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Behavioral and Neural Substrates of Habit Formation in Rats Intravenously Self-Administering Nicotine

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Tobacco addiction involves a transition from occasional, voluntary smoking towards habitual behavior that becomes increasingly resistant to quitting. The development of smoking habits may reflect a loss of behavioral control that can be modeled in rats. This study investigated the behavioral and neural substrates of habit formation in rats exposed to either brief (10 days) or extended (47 days) intravenous (IV) nicotine self-administration training. Following training, the first cohort of rats were exposed to a nicotine devaluation treatment, which involved repeated pairings of IV nicotine with lithium injection. They were then tested for sensitivity of responding to nicotine devaluation under extinction and reinstatement conditions. The second cohort of rats received equivalent self-administration training followed by processing of brain tissue for c-Fos immunohistochemistry. After brief training, devaluation suppressed nicotine-seeking during tests of extinction and reinstatement, confirming that responding is initially sensitive to current nicotine value, and therefore, goal directed. In contrast, after extended training, devaluation had no effect on extinction or reinstatement of responding, indicating that responding had become habitual. Complementary neuroanalysis revealed that extended nicotine self-administration was associated with increased c-Fos expression in brain regions implicated in habitual control of reward seeking, including activation of the dorsolateral striatum and substantia nigra pars compacta. These findings provide evidence of direct devaluation of an IV drug reward, that nicotine self-administration is initially goal-directed but becomes habitual with extended training, and that this behavioral transition involves activation of brain areas associated with the nigrostriatal system.
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INTRODUCTION

Tobacco is highly addictive, with users continuing to smoke despite clear evidence of severe negative health consequences (World Health Organisation, 2011). This insensitivity to the dangers associated with smoking may be partly due to the formation of smoking habits, as cues and environments repeatedly paired with smoking come to trigger the behavior reflexively or automatically. Despite the intuitive association between tobacco and habits, few studies have explicitly examined this relationship; and among those that have, the findings are mixed (Dar *et al*, 2010; Hogarth and Chase, 2011).

Animal studies investigating the behavioral and neural correlates of habit formation have largely focused on responses aimed at procuring a food reward. The logic underpinning these studies is that behaviors that are voluntary or aimed at obtaining a specific reward will be sensitive to changes in its current value: if the reward value is reduced via pre-feeding to satiety or pairings with a nauseating agent (lithium chloride), responses that normally lead to the reward will decline. In contrast, habitual behaviors that develop with repeated training are insensitive to changes in current reward value; they are triggered reflexively or automatically by specific cues or environments previously associated with reward (Adams and Dickinson, 1981; Balleine and Dickinson, 1998).

Extending such findings to the case of intravenously (IV) administered drug rewards has proven difficult due to the absence of gustatory or ingestive reward features; components deemed necessary for effective reward devaluation using standard methods (Belin-Rauscent *et al*, 2012). For this reason, alternative approaches have been employed, including oral drug delivery (Dickinson *et al*, 2002; Miles *et al*, 2003) or the use of seeking–taking schedules to provide indirect measures of reward devaluation (Olmstead *et al*, 2001; Zapata *et al*, 2010). Although informative, such procedures may be limited as the method and route of administration are critical to the long-term behavioral (Leblanc *et al*, 2013) and neurobiological (Chen *et al*, 2008; Stefanski *et al*, 1999) effects of addictive drugs. Here we propose that IV nicotine self-administration may be particularly amenable to devaluation through techniques typically employed with natural rewards (ie, pairing nicotine with lithium-induced nausea) due to the unique

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characteristics of nicotine self-administration. First, rats are relatively poor at titrating nicotine intake with changing infusion dose, but are highly sensitive to non-pharmacological factors, including physical response requirements (nose poke *vs* lever press; Clemens *et al*, 2010) and the presence of nicotine-related cues (Caggiula *et al*, 2009). Second, nicotine produces strong interoceptive cues that can enter into associations with other stimuli (Charntikov *et al*, 2012), such as lithium-induced nausea (Pittenger and Bevins, 2013). Accordingly, the first aim of the current study was to use lithium devaluation to examine habit formation in rats receiving extended nicotine self-administration.

Two distinct neural circuits have been shown to support goal-directed and habitual responding for natural rewards in rats. Goal-directed behavior relies on the integrity of the ventral and dorsomedial striatum (DMS), whereas habitual responding relies on dopaminergic input from the substantia nigra to the dorsolateral striatum (DLS) (Corbit and Janak, 2010; Faure *et al*, 2005; Yin *et al*, 2005). The contribution of these structures to maintaining IV nicotine self-administration, particularly after extended exposure, remains to be explored. Therefore, the second aim of this study was to use c-Fos immunohistochemistry to investigate whether extended nicotine self-administration increases activation of brain regions implicated in the development of habits.

MATERIALS AND METHODS

Subjects

Male Sprague–Dawley rats (175–200 g; Animal Resources Centre, Perth, Australia) were housed four/cage on a 12-h reverse light/dark cycle (lights off at 0600 h). Food and water were available *ad libitum* before surgery and during recovery, and thereafter restricted to 20 g/rat/day. All experiments were approved by the Macquarie University Animal Ethics Committee (ARA2010/039, 2011/007) and were in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes (7th ed.).

Drugs

Nicotine tartrate (Sigma, St Louis, MO, USA) was dissolved in 0.9% sterile saline and administered IV at 30 μg nicotine base/kg/100 μl infusion. Lithium chloride (Sigma) was administered as an intraperitoneal (IP) injection (10 ml/kg) at a concentration of 0.15 M.

Self-Administration

All rats received implantation of a chronic IV catheter into the right jugular vein as described previously (Motbey *et al*, 2013).

Training commenced with two 1-h sessions of habituation to the self-administration chambers (Med Associates, VT, USA) with the nose pokes covered and house-light on. Self-administration training then commenced, with responses in the active nose poke resulting in an infusion of nicotine (3 s), illumination of the nose-poke cue light (3 s), and extinction of the house light (20 s). Active responses made during the 20-s time-out or in the inactive nose-poke hole had no programmed consequences. Rats were considered to have acquired self-administration when they achieved a minimum of six infusions per 60-min session, and demonstrated a preference for the active nose poke (2 active : 1 inactive). Data from rats that did not acquire self-administration were excluded from analysis.

Experiment 1: The Effects of Lithium Devaluation on Nicotine-Seeking Following Brief or Extended Self-Administration

Training and devaluation. Sixty-four rats were subjected to either brief (10 days) or extended (47 days) training. Twenty-four hours after the final training session, all rats underwent devaluation training. Rats were assigned to the Paired or Non-Paired treatment condition based on average performance (active and inactive nose pokes) across the last 5 days of self-administration.

The devaluation procedure was based on previous research using lithium chloride to devalue natural food rewards (Nelson and Killcross, 2006). Devaluation was conducted in a room separate from both the self-administration chambers and the colony room. On days 1, 3 and 5 of devaluation treatment, rats in the 'Paired' group (nicotine paired with lithium) received 1×0.1 ml experimenter-administered infusion of IV nicotine (30 μg/kg/infusion; across 3 s), followed immediately by an IP injection of lithium. Sixty seconds later, rats received a second experimenter-administered IV nicotine infusion. This dose and time-course were selected based on the average interval between the first and second responses made during the self-administration phase, and are sufficient to produce clear physiological (increase in activity) and motivational (reinstatement of nicotine-seeking) effects (Clemens *et al*, unpublished data). Rats in the 'Non-Paired' group (saline paired with lithium) received IV infusions of saline with IP injections of lithium following the same procedure.

On days 2, 4 and 6 of the devaluation treatment, rats in the 'Paired' group received pairings of IV saline with IP saline, whereas rats in the 'Non-Paired' group received pairings of IV nicotine with IP saline. This ensured that Paired and Non-Paired rats received equal exposure to IV nicotine and lithium injections, and differed only in their temporal association.

After the infusion-injection-infusion sequence, rats were monitored in individual holding cages for 10–15 min before they were returned to the home cage and colony room. The daily food ration was delivered 3 h after the devaluation procedure to reduce the possibility of an association forming between the lithium and rat chow.

Extinction and reinstatement. Twenty-four hours after the last devaluation session, all rats were returned to the self-administration chambers for a 30-min test session under extinction conditions (house-light on, nose-poke responses now without consequence) to examine nicotine-seeking in the absence of the reward itself. No cues were presented at any time.

After the extinction test, each rat was removed from the chamber and received two experimenter-administered IV infusions of nicotine (2×0.1 ml at 60 s interval). The rat was then immediately returned to the chamber for a further

30-min session under extinction conditions to test for reinstatement of nose-poke responding.

Reacquisition. The following day, all rats underwent a 30-min reacquisition session, which was identical to previous self-administration sessions: nicotine infusions and associated visual cues were again available in a response-contingent manner.

Experiment 2: c-Fos Immunoreactivity Following Brief or Extended Nicotine Self-Administration

Training and tissue processing. Rats underwent surgery and training as described above (Nicotine-Brief and Nicotine-Extended, $n = 5$/group), with the addition of two control groups that self-administered saline only (Saline-Brief and Saline-Extended, $n = 5$/group).

Thirty minutes after the final training session, rats were anesthetized (pentobarbitone sodium, 325 mg/ml/rat) and transcardially perfused with phosphate-buffered saline (PBS; 0.1 M, pH 7.3) then 4% paraformaldehyde in PBS. Brain sections were postfixed in 4% paraformaldehyde (24 h, 4 °C), then 15% PB sucrose (24 h, 4 °C) followed by 30% PB sucrose until sectioning. Consecutive coronal sections (40 μm) were cut using a vibratome and stored in a freezing solution (30% ethylene glycol, 25% glycerol in PBS) at −20 °C.

Briefly, free-floating sections were washed (3 × tris-PBS (TPBS), Tris–HCl 10 mM, sodium phosphate buffer 10 mM, 0.9% NaCl, pH 7.4), then incubated in the rabbit polyclonal anti-c-Fos; antibody (sc-52; Santa Cruz Biotechnology, CA, USA) for 48 h at 4 °C diluted 1:1000 in TPBS containing 5% normal horse serum (NHS). After washing 3 × 30 min, sections were incubated overnight (4 °C) in biotinylated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories, PA, USA) diluted 1:500 in TPBS containing 2% NHS). The tissue was then washed before incubation for 24 h (4 °C) in ExtraAvidin-horseradish peroxidase (diluted 1:1000 in PB saline, Sigma). Bound antibodies were visualized using a nickel-intensified diaminobenzidine and glucose oxidase reaction, which was terminated after 15 min using dilution with TPBS. Processed sections were washed (3 × TPBS) and then mounted onto gelatinized slides, dehydrated using graded ethanol, xylene cleared and coverslipped using UltraMount (Thermo Fisher Scientific Australia, VIC, Australia).

Neuronal counting. c-Fos immunoreactive (IR) sections were viewed using an Olympus BX53 System Microscope under bright-field illumination (Olympus, Tokyo, Japan) and a ×20 objective by an observer blind to the experimental group. Boundaries of anatomical regions were determined using Paxinos and Watson (1998) and coordinates reported as distance from bregma.

The size and location of regions for counting are illustrated in Figure 1. Regions of interest were selected based on their known involvement in reward-related processes, including expression of goal-directed and habitual behavior. Brain regions were counted on sections 160 μm apart, 3–6 slices for each animal depending on the anterior–posterior extent of the structure. Counts were

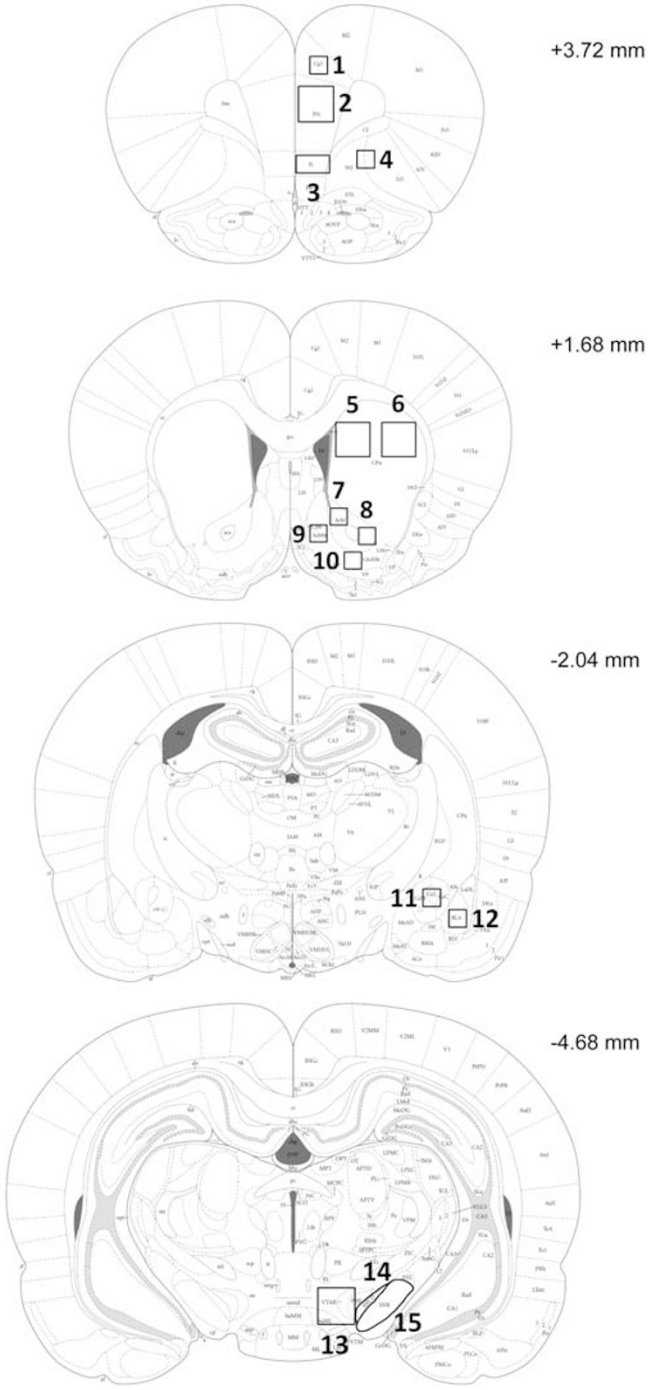

Figure 1 Schematic representations of coronal sections illustrating the brain regions analyzed for c-Fos protein expression in Experiment 2. Counts included sections beginning at +3.72, +1.68, −2.04, and −4.68 mm from the bregma (Paxinos and Watson, 1998). The numbered regions and the size of the area counted are as follows: (1) Cg1 region of the anterior cingulate cortex (Cg1, 0.5 mm^2); (2) prelimbic cortex (PrL, 1.0 mm^2); (3) infralimbic cortex (IL, 1.0 × 0.6 mm); (4) orbitofrontal cortex (OFC, 1.0 mm^2); (5) dorsomedial striatum (DMS, 1.0 mm^2); (6) dorsolateral striatum (DLS, 1.0 mm^2); (7, 8) nucleus accumbens core (NAcC, 2 × 0.4 mm^2); (9, 10) nucleus accumbens shell (NAcSh, 2 × 0.4 mm^2); (11) central amygdala (CEA, 0.5 mm^2); (12) basolateral amygdala (BLA, 0.5 mm^2); (13) ventral tegmental area (VTA, 1.0 mm^2); (14) substantia nigra pars compacta (SNPC, whole structure; (15) substantia nigra pars reticulata (SNPR, whole structure).

averaged for each structure of interest in each animal and normalized to give counts per mm^2 (with the exception of the substantia nigra pars compacta (SNPC) and substantia nigra pars reticulata (SNPR) where the entire structure was counted).

Data Analysis

Nose-poke responses averaged across the last 5 days of acquisition were analysed using a mixed model ANOVA with between-subjects factors of training (brief *vs* extended) and devaluation (paired *vs* non-paired), and a within-subjects factor of operandum (active *vs* inactive response).

Nose-poke responses in extinction and reinstatement tests declined rapidly across each of these sessions and, thus, analyses of these data were confined to data from the first 5 and 15 min of these tests, respectively. To account for variation in levels of baseline responding with increasing training, and to account for responding specific to the active nose poke, we normalized responding using the following ratio: (active–inactive nose pokes at test)/(active–inactive nose pokes on the last day of self-administration).

These normalized data were analyzed using planned orthogonal contrasts (Bird, 2004). The critical contrast examined whether responding in the Brief-Paired group differed from that in the remaining groups (Brief-Non-Paired, Extended-Paired, and Extended-Non-Paired). Remaining contrasts examined whether responding in the Brief-Non-Paired group differed from that in both Extended groups; and whether responding in the Extended-Paired group differed from that in the Extended-Non-Paired group. For each analysis, the criterion for rejection of the null hypothesis (ie, $F_{critical}$) with 1 and 40 degrees of freedom was set at 4.09, $\alpha = 0.05$.

Latency to the first response was analyzed using Mann–Whitney *U*-test as this data was not normally distributed (detected using the Kolmogorov-Smirnov test).

Immunohistochemistry

Nose-poke responses across the last five days of self-administration training were analyzed as described above.

C-Fos-IR neurons were analyzed for each structure using a two-way ANOVA with training (brief *vs* extended) and infusion drug (nicotine *vs* saline) as between-subjects factors. Contrasts were not used here due to the large number of comparisons and the variation in direction of predicted differences across regions. *Post-hoc* analysis was conducted using a Scheffe corrected $F_{critical}$ of 9.02.

RESULTS

Experiment 1: The Effects of Lithium Devaluation on Nicotine-Seeking Following Brief or Extended Self-Administration

Nineteen rats were excluded from data analysis. Eleven did not satisfy the acquisition criteria, two had responding and total nicotine intake beyond 2 SEM, and six lost catheter patency. The final group sizes were Brief-paired, $n = 9$, Brief-non-paired, $n = 10$; Extended-paired, $n = 12$, and Extended-non-paired, $n = 13$.

Acquisition. Data showing acquisition of self-administration is included in Supplementary Materials S1. The mean number of active and inactive responses during the last 5 days of training for each group was: Nicotine-Brief-Paired: 14.60 ± 1.15, 6.27 ± 2.13; Nicotine-Brief-Non-Paired: 16.58 ± 0.99, 5.66 ± 1.37; Nicotine-Extended-Paired: 29.38 ± 2.86, 7.28 ± 1.97, Nicotine-Extended-Non-Paired: 29.72 ± 2.96, 9.03 ± 1.31. Responding was greater in the active hole than the inactive hole (main effect of nose-poke hole: $F_{1,40} = 122.54$, $p < 0.001$) and increased with training (main effect of training: $F_{1,40} = 25.63$, $p < 0.001$). A nose-poke hole by training interaction ($F_{1,40} = 17.46$, $p < 0.001$) indicated that the increase in responding with training was specific to the active hole. There were no main effects or interactions involving the devaluation factor ($Fs < 1$).

Extinction. Lithium-induced devaluation of nicotine had no significant effect on the latency to the first response during the extinction test in either brief or extended training groups (Figure 2a).

Figure 2b shows conditioned responding (active minus inactive responses) relative to baseline for each group in the first 5 min of the extinction test. After this, time responding rapidly extinguished in all groups (Supplementary Materials S2). Rats in the Brief-Paired group responded significantly less than all other groups ($F_{1,40} = 7.34$, $p < 0.05$). There were no significant differences between the Brief-Non-Paired and both Extended groups, or between Extended-Paired and Extended-Non-Paired groups ($Fs < 1$). Hence, nicotine–lithium pairings suppressed responding after brief but not extended self-administration training.

Reinstatement. Following a priming infusion of nicotine, the median latency to the first response was longer in the Brief-Paired group than the Brief-Non-Paired group (two-sample K–S test for normality $D(19) = 0.34$, $p < 0.05$; Mann–Whitney *U*-test $= 20.5$, $n_1 = 9$, $n_2 = 10$, $p < 0.05$ two-tailed, Figure 2c). In contrast, among rats subjected to extended training, the median latency to the first response did not differ between paired and non-paired conditions.

Figure 2d shows conditioned responding (active minus inactive responses) relative to baseline for each group in the first 5 min of the reinstatement test. After this, time responding rapidly extinguished in all groups (Supplementary Materials S2). Similar to the extinction test, responding was supressed in the Brief-Paired group relative to all other groups ($F_{1,40} = 4.19$, $p < 0.05$). There were no differences between the Brief-Non-Paired and Extended groups, or between the Extended-Paired and Extended-Non-Paired groups ($Fs < 1$).

Thus, nicotine–lithium pairings suppressed reinstatement after brief but not extended self-administration training.

Reacquisition. The latency to the first active response during reacquisition was similar for all groups (Figure 2e).

Figure 2f shows conditioned responding (active minus inactive responses) relative to baseline for each group during the 30-min reacquisition test. There was no overall effect of devaluation ($F < 1$); however, a significant

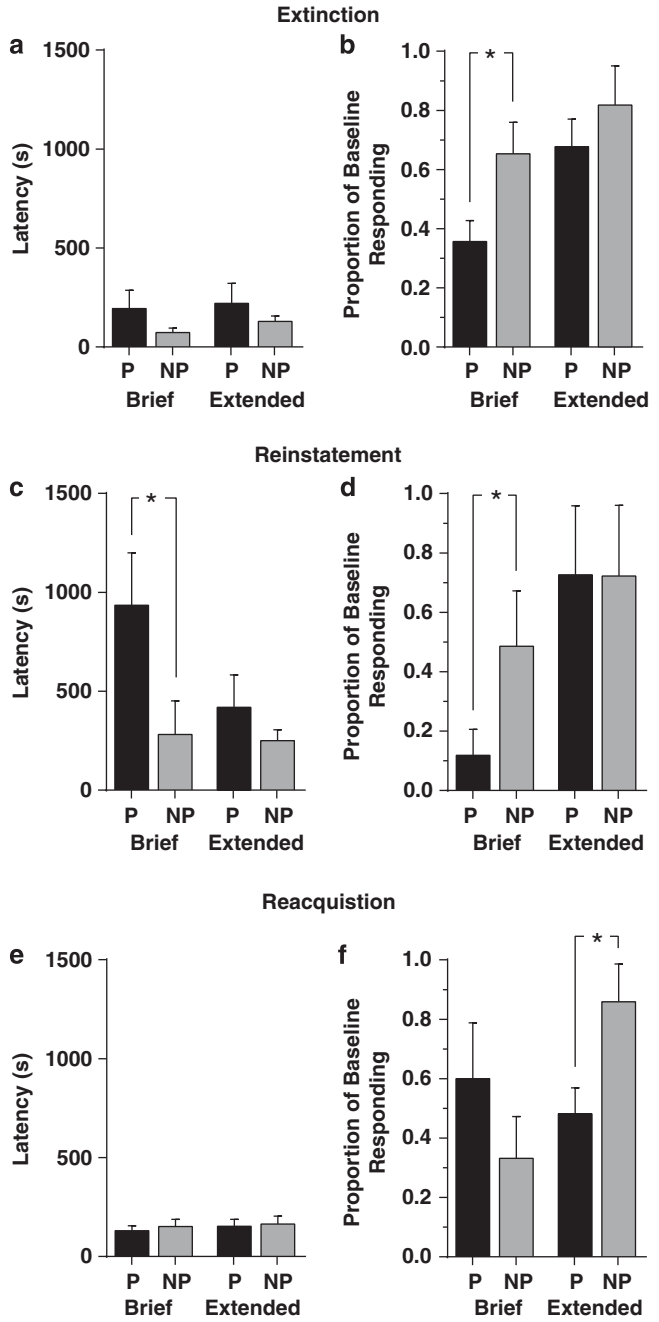

Figure 2 Latency to the first response (seconds) at the beginning of the session and conditioned responses (active minus inactive) as a proportion of baseline during the extinction (a and b), reinstatement (c and d) and reacquisition (e and f) tests. Rats had received nicotine paired with lithium (Paired: P) or with saline (non-paired: NP) after brief or extended self-administration training. Data represents group means ± SEM. Asterisks indicate significant difference between Paired and Non-Paired when $p<0.05$.

devaluation by training interaction ($F_{1,40}=5.86$, $p<0.05$) indicated that the difference between paired and non-paired conditions varied with training history. Responding was suppressed in the Extended-Paired group compared with Extended-Non-Paired ($F_{1,23}=4.28$, $p<0.05$), but there was no such difference among rats in the Brief-Paired and Brief-Non-Paired groups ($F<1$).

Locomotor activity data for all tests is included in Supplementary Materials S3.

Experiment 2: c-Fos Immunoreactivity Following Brief or Extended Nicotine Self-Administration

Self-administration. The acquisition of self-administration across sessions is included in Supplementary Materials S4. The mean number of active and inactive responses during the last 5 days of training for each group was: Saline-Brief: 3.60 ± 1.19, 3.24 ± 0.06; Nicotine-Brief: 20.24 ± 3.10, 9.96 ± 3.19; Saline-Extended: 5.68 ± 1.34, 1.04 ± 0.21; Nicotine-Extended: 29.60 ± 3.44, 5.92 ± 1.62. Rats self-administering nicotine made significantly more responses than those infusing saline (main effect of treatment: $F_{1,16}=48.79$, $p<0.001$) and this responding was specific to the active nose poke (main effect of nose-poke hole: $F_{1,16}=75.80$, $p<0.001$; nose-poke hole by treatment interaction: $F_{1,16}=15.61$, $p<0.001$).

c-Fos immunohistochemistry. Fos IR neurons were observed in all regions of interest (Table 1). Figures 3 and 4 show average counts and photomicrographs of specific regions of interest.

There were no significant group differences in c-Fos expression in any of the prefrontal cortex regions counted (Cg1 region of the anterior cingulate cortex (Cg1), prelimbic cortex (PRL), infralimbic cortex (IL), and orbitofrontal cortex) or the SNPR.

Independently of the extent of training, nicotine self-administration increased c-Fos expression in the nucleus accumbens core (NAcC), nucleus accumbens shell (NAcSh), ventral tegmental area (VTA), basolateral amygdala (BLA), and central amygdala (CEA; main effect of drug; ranged from $F_{1,16}=4.75$ to 21.81, $p=0.045<0.001$).

A significant increase in c-Fos expression that was specific to the nicotine extended training group was detected in three brain regions. This was evident as a significant drug by training interaction in the DMS ($F_{1,14}=4.83$, $p<0.05$) and the SNPC ($F_{1,16}=7.06$, $p<0.05$). A main effect of training in the DLS ($F_{1,14}=8.57$, $p<0.05$) without any interaction suggested higher levels of c-Fos in both the saline and nicotine extended training groups compared to their brief counterparts. Inspection of Figure 3d suggests that this was due to increased c-Fos expression in the nicotine extended group only, and a *post-hoc* test confirmed that this was the case (nicotine extended *vs* all, $F_{1,14}=14.44$, $p<0.01$).

DISCUSSION

This study shows for the first time that an intravenously administered reward can be devalued through pairings with the nauseating agent lithium, and that this method of reward devaluation can be used to indicate habitual seeking of an IV drug reward. It is also the first description of any involvement of habits in the persistence of nicotine-seeking in rats, and of changes in nicotine-dependent neural activation that occur with extended nicotine self-administration.

This study demonstrates clear differences in sensitivity to reward value depending on the extent of training. Pairing

Table 1 Mean Number of Fos-Positive Nuclei for Each Region of Interest in Experiment 2

Training	Brief		Extended	
Drug	**Saline**	**Nicotine**	**Saline**	**Nicotine**
Cingulate cortex	29.20 (8.43)	25.53 (5.74)	16.40 (6.03)	35.60 (9.77)
Prelimbic cortex	48.00 (9.08)	49.33 (6.04)	44.94 (5.72)	57.90 (5.11)
Infralimbic cortex	40.59 (6.97)	35.71 (5.61)	44.40 (8.30)	48.65 (3.87)
Orbitofrontal cortex	91.20 (26.20)	89.00 (11.65)	101.00 (30.36)	112.60 (22.35)
Dorsomedial striatum[a]	35.45 (4.43)	44.77 (1.58)	32.24 (6.77)	69.76 (8.30)
Dorsolateral striatum[b]	1.81 (0.74)	2.42 (0.72)	2.71 (0.84)	6.39 (1.36)
Nucleus accumbens core[c]	64.69 (17.02)	146.75 (29.38)	51.02 (8.07)	133.54 (19.63)
Nucleus accumbens shell[c]	49.06 (22.61)	83.75 (19.22)	44.04 (9.06)	84.06 (12.55)
Central amygdala[c]	7.15 (2.17)	18.01 (4.17)	8.16 (2.34)	12.00 (4.24)
Basolateral amygdala[c]	15.60 (3.75)	31.20 (3.68)	8.56 (2.54)	25.35 (3.75)
Ventral tegmental area[c]	23.50 (6.46)	43.00 (9.45)	20.40 (8.19)	61.90 (9.42)
Substantia nigra				
Pars compacta[a]	5.55 (3.35)	8.33 (3.05)	2.35 (0.90)	24.20 (5.50)
Pars reticulata	0.06 (0.06)	0.06 (0.06)	0.00 (0.00)	1.03 (0.78)

Data indicates group mean and SEM.
[a]Training by treatment interaction.
[b]Main effect of training (brief *vs* extended).
[c]Main effect of infusion drug (nicotine *vs* saline).

nicotine with lithium in rats that had received only brief training resulted in a clear suppression of responding during both the extinction and reinstatement tests relative to all other groups. This reduction indicates that at this stage of training, responding was controlled by a representation of its consequences (ie, IV infusion of nicotine) and associated value, and is therefore goal directed. Importantly, the reduction of responding in the nicotine-brief group was evident despite low rates of responding across the test session, was specific to the active nose poke, and was not associated with indirect effects of devaluation on locomotor activity (see Supplementary Materials). In contrast, responding by rats that had received extended nicotine self-administration training was insensitive to devaluation in both tests; nicotine–lithium pairings did not affect responding during extinction, or following a priming injection during reinstatement. This suggests that with extensive training, nicotine self-administration becomes habitual, triggered automatically or reflexively by its stimulus antecedents (eg, the self-administration context).

Importantly, the absence of a devaluation effect following extended training was not due to ineffective formation of a nicotine-illness association. The extended-paired group exhibited a clear suppression of responding in the reacquisition test, indicating that these rats had formed a nicotine–lithium association, but that this association did not influence responding until nicotine was once again available in a response-contingent manner (see Jonkman *et al*, 2010; Nelson and Killcross, 2006).

In contrast, the effectiveness of devaluation in supressing test responding in the brief-paired group did not persist into reacquisition. This may reflect formation of a weaker nicotine-illness association in the briefly trained rats, which extinguished across the extinction and reinstatement tests, or simply weakened across time. The extended training group may have formed a more robust nicotine-illness association, which was immune to extinction across these tests. This may occur as a consequence of the much greater experience with nicotine in these animals, or because of sensitization to nicotine across training (Clemens *et al*, 2009; Kenny and Markou, 2006), which permitted a richer representation of internal drug effects that were subsequently associated with nausea.

The present findings extend those obtained using natural or oral drug rewards (Corbit *et al*, 2012; Dickinson *et al*, 2002; Miles *et al*, 2003) showing that responding that is initially goal directed becomes habitual with overtraining. However, the increasing influence of habits in driving nicotine-seeking does not preclude involvement of other factors in sustaining responding, including the conditioned reinforcing properties of nicotine-paired cues. The contribution of response-contingent cues in sustaining responding across extended training remains to be determined.

The second major finding of this study is that the neural substrates of nicotine IVSA also change across extended self-administration training. Independently of the amount of training, nicotine self-administration increased activation of regions associated with processing of reward, including the NAcC, NAcSh, and VTA (Ikemoto *et al*, 2006), and cues predictive of reward, including the BLA and CEA (Balleine and Killcross, 2006). In contrast, extended self-administration alone resulted in the additional recruitment of brain regions hypothesised to have a crucial role in the expression of habitual and compulsive drug use, including the DLS (Belin and Everitt, 2008; Jonkman *et al*, 2012) and the SNPC

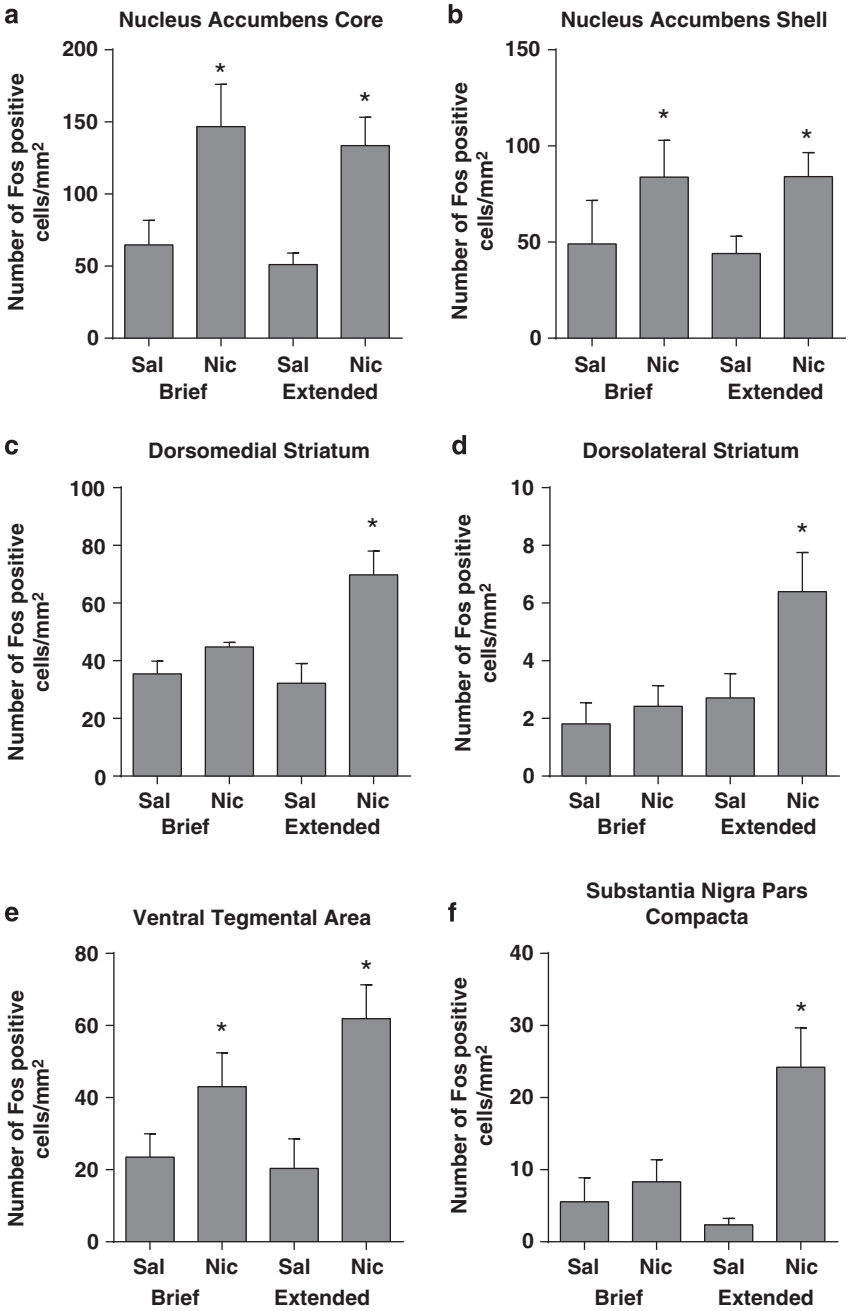

Figure 3 Mean number of Fos-positive nuclei in the (a) nucleus accumbens core, (b) nucleus accumbens shell, (c) dorsomedial striatum, (d) dorsolateral striatum, (e) ventral tegmental area, and (f) substantia nigra pars compacta for rats self-administering saline (SAL) or nicotine (NIC) after either brief (12 days) or extended (42 days) training. Bars indicate group mean ± SEM. Asterisks indicate significant main effect of drug treatment (saline *vs* nicotine) on figures (a, b, and e), main effect of training on figure (d) or drug × treatment interaction on figure (c and f) when $p<0.05$.

(Faure *et al*, 2005). This activation is consistent with contemporary theories of addiction (Belin and Everitt, 2008), suggesting a transition of neuronal control from the mesolimbic dopamine pathway (VTA-NAc) to the nigrostriatal pathway (SNPC-DLS) as behavior becomes increasingly habitual, and eventually, compulsive. Here, for the first time, evidence is provided to indicate increased activation of both the SNPC and DLS with extended nicotine exposure. Further studies are required to address the functional consequences of these changes and to assess whether this behavior corresponds to the onset of compulsive drug-seeking (Deroche-Gamonet *et al*, 2004; Vanderschuren and Everitt, 2004).

Two aspects of the present findings merit further comment. First, whereas past studies have shown that the DMS and DLS express dissociable control over goal-directed and habitual responding, respectively (Corbit and Janak, 2010; Faure *et al*, 2005; Yin *et al*, 2005), we observed an increase in c-Fos activation with extended training in both regions. Second, in contrast to food studies implicating the PRL in maintenance of goal representations and the IL in development of habits (Killcross and Coutureau, 2003),

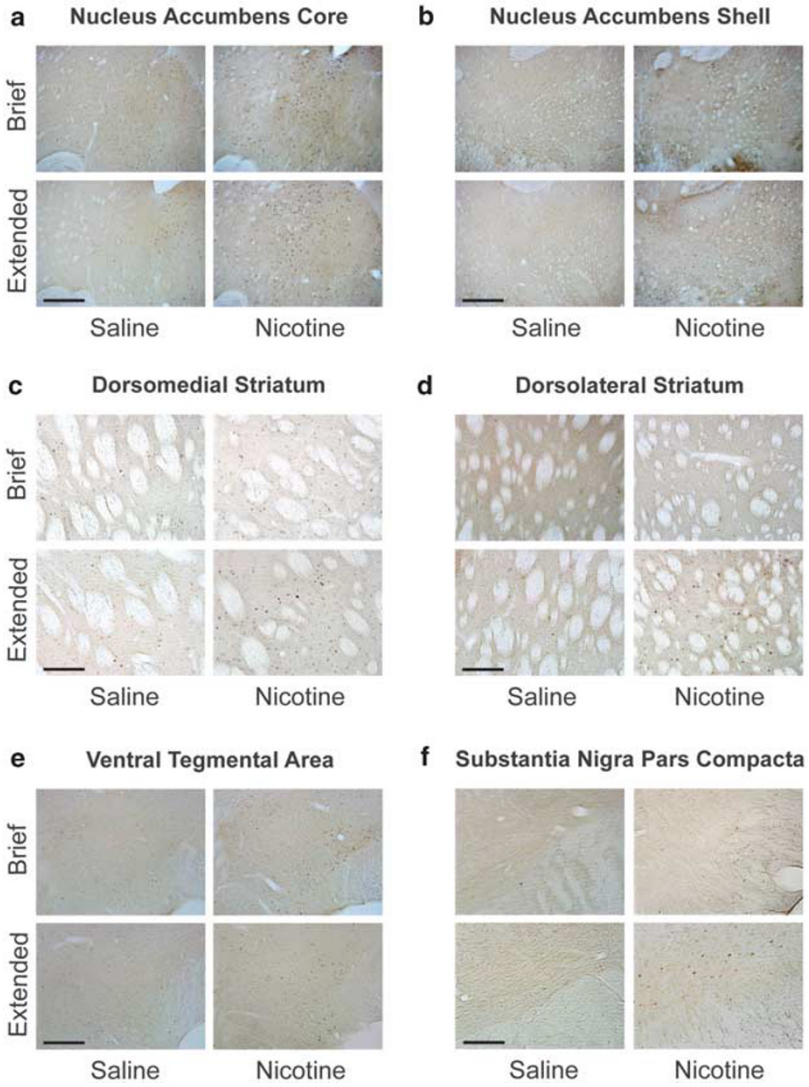

Figure 4 Photomicrographs of Fos-positive nuclei in sections from the (a) nucleus accumbens core, (b) nucleus accumbens shell, (c) dorsomedial striatum, (d) dorsolateral striatum, (e) ventral tegmental area, and (f) substantia nigra pars compacta. Rats intravenously self-administered either saline or nicotine for either brief (12 days) or extended (42 days) training periods. Images were captured at ×20 magnification using an Olympus BX53 Microscope and DP72 Digital Camera. Images were captured using Image-Pro Insight Software (Media Cybernetics, MD, USA) and processed for brightness and contrast using Adobe Photoshop CS6 (Adobe Systems Incorporated, CA, USA). Scale bar represents 500 μm.

there was no evidence for differential c-Fos expression in regions of the prefrontal cortex (but see Pelloux *et al*, 2013). These differences between studies may relate to differences in procedure (eg, reinforcement schedules—see Corbit *et al*, 2012) or specific features of nicotine as a reward. Nicotine primarily exerts its rewarding effects via activation of nicotinic acetylcholine receptors in the VTA (see Markou, 2008 for review); however, receptors are expressed throughout the brain, including the striatum and the SNPC (Keath *et al*, 2007; Marks *et al*, 1992; Wooltorton *et al*, 2003). How changes in nicotine receptor regulation and sensitivity occurring as a consequence of extended drug exposure may interact with neuronal changes associated with the development of habits remains to be determined, but may be crucial in understanding the progressive development of tobacco addiction.

Finally, when studying formation of nicotine habits across time, it is difficult to determine whether a change in sensitivity to devaluation is due to the much higher level of nicotine intake in the extended group, or whether this simply arises through the much higher number of operant responses that have been made in these animals. Extensively pre-treating rats with nicotine before the onset of training may be one approach; however, difficulties associated with varying the method or route of administration, and the potential for producing a conditioned aversion to nicotine (Sellings *et al*, 2008) may limit the interpretation of such results. Indeed, it is likely that both nicotine exposure and overtraining of the nose-poke response contributed to the results achieved here.

In summary, this study demonstrates that pairing of IV administered nicotine with lithium-induced nausea can be

used to reveal that nicotine self-administration is an initially goal-directed behavior that becomes increasingly habitual with extended training. The extended training conditions that led to habitual nicotine-seeking also led to increased activation of brain regions elsewhere implicated in drug-seeking habits and compulsions. This study suggests a new approach for investigating response control in animal models of drug addiction, and clearly implicates a role for habits in sustaining nicotine-seeking behavior following extended nicotine intake.

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The authors declare no conflict of interest.

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