pattern and nicotine dose on brain reward function and somatic signs during chronic nicotine exposure and nicotine withdrawal—

Rats were prepared with ICSS electrodes into the lateral hypothalamus and trained in the ICSS threshold procedure as described above once daily until stable reward thresholds were established, defined as 10% variation in thresholds for five consecutive days. Rats had reward thresholds assessed twice daily for 14 consecutive days to habituate them to multiple daily threshold assessments. Then, rats prepared with osmotic minipumps were exposed to different doses of nicotine administered intermittently for 12 h (1.2, 3.16 and 6.32 mg/kg/12 h,  $n=6-14$ /group) or continuously for 24 h (1.2, 3.16 and 6.32 mg/kg/24 h,  $n=6-9$ /group) for 21 days using modified or standard minipumps. The higher nicotine doses were selected because they were shown previously to produce reliable affective (i.e., threshold elevations) and somatic signs of nicotine withdrawal upon cessation of nicotine exposure (Epping-Jordan et al., 1998, Skjei and Markou, 2003); while the lower nicotine dose was selected to approximate the dose self-administered in a 12 h daily session (Kenny and Markou, 2006). Twenty one days of nicotine/saline exposure were selected because 20–28 days of exposure to nicotine self-administration (Kenny and Markou, 2006) induced threshold lowering upon cessation of nicotine self-administration. The use of modified minipumps allowed us to start and stop the nicotine/saline infusion every 12 h (i.e., intermittent non-contingent nicotine exposure). Rats were tested either once (continuous nicotine exposure) or twice (intermittent nicotine exposure) daily in the ICSS procedure. In rats exposed to nicotine intermittently, the first daily ICSS session assessed effects of 12 h nicotine withdrawal; while the second daily ICSS session assessed the effects of 12 h nicotine/saline self-administration. Thresholds continued to be assessed after pump removal at 12 h and every 24 h thereafter until return to baseline levels. Somatic signs were assessed 12 and 36 h post-nicotine. The experiment involving intermittent nicotine exposure was

conducted in two replications, with each cohort comprised of all experimental groups. << Figure 2 >>

### **2.10.2. Experiments 2 and 3. The effects of administration contingency, temporal pattern and nicotine dose on nAChR binding in various brain areas (Experiment 2), and blood nicotine and cotinine levels (Experiment 3)**

Rats initially trained to respond for food reinforcement were allowed to intravenously self-administer nicotine (0.03 mg/kg/infusion,  $n=12$ ) or saline ( $n=11$ ) for 21–22 days on a FR5 TO 20 s schedule of reinforcement for 12 h/day, 7 days/week. The other rats were exposed to nicotine (1.2 or 3.16 mg/kg/day) for 21–22 days via 28-day standard (nicotine-1.2:  $n=10$ ; nicotine-3.16:  $n=10$ ) or modified (nicotine-1.2:  $n=8$ ; nicotine 3.16:  $n=18$ ) osmotic minipumps. Control rats self-administered saline. Due to the large number of rats and the need to closely control the nicotine exposure regimen (i.e., 12 h after the last nicotine exposure), half of the rats in each experimental group were euthanized after 21 days of nicotine exposure and the other half after 22 days of nicotine exposure.

### **2.11. Statistical Analyses**

Statistical analyses were conducted using the Statistical Package for the Social Sciences version 15.0 (SPSS Inc., Chicago, IL). All behavioral data were analyzed with the appropriate mixed-design ANOVAs, with factors *Temporal pattern* (intermittent vs continuous nicotine exposures) and *Nicotine dose* being a between-subjects factors, and factor *Day of nicotine exposure* (19 levels)/nicotine withdrawal (6 levels for ICSS thresholds/latencies and 2 levels for somatic signs) being a within-subject factors. Thresholds and response latencies were expressed as a percentage of the mean of the last five baseline values before the nicotine/saline exposure. Somatic sign data were expressed as the total number of somatic signs observed during the 10 min observation period. The number of nicotine/saline infusions and the number of active/inactive lever presses during the 21 days of nicotine/saline self-administration were analyzed by two-way repeated measures ANOVAs with *Nicotine dose* as a between-subjects factor and *Day of exposure* and *Lever* as a within-subjects factor. Plasma nicotine/cotinine levels and nAChRs binding data were analyzed using one-way ANOVAs with factors *Experimental Group* (7 levels), *Contingency* (contingent vs non-contingent), or *Temporal pattern* (continuous 24 h exposure vs intermittent 12 h exposure) being a between-subjects factors and a two-way ANOVA with *Nicotine dose* and *Temporal pattern* being a between-subjects factors. *Post hoc* comparisons among means were conducted after significant effects using Fisher's Least Significant Difference (LSD) test after statistically significant effects were observed in the ANOVAs. The level of significance was set at 0.05.

## **3. Results**

### **3.1. Experiment 1. The effects of temporal pattern and nicotine dose on brain reward function and somatic signs during chronic nicotine exposure and nicotine withdrawal**

Before any treatments began, no statistically significant differences were observed among mean absolute values of baseline thresholds among the various treatment groups [average group mean thresholds  $\pm$  SEM: group with intermittent exposure to saline ( $136.30 \pm 12.25$



$\mu\text{A}$ ), nicotine-1.2 ( $132.79 \pm 11.43 \mu\text{A}$ ), nicotine-3.16 ( $132.29 \pm 11.11 \mu\text{A}$ ), nicotine-6.32 ( $131.43 \pm 19.08 \mu\text{A}$ ); group with continuous exposure to saline ( $114.22 \pm 11.92 \mu\text{A}$ ), nicotine-1.2 ( $118.05 \pm 16.89 \mu\text{A}$ ); nicotine-3.16 ( $112.37 \pm 10.93 \mu\text{A}$ ); nicotine-6.32 ( $128.43 \pm 8.68 \mu\text{A}$ )]. No statistically significant differences were observed among mean absolute values of baseline response latencies for the various treatment groups [average group mean latencies  $\pm$ SEM: group with intermittent exposure to saline ( $3.43 \pm 0.10$  s), nicotine-1.2 ( $3.72 \pm 0.13$  s), nicotine-3.16 ( $3.34 \pm 0.09$  s), nicotine-6.32 ( $3.59 \pm 0.23$  s); group with continuous exposure to saline ( $2.95 \pm 0.15$  s), nicotine-1.2 ( $3.15 \pm 0.10$  s), nicotine-3.16 ( $3.27 \pm 0.14$  s); nicotine-6.32 ( $3.15 \pm 0.13$  s)].

**3.1.1. The effects of 20-day intermittent and continuous nicotine/saline exposure on reward thresholds and response latencies**—During the 20 days of either 12 h intermittent or 24 h continuous nicotine/saline administration, there were no significant changes in ICSS thresholds or response latencies for any of the treatment groups (data not shown). The ANOVA revealed no significant effect of *Temporal pattern*, *Nicotine dose*, *Day* or any interactions.

During the 20 days of repeated 12 h withdrawal from intermittent nicotine/saline administration there were no significant changes in ICSS thresholds or response latencies in any of the treatment groups (data not shown). The ANOVA revealed no effect of *Nicotine dose*, *Day*, *Temporal pattern* or their interaction.

**3.1.2. The effects of spontaneous withdrawal from intermittent and continuous nicotine exposure on reward thresholds and response latencies**—The reward thresholds and response latencies during spontaneous withdrawal from chronic non-contingent nicotine exposure (intermittent and continuous) are presented in Figure 3. The ANOVA revealed statistically significant effect of *Temporal pattern* (thresholds:  $F_{(1,73)}=11.53$ ,  $p<0.001$ ; latencies:  $F_{(1,73)}=24.27$ ,  $p<0.001$ ), *Day of withdrawal* (thresholds:  $F_{(5,365)}=17.46$ ,  $p<0.05$ ; latencies:  $F_{(5,365)}=12.57$ ,  $p<0.01$ ) and *Nicotine dose* for latencies ( $F_{(3,73)}=3.06$ ,  $p<0.05$ ), but no effect of *Nicotine dose* for thresholds, and no 3-way interaction effect for either thresholds or latencies.

To further analyse the effects of each temporal pattern on nicotine withdrawal, two-way ANOVAs were performed on thresholds and response latencies. Significant differences between rats receiving nicotine intermittently or continuously were found. Withdrawal from intermittent nicotine exposure had no effect on reward thresholds (Fig. 3A) or response latencies (Fig. 3B) for any nicotine treatment dose as demonstrated by the ANOVA (no significant effect of *Nicotine Dose*, or *Day of withdrawal* and no interaction effect). In contrast and in replication of our previous findings (Epping-Jordan et al., 1998, Semenova and Markou, 2003, Skjei and Markou, 2003), rats that received continuous nicotine infusions exhibited threshold elevations (Fig. 3C) and increases in response latencies (Fig. 3D) during nicotine withdrawal. The ANOVA revealed significant main effects of *Nicotine dose* (thresholds:  $F_{(3,28)}=3.06$ ,  $p<0.05$ ; latencies:  $F_{(3,28)}=4.77$ ,  $p<0.01$ ) and *Day of withdrawal* (thresholds:  $F_{(5,140)}=3.06$ ,  $p<0.05$ ; latencies:  $F_{(5,140)}=7.90$ ,  $p<0.05$ ). There was no significant *Nicotine dose* by *Day of withdrawal* interaction effect for thresholds and latencies. Pre-planned comparisons revealed that rats that received continuous nicotine

infusions at the doses of 3.16 and 6.32 mg/kg/day exhibited threshold elevations at all withdrawal times between 12–84 h post-nicotine. Significantly increased response latencies were also observed 12 h and 36 h post-nicotine compared to thresholds and latencies of the saline control group for rats that received continuous nicotine infusions at the doses of 3.16 and 6.32 mg/kg/day.

**3.1.3. The effects of spontaneous withdrawal from intermittent and continuous nicotine exposure on somatic signs**—Figure 4 shows number of somatic signs during spontaneous withdrawal from chronic non-contingent nicotine exposure. The ANOVA revealed a statistically significant effect of *Temporal pattern* ( $F_{(1,73)}=10.01, p<0.05$ ), *Day of withdrawal* ( $F_{(1,73)}=22.55, p<0.05$ ) and *Nicotine dose* ( $F_{(3,73)}=4.67, p<0.05$ ). Furthermore, there were significant interaction effects of *Temporal pattern* by *Day of withdrawal* ( $F_{(1,73)}=18.75, p<0.05$ ), and *Temporal pattern* by *Nicotine dose* ( $F_{(3,73)}=3.24, p<0.05$ ).

To further analyse the effects of each temporal pattern on the expression somatic signs after spontaneous nicotine withdrawal, separate two-way ANOVAs were performed. No effect on the number of somatic signs were observed following withdrawal from intermittent nicotine exposure to any nicotine dose (Fig. 4A) as reflected by non-significant effect of *Day of withdrawal*, *Nicotine dose* and *Day by Nicotine dose* interaction. In contrast, withdrawal from continuous nicotine exposure resulted in increased number of somatic signs (Fig. 4B) as reflected in significant effect of *Day of withdrawal* ( $F_{(1,28)}=23.46, p<0.05$ ) and *Nicotine dose* ( $F_{(3,28)}=4.87, p<0.05$ ) effects. However, no significant *Day by Nicotine dose* interaction was observed. *Post hoc* comparisons revealed that continuous exposure to all nicotine doses induced increases in somatic signs 12 h post-nicotine compared to saline exposure (Fig. 4B). Furthermore, somatic signs were significantly increased 36 h post-nicotine after exposure to the highest dose (6.32 mg/kg/day; Fig. 4B).

## 3.2. Experiment 2. The effects of administration contingency, temporal pattern and nicotine dose on nAChR bindings in various brain areas

**3.2.1. Nicotine self-administration**—Figure 5 presents the time courses for both active and inactive lever presses of rats self-administering either saline (n=21) or nicotine (n=22) for 12 h per day for 21 days. Those rats self-administering nicotine received an average dose of  $1.04\pm 0.06$  mg/kg per day of nicotine base ranging from 0.77 to 1.27 mg/kg during days 1–21 of self-administration. A two-way ANOVA on the number of nicotine/saline infusions revealed significant main effects of *Infusions* ( $F_{(1,41)}=88.9, p<0.0001$ ), *Day* ( $F_{(20,820)}=17.26, p<0.0001$ ), and an *Infusions by Day* interaction ( $F_{(20,820)}=2.49, p<0.001$ ). *Post hoc* comparisons showed that the number of nicotine infusions was significantly higher than the number of saline infusions during 12 h daily sessions from day 3 to day 21 of self-administration ( $p<0.001$ ). The relatively high rates of responding on the first day resulted from initial food training prior to nicotine self-administration. A two-way repeated measures ANOVA on the number of active and inactive lever presses revealed significant effects of *Nicotine/saline exposure* ( $F_{(1,82)}=79.45, p<0.0001$ ), *Lever* ( $F_{(1,82)}=162.15, p<0.0001$ ), *Day* ( $F_{(20,1640)}=23.02, p<0.0001$ ) as well as *Nicotine/saline exposure by Lever* ( $F_{(1,82)}=45.54, p<0.0001$ ) and *Day by Lever* ( $F_{(20,1640)}=9.22, p<0.001$ ) interactions. Both saline and

nicotine maintained more responding on the active than the inactive lever, and nicotine maintained significantly more active lever pressing than saline (Fig. 5A&B).

**3.2.2. Nicotinic acetylcholine receptor binding analyses**—This experiment was performed in two replications because several samples were spoiled during sample collection and processing. Cytisine-sensitive and resistant nAChR binding was measured in the PLC, AMY, Acb, VTA, HIP, HYP, HAB and IPN 12 h after the termination of nicotine exposure via pumps (continuously or intermittently) or self-administration. Rats were exposed to nicotine at doses equal to both contingently self-administered (1.2 mg/kg/12 h) and non-contingently administered via minipumps (1.2 mg/kg/12 h and 1.2 mg/kg/24 h), as well as the minimal nicotine dose that produced nicotine withdrawal signs (3.16 mg/kg/12 h and 3.16 mg/kg/24 h). nAChRs binding assays were not conducted on brain samples from rats treated with the highest nicotine dose (6.32 mg/kg/24 h or 6.32 mg/kg/12 h base) because medium nicotine dose (3.16 mg/kg/12 h and 3.16 mg/kg/24 h) produced upregulation of nAChRs in all brain areas assessed independent of contingency.

Figure 6 presents the levels of cytosine-sensitive epibatidine binding in corticolimbic brain areas. One-way ANOVAs for all seven treatment groups revealed significant effects of *Treatment Group* in PLC ( $F_{(6,60)}=4.35$ ,  $p<0.001$ ), AMY ( $F_{(6,55)}=15.86$ ,  $p<0.001$ ), Acb ( $F_{(6,51)}=4.43$ ,  $p<0.01$ ), VTA ( $F_{(6,60)}=4.35$ ,  $p<0.001$ ), HIP ( $F_{(6,125)}=27.01$ ,  $p<0.001$ ) and HYP ( $F_{(6,131)}=9.67$ ,  $p<0.001$ ), but not in HAB ( $F_{(6,59)}=0.89$ ,  $p>0.05$ ) or IPN ( $F_{(6,76)}=0.79$ ,  $p>0.05$ ). There were no significant differences in nAChR binding between control groups exposed to saline via self-administration or pump.

Analysis of the effect of nicotine treatment for rats self-administering nicotine was assessed using t-tests (Figure 6). These analyses revealed a significant effect of nicotine treatment on cytosine-sensitive [ $^{125}$ I]-epibatidine binding in AMY ( $t_{14} = 5.54$ ,  $p<0.001$ ), HIP ( $t_{37} = 4.35$ ,  $p<0.001$ ), HYP ( $t_{39} = 3.35$ ,  $p<0.001$ ), Acb ( $t_{14} = 2.48$ ,  $p=0.026$ ), PLC ( $t_{39} = 4.32$ ,  $p<0.001$ ), but not in HAB ( $t_{21} = 0.34$ ,  $p>0.05$ ), IPN ( $t_{21} = 0.08$ ,  $p>0.05$ ) or VTA ( $t_{21} = 0.02$ ,  $p>0.05$ ).

Additional statistical analyses compared nAChR binding after exposure to the low 1.2 mg/kg nicotine dose via self-administration and minipumps (intermittently). Consistent with the results of the overall one-way ANOVA, significant effects of nicotine exposure on cytosine-sensitive [ $^{125}$ I]-epibatidine binding were revealed by two-way ANOVA (with nicotine dose and route of administration as the independent variables): AMY ( $F_{(1,28)}=45.11$ ,  $p<0.001$ ), HIP ( $F_{(1,60)}=46.39$ ,  $p<0.001$ ), HYP ( $F_{(1,63)}=45.27$ ,  $p<0.001$ ), Acb ( $F_{(1,24)}=6.99$ ,  $p=0.014$ ), PLC ( $F_{(1,62)}=69.08$ ,  $p<0.001$ ). However, the main effect of nicotine dose was not significant in VTA ( $F_{(1,32)}=3.67$ ,  $p=0.064$ ), HAB ( $F_{(1,28)}=0.88$ ,  $p>0.05$ ) and IPN ( $F_{(1,34)}=0.27$ ,  $p>0.05$ ). No significant difference between the treatment groups on cytosine-sensitive [ $^{125}$ I]-epibatidine binding (self-administration vs. minipump) was detected in any brain region. However, a significant treatment group by nicotine dose interaction was noted for HIP ( $F_{(1,60)}=7.83$ ,  $p=0.007$ ), HYP ( $F_{(1,63)}=8.01$ ,  $p=0.006$ ) and PLC ( $F_{(1,62)}=8.46$ ,  $p = 0.005$ ) reflecting difference in the magnitude of increase between for the self-administration and minipump groups elicited by nicotine.

Further analyses compared nAChR binding after intermittent and continuous exposure to nicotine via minipumps. A two-way ANOVA revealed a significant difference between rats treated for 12 h or 24 h in three brain regions with higher binding noted for the 24 h treatment group: HIPP ( $F_{(1,75)}=11.51, p<0.001$ ), Acb ( $F_{(1,28)}=8.65, p=0.006$ ), and PLC ( $F_{(1,62)}=9.32, p=0.006$ ). The two-way ANOVA also indicated a significant effect of nicotine dose in HIPP ( $F_{(1,75)}=7.88, p=0.006$ ) where higher binding was noted following treatment with 3.6 mg/kg/day: HIPP ( $F_{(1,75)}=7.88, p=0.006$ ).

The levels of cytosine-resistant binding were relatively small compared to cytosine-sensitive epibatidine binding in all brain structures except the AMY, VTA, HAB and IPN (Table 1). All analyses revealed no effect of *Experimental group* for any of these four brain structures.

### 3.3. Experiment 3. The effects of administration contingency, temporal pattern and nicotine dose on plasma nicotine and cotinine levels—

After continuous or intermittent exposure to nicotine for 21 days, levels of both nicotine and cotinine were detected in plasma (Table 2) immediately after nicotine exposure for 12 h. One-way ANOVA revealed a significant effect of *Treatment Group* on level of nicotine ( $F_{(4,48)}=3.46, p<0.05$ ) and cotinine ( $F_{(4,48)}=3.66, p<0.01$ ). During exposure to the similar nicotine dose 1.2 mg/kg/day intermittently via self-administration and pump or continuously via pump, ANOVA revealed a significant effect of *Contingency* for nicotine ( $F_{(2,29)}=5.58, p<0.01$ ), but not for cotinine. *Post hoc* comparisons revealed that nicotine levels were significantly higher after nicotine self-administration compared to intermittent exposures to a similar nicotine dose via pumps; while cotinine levels did not differ between these experimental groups (Table 2). During intermittent and continuous exposure to low and high nicotine doses via pumps, an ANOVA revealed no effect of *Temporal Pattern* or *Temporal Pattern* by *Nicotine dose* interaction, although there was a significant effect of *Nicotine dose* for nicotine ( $F_{(1,36)}=11.45, p<0.01$ ) and cotinine ( $F_{(1,36)}=8.78, p<0.01$ ) levels in plasma. Group comparisons showed that intermittent or continuous exposure to low nicotine dose via pumps resulted in significantly lower levels of both nicotine and cotinine compared to intermittent or continuous exposure to high nicotine dose via pumps (Table 2).

ANOVA on cotinine levels at the 12 h nicotine withdrawal time-point, revealed a significant effect of *Treatment Group* ( $F_{(4,48)}=20.82, p<0.001$ ). During exposure to a similar nicotine dose (1.2 mg/kg/day) administered either intermittently via self-administration or pump, an ANOVA revealed no effect of *Contingency* for cotinine levels. During intermittent and continuous exposure to low and high nicotine doses via pumps, the ANOVA revealed a significant effect of *Temporal Pattern* ( $F_{(1,36)}=12.91, p<0.001$ ), Nicotine dose ( $F_{(1,36)}=40.71, p<0.001$ ) and their interaction ( $F_{(1,36)}=5.16, p<0.05$ ). Group comparisons showed that intermittent or continuous exposure to a low nicotine dose via pumps resulted in significantly lower levels of cotinine compared to intermittent or continuous exposure to the high nicotine dose administered via pumps during withdrawal (Table 2).

## 4. Discussion

### 4.1. Main findings

The results of the present studies showed that the termination of intermittent non-contingent nicotine exposure (1.2, 3.16 and 6.32 mg/kg/12 h) had no effect on reward thresholds, response latencies or somatic signs indicating no nicotine withdrawal after this intermittent pattern of nicotine exposure. In contrast and in replication of our previous findings, withdrawal from continuous non-contingent nicotine exposure at higher doses (3.16 and 6.32 mg/kg/24 h) induced threshold elevations and increased number of somatic signs. Termination from continuous exposure to a low nicotine dose (1.2 mg/kg/24 h) did not induce threshold elevations but increased number of somatic signs. Chronic nicotine exposure induced upregulation of  $\alpha 4\beta 2^*$  nAChR in contingency-dependent manner that varied among brain regions. Contingent intermittent nicotine exposure resulted in a higher plasma nicotine levels compared to levels seen after nicotine exposure at the same dose non-contingently independent of temporal pattern. Plasma nicotine levels after non-contingent exposure depended on nicotine dose but not on temporal pattern. The same nicotine dose resulted in higher cotinine levels after intermittent exposure compared to continuous exposure via minipumps.

### 4.2. Expression of nicotine withdrawal

We showed previously that dose and duration of nicotine exposure significantly influenced the duration and/or overall severity of the subsequent nicotine withdrawal (Epping-Jordan et al., 1998, Skjei and Markou, 2003). In replication of these findings, withdrawal from continuous nicotine exposure administered at the doses of 3.16 or 6.32 mg/kg/24 h elevated reward thresholds, increased response latencies and the number of somatic signs of withdrawal. Importantly, these same nicotine doses (3.16 or 6.32 mg/kg/12 h) administered intermittently did not induce threshold elevations or increases in somatic signs. Further, withdrawal from the lowest nicotine dose administered continuously (1.2 mg/kg/24 h) or intermittently (1.2 mg/kg/12 h) via minipumps had no effect on reward threshold, response latencies or somatic signs of withdrawal. In contrast to our findings, one published study (Brynildsen et al., 2016), using a different intermittent pattern of exposure (1 h ON, 1 h OFF) via a modified minipump, reported lasting effects of intermittent nicotine (1.2, 2,4 and 4.8 mg/kg/day for 14 days) on mecamylamine-precipitated somatic withdrawal signs. Similarly, moderate increases in somatic signs were observed during spontaneous or mecamylamine-precipitated nicotine withdrawal in rats exposed to daily nicotine self-administration for 1 h or 6 h (Paterson and Markou, 2004), or 23 h (Harris et al., 2011, O'Dell et al., 2007). These results demonstrate the importance of temporal pattern of nicotine exposure in inducing the negative affective and somatic signs of spontaneous, but not mecamylamine-precipitated nicotine withdrawal. The reported mecamylamine-precipitated withdrawal after exposure to low nicotine doses may be attributed to acute blockade of nAChRs; while during spontaneous nicotine withdrawal other neuroplasticity mechanisms are involved.

The findings that intermittent non-contingent nicotine administration does not affect measures of nicotine withdrawal are in contrast to the effects observed following contingent

12 h nicotine self-administration of the same nicotine dose reported previously (Kenny and Markou, 2006) that produced long-lasting enhancement of brain reward function. However, withdrawal from unlimited 23 h access to nicotine self-administration resulted in transient small threshold elevations (below 10 % of baseline levels) during the first three days of withdrawal followed by modest short-lasting threshold lowering on days 5–6 of withdrawal/extinction (Harris et al., 2011), a result that contrasts with the current study and the previous report (Kenny and Markou, 2006). Thus, it appears that intermittent access to nicotine self-administration produced differential effects on brain reward function compared to unlimited access.

The expression of nicotine withdrawal in rodents is complex (for review, Jackson et al., 2015). Studies with genetically modified mice have established that somatic signs of withdrawal are mediated by  $\beta 4$  (Salas et al., 2004; Stoker et al., 2012),  $\alpha 2$  (Salas et al., 2009) and  $\alpha 5$  nAChR subunits (Jackson et al., 2008; Salas et al., 2009), and also involve receptors in the medial habenula-interpeduncular nucleus system (Salas et al., 2009). While the  $\beta 2$  nAChR subunit is not implicated in mediating somatic signs of withdrawal, this subunit is involved in affective signs of withdrawal including reward deficits or anhedonia (Stoker et al., 2015), fear conditioning (Portugal et al., 2008) and anxiety-like behavior (Jackson et al., 2008). Nicotine withdrawal is also influenced by age, sex, the environment indicating that, in addition to genetic factors, complex neuropsychological mechanisms mediate the withdrawal syndrome (for review, Jackson et al., 2015). Our findings indicate that both contingency and a temporal pattern of exposure are important determinants in modeling nicotine dependence in Wistar male rats. These results may differ somewhat from results in human smokers who experience well-characterized withdrawal syndrome following smoking cessation (Hughes, 2007). The differential effects of temporal pattern of non-contingent nicotine exposure on signs of nicotine withdrawal may be attributed to different pharmacokinetic mechanisms of exposure. However, limited assessment of nicotine levels at one time point was not sufficient to draw meaningful conclusions. Further, considering that chronic nicotine exposure produces a wide range of adaptations in neuroplasticity, the absence of spontaneous withdrawal signs from intermittent non-contingent nicotine exposure can be attributed to multiple factors. The role of these factors in the expression of nicotine withdrawal induced by non-contingent intermittent nicotine exposure was not investigated in the present studies. Future work in this area is warranted.

### 4.3. Upregulation of $\alpha 4\beta 2^*$ nAChR

Cytisine-sensitive [ $^{125}\text{I}$ ]-epibatidine binding results demonstrated that  $\alpha 4\beta 2^*$  nAChR upregulation was brain region-specific and depended on both contingency and temporal pattern of nicotine exposure. Consistent with previous reports, the cytisine-sensitive [ $^{125}\text{I}$ ]-epibatidine binding in HAB and IPN were unaffected by any of the nicotine treatments performed in the present study (Marks et al., 2011, Nguyen et al., 2003). In contrast, nicotine treatment elicited up-regulation of cytisine-sensitive [ $^{125}\text{I}$ ]-epibatidine binding in the other brain regions tested, although some differences in the response to nicotine exposure among these regions were noted. Self-administration of nicotine elicited significant up-regulation of cytisine-sensitive [ $^{125}\text{I}$ ]-epibatidine binding in PLC, AMY, Acb, HIP and HYP but not in VTA, HAB or IPN. When the extent of up-regulation of cytisine-sensitive



[<sup>125</sup>I]-epibatidine binding elicited by contingent nicotine administration was compared to that elicited by the most comparable non-contingent nicotine treatment (1.2 mg/kg/12 h), significant effects of nicotine in PLC, AMY, Acb, HIPPO and HYP were noted, but there were no main effects of treatment pattern were found. However, the significant treatment by nicotine interactions observed for HIPPO, HYP and PLC indicated a different pattern of response in these brain regions. Of particular interest was the difference in response to nicotine treatment between VTA and Acb, brain structures directly involved in nicotine reward and withdrawal (D'Souza and Markou, 2011, Koob and Volkow, 2010). Nicotine self-administration produced  $\alpha 4\beta 2^*$  nAChR upregulation in the Acb, but not in the VTA. Consistent with our results, upregulation of  $\alpha 4\beta 2^*$  nAChR was observed in the Acb after 1 h (Moretti et al., 2010) and 23 h (Parker et al., 2004) of nicotine self-administration in rats. Interestingly, however, exposure to 23 h of nicotine self-administration also up-regulated  $\alpha 4\beta 2^*$  nAChR in the VTA (Parker et al., 2004), indicating that longer duration of contingent nicotine exposure was needed to produce  $\alpha 4\beta 2^*$  nAChR up-regulation in the VTA. The opposite pattern of results was observed in studies with mice, where increased levels of  $\alpha 4\beta 2^*$  nAChR binding were observed in the VTA, but not in the Acb, after 1 h self-administration (Metaxas et al., 2010). These discrepancies between mouse and rat studies may be attributed to species or procedural differences (e.g., the lower nicotine dose self-administered by mice compared to those self-administered by rats; shorter duration of nicotine exposure).

In contrast to contingent nicotine self-administration, intermittent non-contingent exposure to the same nicotine dose (1.2 mg/kg/ 12 h) upregulated  $\alpha 4\beta 2^*$  nAChR in the VTA and PLC, but not in the Acb. Similar to our findings, non-contingent repeated nicotine injections (4 x 0.4 mg/kg base for 7 days) upregulated  $\alpha 4\beta 2^*$  nAChRs in the VTA, but not in the Acb or prefrontal cortex (Baker et al., 2013). Thus, it appears that contingent nicotine self-administration is associated with specific upregulation of  $\alpha 4\beta 2^*$  nAChR in the Acb; while non-contingent intermittent nicotine exposure upregulates  $\alpha 4\beta 2^*$  nAChR in the VTA and the cortex. Non-contingent exposure to the higher nicotine doses tested, administered continuously or intermittently up-regulated  $\alpha 4\beta 2^*$  nAChR in all six brain areas. These findings are consistent with earlier work showing increased ligand binding after non-contingent intermittent or continuous nicotine exposure in various brain regions including the prefrontal cortex, the VTA and the Acb (for review see (Vezina et al., 2007)).

Associations between nAChRs upregulation and expression of nicotine withdrawal signs are not well-understood. Our finding demonstrated that  $\alpha 4\beta 2^*$  nAChRs upregulation can be observed in the absence of reward deficit and somatic withdrawal after exposure to non-contingent intermittent nicotine exposure. Consistent with our findings,  $\beta 2^*$  nAChR availability in abstinent smokers during early abstinence did not correlate with the severity of nicotine withdrawal assessed with the Minnesota Withdrawal Questionnaire (Staley et al., 2006). Nevertheless, these data doesn't preclude a more direct relationship with individual features of nicotine withdrawal such as depression, anxiety or other symptoms because the severity of nicotine withdrawal is likely determined by the complex interplay between  $\alpha 4\beta 2^*$  nAChRs and multiple neurochemical systems as well as the large number of signaling pathways (for review, Jackson et al., 2015).

Cytisine-resistant [ $^{125}$ I]-epibatidine binding sites are related to other nAChRs subtypes that may contain  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 6$ ,  $\beta 2$  and  $\beta 4$  subunits (Baddick and Marks, 2011, Marks et al., 2006). Independent of contingency or temporal pattern, exposure to nicotine had no effect in the four brain areas expressing reliably measurable cytosine-resistant [ $^{125}$ I]-epibatidine binding. Consistent with our findings, no differences in cytosine-resistant nAChRs binding between self-administering mice and yoked control mice in corticolimbic brain areas were reported (Metaxas et al., 2010). Altogether, consistent with previous work (Nguyen et al., 2003), our findings on cytosine-sensitive (primarily  $\alpha 4\beta 2^*$ -nAChR sites) and cytosine-resistant nAChRs (non-  $\alpha 4\beta 2^*$ -nAChR sites, primarily  $\alpha 3\beta 4^*$ -nAChR sites in HAB and IPN) binding suggest that chronic nicotine exposure upregulates mainly  $\alpha 4\beta 2^*$  nAChRs, but not nAChRs subtypes containing  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 6$ ,  $\beta 2$  and  $\beta 4$  subunits in the corticolimbic brain areas, but the effects on  $\alpha 4\beta 2^*$ -nAChR sites depend on contingency and temporal pattern of nicotine exposure. Our observation that chronic nicotine treatment has differential effects on  $\alpha 4\beta 2^*$ -nAChR sites in various brain regions is also consistent with previous reports (Marks et al., 2011, Nguyen et al., 2003). The reasons underlying these differential responses to chronic nicotine remain unclear. However, differences in receptor composition, such as the resistance of  $\alpha 4\beta 2\alpha 5$ -nAChR subtype to up-regulation following nicotine treatment (Mao et al., 2008) and potential differences in responses of receptors differing in  $\alpha 4/\beta 2$  stoichiometry (Moroni et al., 2006) may contribute to the differential responses among brain regions.

#### 4.4. Plasma nicotine and cotinine levels

Our results extend previous findings showing differential effects of contingency of nicotine exposure on nicotine/cotinine levels in plasma. In our studies, nicotine plasma levels after the 12 h self-administration session ( $39.8 \pm 2.8$  infusions/12 h,  $35.4 \pm 7.1$  ng/ml) were lower compared to previously reported plasma nicotine levels measured after fewer infusions obtained during 1 h (7 infusions,  $\sim 95$  ng/ml nicotine arterial levels (Donny et al., 2000)) or 2 h (10 infusions, 65.4 ng/ml nicotine venous levels (Shoaib and Stolerman, 1999)) self-administration sessions. These results are not surprising because the highest nicotine intake usually occurs during the first hour of the self-administration session, and then a steady low intake is maintained during 22 h unlimited access to nicotine (O'Dell et al., 2007). When nicotine was delivered continuously via minipumps, plasma nicotine levels depended on nicotine dose but not on temporal pattern. The highest nicotine plasma levels ( $38.7 \pm 2.1$  ng/ml) were achieved during continuous nicotine exposure to the highest nicotine dose (3.16 mg/kg/24 h) via minipumps. Similarly, dose-dependent increases in plasma nicotine levels can also be gathered from the literature reporting nicotine levels during continuous exposure to nicotine at different doses via minipumps (Ghosheh et al., 2001, O'Dell et al., 2006, Winders et al., 1998). Interestingly, in the present work, the plasma nicotine levels after 12 h of self-administration were higher compared to levels seen after nicotine exposure at the same dose via minipumps (intermittently or continuously). To reach plasma nicotine levels comparable to those observed during nicotine self-administration, nicotine dose delivered via minipumps had to be 3-fold higher than the dose administered non-contingently independent of temporal pattern of administration (intermittent or continuous). Consistent with our findings, nicotine plasma levels in smokers usually peak at 15–40 ng/ml following cigarette smoking and the levels decline thereafter to around 10 ng/ml (Feyerabend and

Russell, 1990, Russell et al., 1986). However, some smokers can achieve nicotine plasma levels as high as 100 ng/ml depending on how quickly the cigarette is smoked (Benowitz et al., 1983), as well as the time of blood sampling (Rose et al., 1999).

Further, in smokers, the plasma cotinine levels are in average approximately 300 ng/ml (Benowitz et al., 1997), and increase during the day proportionally to the number of cigarettes smoked reaching up to 600 ng/ml in some smokers (Benowitz et al., 1983). In the present study, the highest cotinine levels were detected during continuous exposure to nicotine at the highest dose via minipumps (1211.1±40.6 ng/ml); while all other nicotine exposures yielded plasma cotinine levels ranging from 450 to 666 ng/ml. Similar to nicotine levels, dose-dependent increases in plasma cotinine levels were reported in the literature during continuous exposure to various nicotine doses via minipumps (Ghosheh et al., 2001, O'Dell et al., 2006, Winders et al., 1998). During nicotine withdrawal, plasma cotinine levels were decreased in all experimental groups. However, the decreases in cotinine levels during nicotine withdrawal were dose-dependent within each temporal pattern with no effect of contingency. Interestingly, however, the same nicotine dose resulted in higher cotinine levels after intermittent exposure compared to continuous exposure via minipumps.

## 5. Conclusions

Our findings demonstrated that contingency and temporal pattern of nicotine exposure induced differential behavioral adaptations and neuroplasticity related to  $\alpha 4\beta 2^*$  nAChRs expression. Although intermittent pattern of nicotine exposure closely mimics smoking patterns in humans, cessation of non-contingent intermittent nicotine exposure via minipumps did not result in reward deficit or somatic withdrawal compared to non-contingent continuous nicotine exposure in rats. Thus, temporal pattern of nicotine exposure should be taken into consideration when modeling reward deficit and somatic aspects of nicotine withdrawal in rats. Upregulation of  $\alpha 4\beta 2^*$  nAChRs was brain site-specific and depended on both temporal pattern and contingency of nicotine exposure suggesting that different brain circuits may mediate behavioral adaptations in response to different patterns of nicotine exposure. Future research is warranted to understand potential mechanisms underlying differential effects of temporal pattern of nicotine exposure on reward deficit and somatic aspects of nicotine withdrawal in rats.

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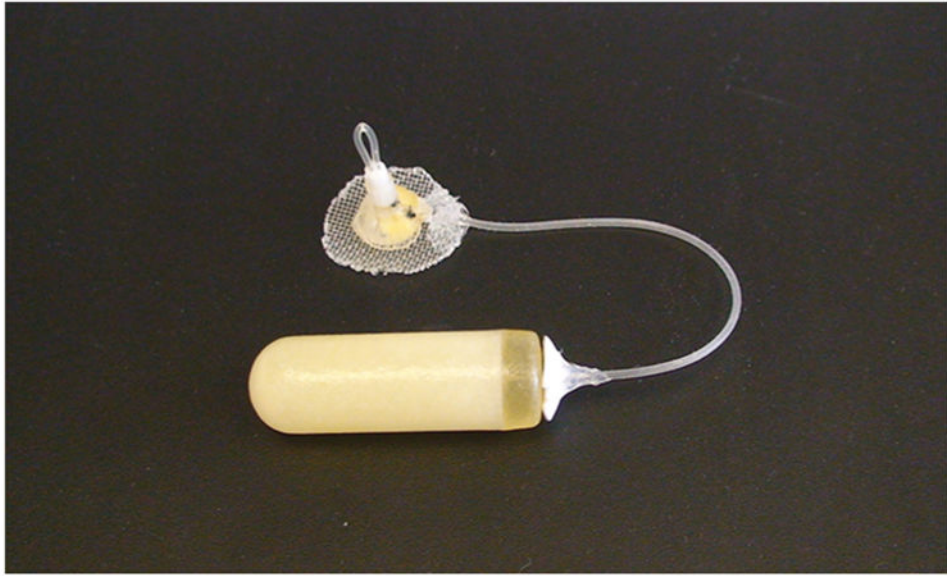
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**Highlights**

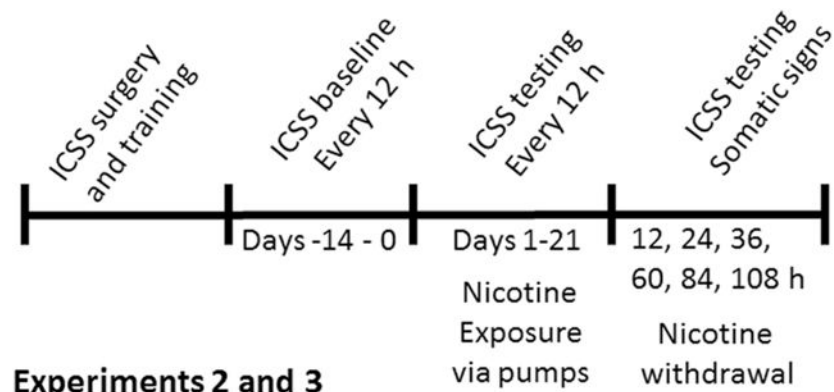
- Non-contingent continuous nicotine produced withdrawal and upregulated  $\alpha 4\beta 2$  nAChRs.
- Non-contingent intermittent nicotine exposure did not produce nicotine withdrawal.
- Non-contingent intermittent nicotine upregulated  $\alpha 4\beta 2$  nAChRs in the VTA and PLC.
- Contingent intermittent nicotine exposure upregulated  $\alpha 4\beta 2^*$  nAChRs in the Acb.



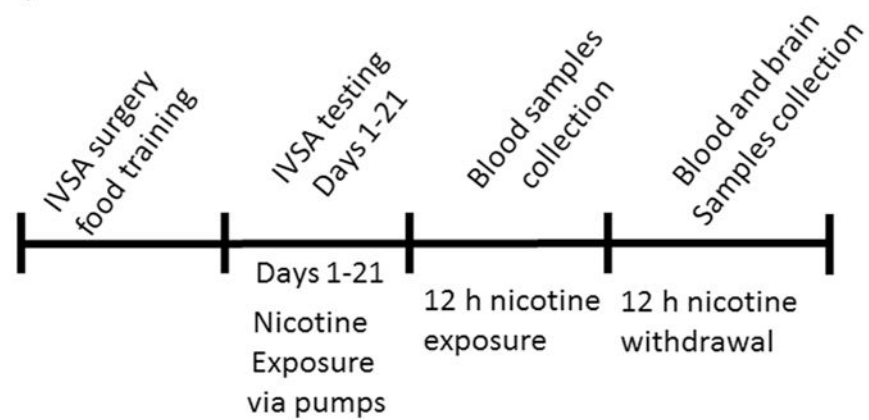
**Figure 1.** Photograph of a modified osmotic minipump showing an assembled gating device connected to a standard osmotic minipump (model 2ML4, Alzet Osmotic Pumps, Cupertino, CA).

## Experimental design

### Experiment 1

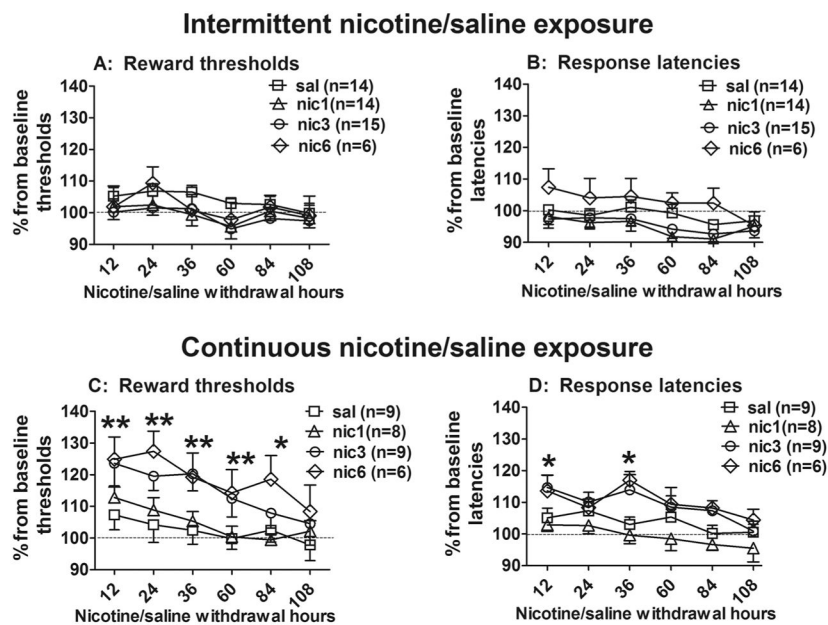


### Experiments 2 and 3

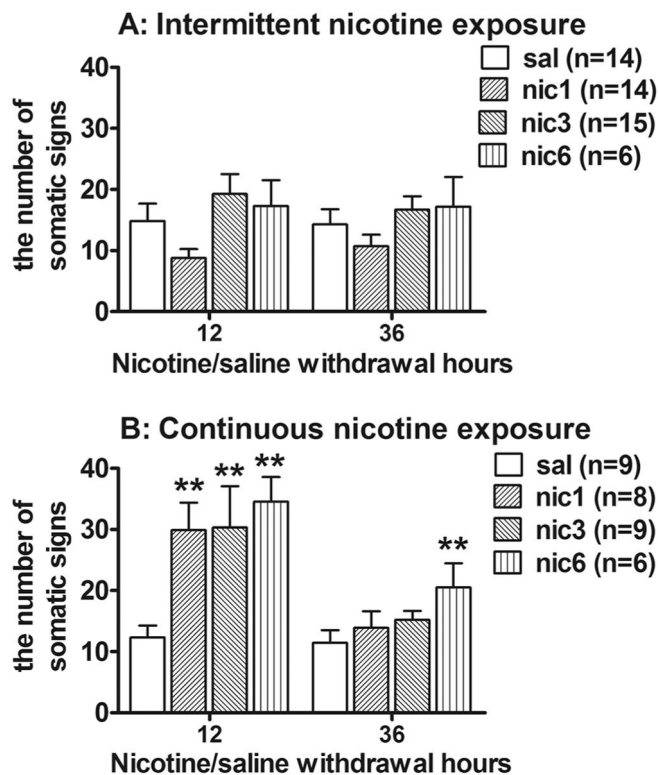


#### Figure 2. Experimental design

Timeline of experimental design for Experiment 1 (top panel) and Experiments 2 and 3 (bottom panel) showing the sequence of experimental procedures, nicotine exposure and blood/brain samples collection. See text for details. ICSS, intracranial self-stimulation; IVSA, intravenous self-administration; h, hour.

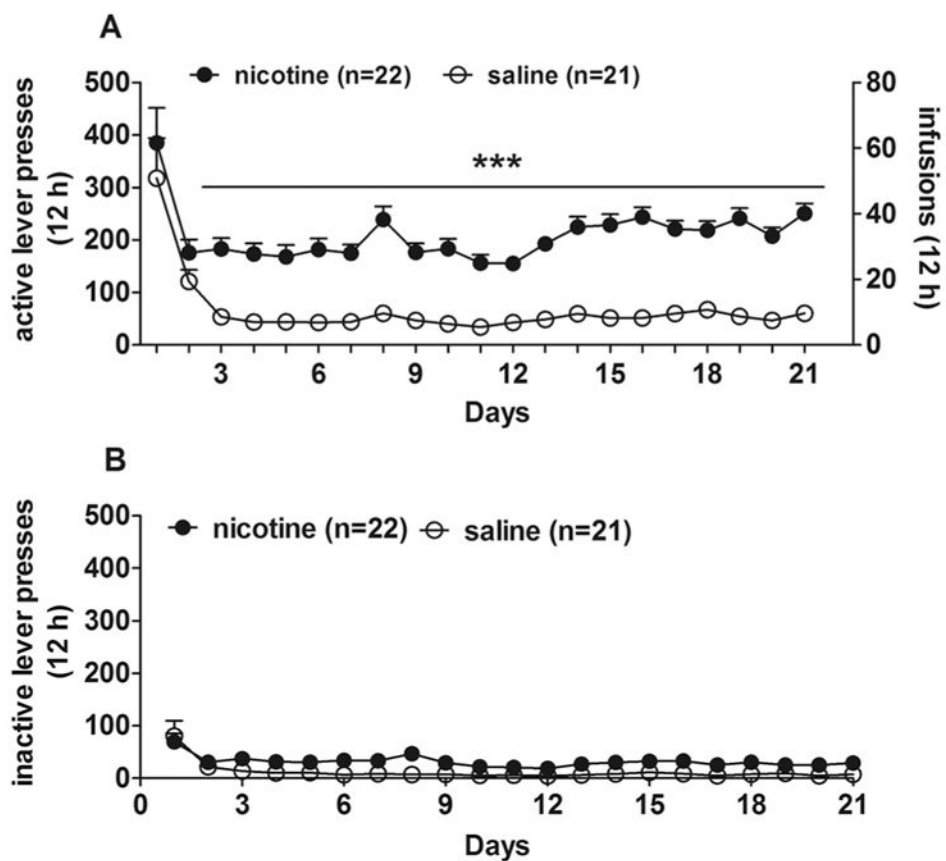


**Figure 3.** The effects of withdrawal from intermittent and continuous nicotine exposure on brain reward thresholds (A and C, respectively) and response latencies (B and D, respectively). Data are presented as a percentage of baseline thresholds/latencies (mean  $\pm$  SEM) before nicotine/saline exposure. Asterisks denote statistically significant differences between corresponding groups of nicotine- and saline-exposed rats (\*\* $p < 0.01$ , \* $p < 0.05$ , LSD *post hoc* test).

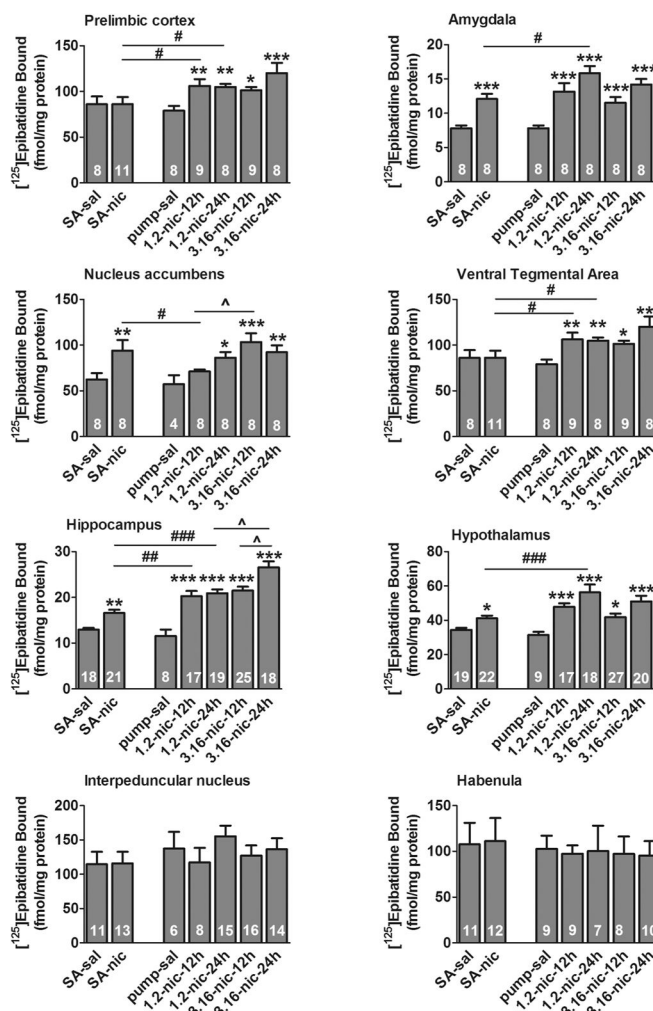


**Figure 4.** The effects of withdrawal from the intermittent (A) and continuous (B) nicotine exposure on somatic signs of nicotine withdrawal (mean  $\pm$  SEM). Asterisks denote statistically significant differences between nicotine- and saline-exposed rats (\*\* $p < 0.01$ , \* $p < 0.05$ , LSD *post hoc* test).





**Figure 5.** Nicotine self-administration in rats. (A) The number of infusions and active lever presses obtained daily during the 21-day self-administration period. (B) The number of inactive lever presses obtained daily during the 21-day self-administration period. Data are expressed as mean  $\pm$  SEM. \*\*\* $p < 0.001$ , compared with saline using LSD *post hoc* comparisons.



**Figure 6.** Cytisine-sensitive nicotinic acetylcholine receptor binding data in different brain regions. Data are expressed as mean  $\pm$  SEM. Asterisks denote a significant difference between exposures to nicotine (nic) and saline (sal) in corresponding treatments groups (\*,  $p < 0.05$ , \*\* ,  $p < 0.01$ , \*\*\* ,  $p < 0.001$ , LSD *post hoc* test). (#) denotes a significant difference between exposures to low nicotine dose via self-administration (SA) and pumps with intermittent (12 h) or continuous (24 h) nicotine delivery (#,  $p < 0.05$ , ## ,  $p < 0.01$ , ### ,  $p < 0.001$ , LSD *post hoc* test). (^) denotes a significant difference between intermittent exposure to 1.2 mg/kg/12 h and 3.16 mg/kg/12 h nicotine doses via pumps (^,  $p < 0.05$ , LSD *post hoc* test).

Table 1

Cytisine-resistant nicotinic acetylcholine receptor binding (fmol/mg protein) in different brain structures at 12 h post-nicotine/saline exposure.

Group	PLC	AMY	Acb	VTA	HYP	HIPP
SA-sal	81.2±14.2	1.89±0.3	-0.38±0.9	37.5±14.2	1.78±0.8	2.10±0.1
SA-nic	57.7±10.1	2.36±0.4	0.00±0.0	32.04±10.1	1.52±0.9	2.51±0.1
Pump-sal	64.3±9.7	1.89±0.3	1.3±1.7	25.78±9.7	3.40±0.4	1.3±0.2 <sup>#</sup>
1.2-nic-12h	77.0±7.4	2.79±0.4	0.00±0.0	21.04±7.4	0.98±1.0	2.81±0.2 <sup>*</sup>
1.2-nic-24h	74.8±10.1	2.65±0.3	0.00±0.0	26.8±10.1	0.35±1.5	3.23±0.2 <sup>*</sup>
3.16-nic-12h	76.1±7.3	2.58±0.3	0.00±0.0	20.76±7.3	1.11±1.05	3.00±0.2 <sup>*</sup>
3.16-nic-24h	71.6±18.5	2.90±0.3	0.00±0.0	48.92±18.5	1.16±0.8	3.88±0.3 <sup>*^</sup>

Data are expressed as mean ± SEM.3

<sup>\*</sup>  $p < 0.001$  nicotine exposure via pumps compared to saline exposure via pumps;

<sup>#</sup>  $p < 0.05$  saline exposure via self-administration compare to saline exposure via pumps;

<sup>^</sup>  $p < 0.01$  intermittent (12 h) compared to continuous (24 h) exposure to 3.16 mg/kg/day nicotine dose via pumps.

Abbreviations: PLC, prefrontal cortex, AMY-amygdala, Acb, nucleus accumbens, VTA, ventral tegmental area, HYP, hypothalamus, HIPP, hippocampus, nic, nicotine, sal, saline, SA, self-administration.

**Table 2**

Plasma nicotine and cotinine levels following exposure to nicotine or nicotine withdrawal in rats exposed to nicotine/saline for 21 days.

Temporal Pattern	Nicotine dose	N	Cotinine (ng/ml)	Nicotine (ng/ml)
<i>Exposure to nicotine</i>				
Intermittent-SA	1.2 mg/kg/12h	12	666.4±78.2 <sup>a</sup>	35.4±7.1
Intermittent-pump	1.2 mg/kg/12h	9	510.25±158.4 <sup>a</sup>	14.45±5.2 <sup>ab</sup>
Continuous-pump	1.2 mg/kg/24h	9	450.73±30.9 <sup>a</sup>	13.53±1.44 <sup>ab</sup>
Intermittent-pump	3.16 mg/kg/12h	9	825.04±358.5 <sup>a</sup>	29.69±11.6
Continuous-pump	3.16 mg/kg/24h	10	1211.12±40.6	38.69±2.1
<i>Exposure to 12h nicotine withdrawal</i>				
Intermittent-SA	1.2 mg/kg/12h	12	141.6±17.1	
Intermittent-pump	1.2 mg/kg/12h	9	162.6±32.4 <sup>ac</sup>	
Continuous-pump	1.2 mg/kg/24h	9	104.9±9.2 <sup>ac</sup>	
Intermittent-pump	3.16 mg/kg/12h	9	540.8±87.7 <sup>a</sup>	
Continuous-pump	3.16 mg/kg/24h	10	284.5±14.4	

Data are expressed as mean ± SEM.

<sup>a</sup>denotes a significant group differences from continuous exposure to 3.16 mg/kg/24h nicotine dose (a,  $p < 0.05$ , LSD test);

<sup>b</sup>denotes a significant group differences from nicotine self-administration (b,  $p < 0.05$ , LSD test);

<sup>c</sup>denoted a significant group differences from intermittent exposure to 3.16 mg/kg/12h nicotine dose (c,  $p < 0.05$ , LSD test).

During nicotine withdrawal, no nicotine was detected in plasma. No nicotine or cotinine were detected in plasma in control rats exposed to saline either via minipumps or self-administration. SA, self-administration, N, number of samples per group.