New operant model of nicotine-seeking behaviour in mice

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Abstract
Nicotine addiction represents a major health problem in the world with dramatic socio-economic consequences. Recent studies using genetically modified mice have provided a better understanding of the neurobiological mechanisms involved in nicotine responses. However, the study of nicotine addiction requires sophisticated behavioural models that are still not fully developed in mice. Here, we report the validation of a new reliable operant model of nicotine-seeking behaviour in mice. C57BL/6 mice were trained to self-administer nicotine (0.03 mg/kg per infusion) under a fixed ratio 1 schedule of reinforcement for 10 d. A light cue was contingently associated with the nicotine infusion. After reaching the acquisition criteria of nicotine self-administration, mice were exposed to extinction sessions similar to the self-administration training except that nicotine was not available and the associated cues were not presented. Nicotine-seeking behaviour was then reinstated by exposure to nicotine-associated environment cues, a priming injection of nicotine or stress, the three main conditions leading to nicotine relapse in humans. The exposure to the cues associated with nicotine infusion was the most effective stimulus reinstating nicotine-seeking behaviour in 90% of mice. A priming injection of nicotine (0.18 mg/kg) produced nicotine reinstatement in 30% of the animals, whereas stress exposure (0.22 mA footshock) reinstated nicotine-seeking behaviour in 50% of mice. The validation of this new model of nicotine-seeking behaviour and reinstatement in mice provides an important tool to help clarify the genetic and neurochemical bases of nicotine addiction.

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Introduction
Tobacco addiction is one of the most important socio-economic health problems in developed countries and the prevalence of this disease is dramatically increasing in developing countries. Tobacco use is the leading preventable cause of death in the USA, and only 6% of people trying to quit smoking reached abstinence for more than 1 month (NIDA, 1998). The neurobiological mechanisms involved in nicotine pharmacological responses and the adaptive changes occurring in the brain after nicotine exposure have been widely studied (Benowitz, 2008). A broad range of studies have investigated the high vulnerability to relapse to nicotine consumption even after extended periods of abstinence. Indeed, three different causes that effectively induce reinstatement of nicotine-seeking behaviour in humans, and in laboratory animals have been identified. These events are the presence of a stressful situation, the re-exposure to the drug itself, and the exposure to environmental stimuli previously associated with drug taking (Self and Nestler, 1998; Stewart, 2000). In spite of the strong addictive properties of nicotine in humans, nicotine addictive effects are difficult to demonstrate in laboratory animals. Sophisticated operant behavioural techniques have been used to validate models of nicotine-seeking behaviour in rats (Shaham et al., 1997). In the case of mice, which represent excellent tools for investigation of addictive processes due to the advances in genetic techniques employed in this species, only one study concerning nicotine relapse has been published so far (Bilkei-Gorzo et al., 2008).

The reinforcing effects of nicotine are thought to be the primary reason for inhalation of tobacco...
smoke by humans (Shoaib, 2006). The most reliable and predictive experimental model for evaluation of drug-reinforcing effects in animals is the operant intravenous self-administration procedure (Deroche-Gamonet et al., 2004; Koob and Le Moal, 2005). In this model, the animal learns to self-administer a drug by an instrumental response in an active manipulanda contingently associated with delivery of the drug. This procedure allows evaluation of the acquisition of an operant behaviour to obtain the drug, and maintenance, extinction, and reinstatement of such behaviour. The intravenous route is a valid method for evaluating the reinforcing properties of nicotine, using a short infusion duration which promotes abuse-related effects of the drug under comparable conditions to smoke in humans (Thomsen and Caine, 2007). The high predictive value of nicotine self-administration procedure in rodents has been widely demonstrated in multiple studies (Shoaib and Stolerman, 1999; Stolerman et al., 1999).

In the present study, we validated a reliable behavioural model to evaluate nicotine-seeking behaviour in mice, which is the main behavioural trait for investigation of nicotine addiction and relapse. For this purpose, we used the reinstatement model after intravenous self-administration training and extinction, which has been widely used to evaluate relapse in rats (Di Ciano et al., 2008; Gerrits et al., 2005). The reinstatement of drug-seeking was evaluated in three different experimental conditions: the presentation of environmental cues associated with drug delivery, a priming injection of nicotine administered at the home cage, and exposure to a stressful situation (an electric foot-shock) (Le et al., 1998; Shalev et al., 2002). This new model will be extremely useful in combination with genetic and neurochemical tools in elucidating the neurobiological mechanisms underlying nicotine addiction and in identifying new therapeutic targets.

Materials and methods

**Animals**

Male C57BL/6 (Charles River, L’Arbresle, France) mice, weighing 24–26 g at the beginning of the experiments were used in the study. Mice were housed individually in controlled laboratory conditions with the temperature maintained at 21 ± 1 °C and humidity at 55 ± 10%. Mice were tested during the dark phase of a 12-h light/dark cycle (lights off 07:30 hours). Food and water were available ad libitum except during the experimental sessions. Animal procedures were conducted in strict accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and were approved by the local ethical committee (CEEA-PRBB).

**Drugs**

(–)-Nicotine hydrogen tartrate salt [(–)-1-methyl-2(3-pyridyl)pyrrolidine] (Sigma, Madrid, Spain) was dissolved in physiological saline (0.9%). During self-administration training, the pH of nicotine solutions was adjusted to 7.4 with sodium hydroxide. Nicotine doses were reported as free base concentration. In the priming-induced reinstatement test, nicotine (0.18 mg/kg) was administered by subcutaneous route in a volume of 10 ml/kg body weight. Ketamine hydrochloride (100 mg/kg) (Imalgène 1000; Rhône Mérieux, Lyon, France) and xylazine hydrochloride (20 mg/kg) (Sigma) were mixed and dissolved in ethanol (5%) and distilled water (95%). This anaesthetic mixture was administered intraperitoneally in an injection volume of 20 ml/kg body weight. Thiopental sodium (5 mg/ml) (Braun Medical S.A., Barcelona, Spain) was dissolved in distilled water and delivered by infusion of 0.1 ml through the intravenous catheter.

**Operant self-administration apparatus**

The self-administration experiments were conducted in mouse operant chambers (Model ENV-307A-CT; Med Associates Inc., Georgia, VT, USA) equipped with two holes, one randomly selected as the active hole and the other as the inactive hole. The chambers were made of aluminum and acrylic, with grid floors connected to an electrical shocker (EVV-414, Med Associates Inc., St Albans, VT, USA), and were housed in sound- and light-attenuated boxes equipped with fans to provide ventilation and white noise. Pump noise and stimuli lights (cues), one located inside the active hole and the other above it were paired contingently with the delivery of the reinforcer. Nicotine was infused via a syringe that was mounted on a microinfusion pump (PHM-100A; Med Associates) and connected, via Tygon tubing (0.96 mm o.d., Portex Fine Bore Polythene Tubing, Portex Ltd, Kent, UK) to a single-channel liquid swivel (375/25, Instech Laboratories, Plymouth Meeting, PA, USA) and to the mouse intravenous catheter. The swivel was mounted on a counter-balanced arm above the operant chamber.

**Surgery**

Mice were anaesthetized with a ketamine/xylazine mixture and then implanted with indwelling i.v.
Nicotine self-administration training

Nicotine self-administration sessions were performed in accordance to protocols previously described (Soria et al., 2005). Briefly, a 6 cm length of silastic tubing (0.3 mm inner diameter, 0.6 mm outer diameter) (Silastic, Dow Corning, Houdeng-Goegnies, Belgium) was fitted to a 22-gauge steel cannula (Semat, Herts, UK) that was bent at a right angle and then embedded in a cement disk (Dentalon Plus, Heraeus Kulzer, Germany) with an underlying nylon mesh. The catheter tubing was inserted 1.3 cm into the right jugular vein and anchored with suture. The remaining tubing ran subcutaneously to the cannula, which exited at the midscapular region. All incisions were sutured and coated with antibiotic ointment (Bactroban; GlaxoSmithKline, Madrid, Spain). After surgery, animals were allowed to recover for 3 d prior to initiation of self-administration sessions. The catheters were flushed daily with a saline solution in order to maintain patency. The patency of intravenous catheters was evaluated periodically (approximately every 6 d) and at the end of nicotine self-administration training. Verification of the catheter patency was not necessary for the extinction and reinstatement phases since nicotine was not available. The evaluation consisted of an infusion of thiopental sodium through the catheter, if prominent signs of anesthesia were not apparent within 3 s of the infusion the mouse was removed from the experiment. The success rate for maintaining patency of the catheter (mean duration 12 d) until the end of the nicotine self-administration training was 87%.

Reinstatement phase

Three different experimental conditions were evaluated to reinstate nicotine-seeking behaviour: the presentation of conditioned environmental cues, a priming injection of nicotine and the exposure to a stressful situation. The reinstatement criterion for the three experiments was achieved when nose-pokes in the active hole were double that of nose-pokes in the inactive hole and all the responses elicited during the 10-s timeout period were also recorded. The session was terminated after 50 reinforcers were delivered or after 1 h, whichever occurred first. As previously described (Soria et al., 2005; Trigo et al., 2006), the criteria for self-administration behaviour were achieved when all of the following conditions were met: (1) mice maintained a stable responding with <20% deviation from the mean of the total number of reinforcers earned in three consecutive sessions (80% of stability); (2) at least 65% responding on the active hole, and (3) a minimum of four reinforcers per session. After each session, mice were returned to their home cages. Each chamber was cleaned at the end of each session to prevent the presence of odour of the previous mouse. On day 11, mice that achieved the acquisition criteria were moved from the nicotine self-administration/training phase to the extinction phase. Only animals that met all the acquisition criteria were moved to the extinction phase.

Extinction phase

The experimental conditions during the extinction phase were similar to the nicotine self-administration sessions except that nicotine was not available and stimuli light and pump noise (environmental cues) were not presented after nose-poking in the active hole. Mice were given 1-h daily sessions (6 d/wk) until reaching the extinction criterion. The extinction criterion was achieved when during three consecutive sessions mice made a mean number of nose-poking responses in the active hole of <30% of the responses obtained during the mean of the 3 d taken to achieve the acquisition criteria.

All animals were tested during a minimum of 15 d or until reaching the criterion with a maximum of 50 extinction sessions. The following day after reaching the extinction criterion, mice were tested under reinstatement conditions. Only animals that reached the extinction criterion were evaluated for reinstatement induced by the different stimuli.
Cue-induced reinstatement

The test for cue-induced reinstatement was conducted under the same conditions used in the extinction phase (nicotine and cues were not available after nose-poking response in the active hole). A subcutaneous injection of saline was given on day 1, 5 min before the session. This control test allowed verification that the nicotine priming-induced reinstatement was specific to nicotine administration and not triggered by other factors such as the injection procedure. On day 2 mice received a priming subcutaneous injection of nicotine 5 min before the reinstatement test. A group of mice received a fixed amount of 0.18 mg/kg of nicotine (free base). Another group received the mean dose of nicotine that each mouse had self-administered during the 3 d in reaching the acquisition criteria. In this group, the dose was therefore calculated individually for each animal.

Nicotine-induced reinstatement

Tests for nicotine-induced reinstatement were conducted under the same conditions used in the extinction phase (nicotine and cues were available after nose-poking response in the active hole). A subcutaneous injection of saline was given on day 1, 5 min before the session. This control test allowed verification that the nicotine priming-induced reinstatement was specific to nicotine administration and not triggered by other factors such as the injection procedure. On day 2 mice received a priming subcutaneous injection of nicotine 5 min before the reinstatement test. A group of mice received a fixed amount of 0.18 mg/kg of nicotine (free base). Another group received the mean dose of nicotine that each mouse had self-administered during the 3 d in reaching the acquisition criteria. In this group, the dose was therefore calculated individually for each animal.

Stress-induced reinstatement

Tests for stress-induced reinstatement were conducted under the same conditions used in the extinction phase (nicotine and cues were not available after nose-poking response in the active hole). Immediately before the reinstatement session, intermittent electric footshock stimuli were applied during 5 min. Mice received five electric footshocks separated by a 1-min period without shock. Different intensities of footshock were evaluated in order to obtain the most appropriate conditions for reinstatement of nicotine-seeking behaviour while avoiding the freezing behaviour currently associated with high shock intensities. In the first group mice received electric footshocks of 0.22 mA for 2 s and in the second group mice received 0.5 mA for 100 ms. The intensity of 0.22 mA was selected based on previous studies conducted in our laboratory on stress-induced reinstatement of cocaine-seeking behaviour in mice (Soria et al., 2008). The intensity of 0.5 mA was chosen because it was found to be effective in reinstating nicotine-seeking behaviour in mice with a specific phenotype of high responsiveness to stress (Bilkei-Gorzo et al., 2008).

Statistical analysis

Two-way analysis of variance (ANOVA) with repeated measures in the factors day/experimental phase (daily sessions) and hole (active/inactive) was used to analyse the difference between active and inactive nose-poking responses during the 10 d of nicotine self-administration, the first 15 d of extinction, and the day of test for cue-, priming- or stress-induced reinstatement. One-way ANOVA was then performed to analyse differences between the responses on the active and inactive holes (discrimination) in each daily session and the changes across daily sessions. Subsequent post-hoc analyses were performed as required (Newman–Keuls). In order to evaluate reinstatement of drug-seeking behaviour, animals were divided in different groups corresponding to each experimental condition for reinstatement (associated cues, different doses of priming and different stress-exposure procedures). Similarly, two-way ANOVA followed by corresponding one-way ANOVA and post-hoc analyses were performed as required (Newman–Keuls). All animals reaching the reinstatement phase were included in this analysis.

Pearson’s $\chi^2$ test was used to compare the percentage of animals that reinstated nicotine-seeking behaviour in the different experimental conditions, in accordance with the reinstatement criterion established.

Correlation between the total amount of nicotine intake or the active nose-poking responses during 3 d of self-administration acquisition criteria and the active nose-poking responses in session 1 of extinction was performed using a Pearson correlation analysis.

Differences were considered significant at $p<0.05$. All results are expressed as mean ± s.e.m. The statistical analysis was performed using SPSS software version 15.0 (SPSS Inc., Chicago, IL, USA).

Results

In a preliminary study, a dose–response curve for the acquisition of nicotine self-administration (0.03, 0.0075 and 0.00185 mg/kg per infusion) was performed in C57BL/6 mice to determine the appropriate dose of nicotine to be used in this paradigm. In this experiment, the mean drug intake during the 3 d that the animals took to achieve the self-administration acquisition criteria was $0.500 ± 0.141$ mg/kg ($17.000 ± 4.723$ infusions) with the dose of 0.03 mg/kg; $0.157 ± 0.028$ mg/kg ($20.969 ± 3.796$ infusions) with the dose of 0.0075 mg/kg; and $0.020 ± 0.004$ mg/kg ($10.888 ± 2.584$ infusions) with the dose of 0.00185 mg/kg. Therefore,
the dose of 0.03 mg/kg per infusion was chosen for the self-administration and reinstatement experiments. Previous studies have also reported the use of 0.03 mg/kg per infusion as the most appropriate dose to obtain nicotine self-administration acquisition in mice (Bilkei-Gorzo et al., 2008; Picciotto et al., 1998).

Acquisition and maintenance of nicotine self-administration

Different groups of mice were trained to self-administer nicotine, in order to test the ability of environmental cues, nicotine-priming injections and footshock stress to induce reinstatement. One-way ANOVA was performed in these groups to evaluate any possible difference during the training phase. One-way ANOVA showed that there were no significant differences on active \( F_{12,32} = 3.02, \text{n.s.} \) or inactive \( F_{12,32} = 0.80, \text{n.s.} \) nose-poking during the 10-d self-administration training among the different groups. The results of training were then pooled for all the experimental groups. (Figure 1a). Two-way ANOVA showed significant main effects of hole \( F_{2,98} = 154.94, p < 0.001 \) and interaction between day and hole \( F_{9,810} = 10.39, p < 0.001 \) whereas non-significant main effects of day \( F_{9,810} = 1.52, \text{n.s.} \) were obtained. Mice discriminated between the active and the inactive holes during the whole period of self-administration training as shown by one-way ANOVA \( p < 0.001 \) every day of training. The acquisition criteria were achieved in 8.33 ± 0.39 d by 76% of mice. The mean number of active nose-poking responses for nicotine reinforcement during this stable phase of self-administration was 12.90 ± 0.79. This value corresponded to a mean nicotine intake of 0.37 ± 0.02 mg/kg. The mean number of inactive nose-pokes during this stable phase was 2.26 ± 0.21. Polynomial post-hoc analysis revealed an increase in the number of active nose-poking responses across sessions during the 10-d self-administration training [polynomial (linear): \( F_{1,30} = 16.13, p < 0.001 \)]. In addition, a significant increase in the number of active nose-poking responses

Figure 1. Acquisition of nicotine self-administration in C57BL/6 mice. (a) Mean number of nose-poking responses in the active (– – ) and inactive (– – ) holes for the complete group of animals used in the different experiments of reinstatement. Mice were trained daily in 1-h sessions to obtain nicotine (0.03 mg/kg per infusion) during 10 d under a fixed ratio 1 schedule of reinforcement. Data are expressed as mean ± s.e.m. *** \( p < 0.001 \) comparison between holes. +++ \( p < 0.001 \) comparison between days 5 and 6 (Newman–Keuls). Only mice that reached acquisition criteria (76%) are shown \( (n = 91) \). (b) Representatives patterns of active and inactive nose-poking for nicotine self-administration on sessions 1 and 10 of nicotine self-administration training. Each vertical line represents one active or inactive nose-poke. The horizontal line represents the 1-h session; the upper pattern corresponds to the active and the lower to the inactive nose-poking responses.
compared with the previous day was seen on day 6 \( [F_{1,90} = 4.92, p < 0.05] \). Interestingly, the number of inactive nose-poking responses decreased significantly across sessions [polynomial (linear): \( F_{1,90} = 27.57, p < 0.001 \)]. A pattern of responses performed on the active and inactive holes (days 1 and 10 of training) for a representative mouse is depicted in Figure 1b.

**Extinction**

Animals that accomplished the nicotine self-administration acquisition criteria underwent extinction schedule. One-way ANOVA did not show significant differences on active \( [F_{1,32} = 3.33, \text{n.s.}] \) and inactive \( [F_{1,32} = 1.68, \text{n.s.}] \) nose-poking during extinction training among the different groups of reinstatement. The extinction criterion (\(<30\%\) of previous active responses during three consecutive sessions of the stable phase of self-administration training) was achieved by 38\% of mice. This criterion was reached after an average of 22.17 ± 2.53 daily sessions of extinction. Two-way ANOVA showed significant main effects of hole \( [F_{1,14} = 40.83, p < 0.001] \), day \( [F_{14,40} = 5.40, p < 0.001] \) and interaction between these two factors \( [F_{14,40} = 5.78, p < 0.001] \). Subsequent one-way ANOVA showed that mice discriminated between the active and the inactive holes during the first 13 sessions of extinction \( (p < 0.001) \) (Figure 2a). At the end of the extinction period, discrimination between holes diminished as shown by the absence of significant differences between holes on sessions 14 and 15. During session 1 of extinction mice showed an enhancement of the number of responses in the active hole \( (20.42 ± 2.11) \) in comparison to the responses of the last day of nicotine self-administration training \( (12.30 ± 0.89) \). These responses were mainly observed during the initial period of the first extinction session (Figure 2b). The resistance to extinction was also
extended to the inactive nose-poking responses (last day of training: 2.14 ± 0.25; day 1 of extinction: 8.57 ± 1.06). Thereafter, the responses in the active hole went down across sessions [polynomial (linear): \( F_{1,34} = 10.09, p < 0.01 \)]. Furthermore, the number of active nose-poking responses in day 1 of extinction was positively correlated with the total amount of nicotine intake during 10 d training (\( R = 0.50, p < 0.01 \)) and active nose-poking responses during the 3 d required to achieve self-administration acquisition criteria (\( R = 0.44, p < 0.01 \)). Therefore, the resistance to extinction at the beginning of the extinction training was directly related to the previous history of drug consumption. Only the animals that met the criterion for extinction were evaluated for cue-, priming- and stress-induced reinstatement.

**Cue-induced reinstatement**

After the achievement of the extinction criterion, the cue-light and the pump noise were presented in order to evaluate cue-induced reinstatement. On the test day, exposure to the cues reinstated drug-seeking behaviour in 90% of the animals (Figure 3a). Two-way ANOVA showed significant main effects of hole [\( F_{1,9} = 46.02, p < 0.001 \)], experimental phase [\( F_{2,18} = 7.05, p < 0.01 \)] and interaction between these two factors [\( F_{2,18} = 7.60, p < 0.01 \)]. Subsequent one-way ANOVA showed that mice discriminated between the active and inactive holes during the reinstatement session (\( p < 0.05 \)), as they did during the 3 d of self-administration acquisition criteria (\( p < 0.001 \)). The number of active nose-poking responses when reaching the extinction criterion was significantly lower than the responses during the acquisition of self-administration criteria [\( F_{1,9} = 63.53, p < 0.001 \)]. During the reinstatement test, the number of active nose-poking responses was significantly higher than that obtained on the day of achieving the extinction criterion [\( F_{1,9} = 11.62, p < 0.01 \)] and animals reached the same level of responses as that during acquisition training. No significant changes on the number of inactive nose-poking responses were found when comparing the acquisition, extinction and reinstatement phases [one-way ANOVA: \( F_{2,18} = 1.70, \text{n.s.} \)]. Nicotine-seeking behaviour was a long lasting process since it occurred 26.2 ± 6.60 d after withdrawal from nicotine self-administration training. A pattern of responses performed on the active and the inactive holes for a representative mouse under cue-induced reinstatement is depicted in Figure 3b.
Priming-induced reinstatement

In order to evaluate priming-induced reinstatement, a dose of 0.18 mg/kg s.c. was administered before the reinstatement session. Two-way ANOVA showed significant main effects of hole \(F_{1,12}=25.26, p<0.001\), experimental phase \(F_{2,24}=13.08, p<0.001\) and interaction between these two factors \(F_{2,24}=43.24, p<0.001\). Subsequent one-way ANOVA showed that animals discriminated between the active and inactive holes during the reinstatement testing \((p<0.05)\) as well as they did during the training \((p<0.001)\) (Figure 4a). When administered at this dose, nicotine priming induced a reliable reinstatement of drug-seeking behaviour in 30% of mice. Active nose-poking responses when reaching self-administration criteria was significantly higher than the responses during acquisition of extinction criterion \([\text{repeated one-way ANOVA: } F_{1,12}=70.50, p<0.001]\), the saline-priming session \([\text{repeated one-way ANOVA: } F_{1,12}=41.50, p<0.001]\) and the nicotine-priming session \([\text{repeated one-way ANOVA: } F_{1,12}=19.82, p<0.01]\). No significant changes on the number of inactive nose-poking responses were found when comparing the acquisition, extinction and saline- and nicotine-reinstatement phases \([\text{one-way ANOVA: } F_{2,24}=0.84, \text{n.s.}]\).

A pattern of responses performed on the active and inactive holes for a representative mouse under priming-induced reinstatement is depicted in Figure 4b. In a second experiment, the mean dose of nicotine intake during the 3 d training during which mice acquired the criteria of self-administration behaviour was calculated for each animal. This individualized dose of nicotine was administered as a priming stimulus to induce reinstatement in the testing session. The dose of nicotine used in this second group was higher than that used in the first experiment (0.18 mg/kg) in 75% of the animals and failed to significantly produce reinstatement of drug-seeking behaviour (data not shown).

Figure 4. Priming-induced reinstatement. (a) Mean number of nose-poking responses in the active (■) and inactive (□) holes during the different experimental phases: acquisition of nicotine self-administration behaviour (mean of 3-d acquisition criteria), extinction (mean of 3-d extinction criterion) and the reinstatement induced by the administration of saline or nicotine \(n=13\). Data are expressed as mean ± S.E.M., * \(p<0.05\), *** \(p<0.001\) differences between holes within the same experimental phase. ++ \(p<0.01\), +++ \(p<0.001\) significant differences between experimental phases when considering the same hole (Newman–Keuls). (b) Representative patterns of active and inactive nose-poking on sessions of saline and priming-induced reinstatement. Each vertical line represents one active or inactive nose-poke. The horizontal line represents the 1-h session; the upper pattern corresponds to the active and the lower to the inactive nose-poking responses.
Stress-induced reinstatement

A brief exposure to intermittent electric footshock at the intensity of 0.22 mA was first used to evaluate stress-induced reinstatement. The exposure to this stress condition reinstated drug-seeking behaviour in 50% of the animals. Two-way ANOVA showed significant main effects of hole \( F_{1,11} = 18.36, p < 0.01 \), experimental phase \( F_{2,22} = 4.40, p < 0.05 \) and interaction between these two factors \( F_{2,22} = 13.28, p < 0.001 \). The number of active nose-poking responses when reaching the extinction criterion was significantly lower than the responses obtained during the acquisition of self-administration criteria [repeated one-way ANOVA: \( F_{1,11} = 22.01, p < 0.01 \)]. Subsequent one-way ANOVA showed that mice did not discriminate between the active and the inactive holes during the extinction session, in contrast to the significant discrimination seen during the 3-d acquisition of self-administration criteria (\( p < 0.001 \)). However, mice significantly increased drug-seeking behaviour during the reinstatement session when compared with the extinction session [one-way ANOVA: \( F_{1,11} = 7.05, p < 0.05 \)] (Figure 5a). A pattern of responses performed on the active and the inactive holes for a representative mouse under stress-induced reinstatement (0.22 mA) is depicted in Figure 5c.

In another group of mice, the reinstatement produced by a brief exposure to intermittent footshock at 0.5 mA intensity was evaluated. The exposure to this stress condition failed to reinstate drug-seeking behaviour in mice. Two-way ANOVA showed significant main effects of hole \( F_{1,11} = 1.36, p < 0.01 \), experimental phase \( F_{2,22} = 5.42, p < 0.05 \) and interaction between

**Figure 5.** Stress-induced reinstatement. (a) Mean number of nose-pokes in the active (■) and inactive (□) holes during the different experimental phases: acquisition of nicotine self-administration behaviour (mean of 3-d acquisition criteria), extinction (mean of 3-d extinction criterion) and reinstatement induced by 0.22 mA footshock stress (\( n = 12 \)). (b) Mean number of nose-pokes in the active (■) and inactive (□) holes during the different experimental phases: acquisition of the nicotine self-administration behaviour (mean of 3-d acquisition criteria), extinction (mean of 3-d extinction criterion) and reinstatement induced by 0.5 mA footshock stress (\( n = 12 \)). Data are expressed as mean ± S.E.M. *** \( p < 0.001 \) differences between holes within the same experimental phase. + \( p < 0.05 \), + + \( p < 0.01 \) significant differences between experimental phases when considering the same hole (Newman–Keuls). (c) Representative patterns of active and inactive nose-poking on sessions of stress-induced reinstatement. Each vertical line represents one active or inactive nose-poke. The horizontal line represents the 1-h session; the upper pattern corresponds to the active and the lower to the inactive nose-poking responses.
these two factors \(F_{2,32}=19.25, p<0.001\). However, subsequent one-way ANOVA only showed a significant discrimination between the active and inactive holes during the 3-d acquisition of self-administration criteria \((p<0.001)\). Active nose-poking responses when animals reached the self-administration criteria was significantly higher than the responses obtained when achieving the extinction criterion \(F_{1,11}=22.01, p<0.01\) and during the foot-shock reinstatement \(F_{1,11}=8.96, p<0.05\) (Figure 5b).

**Comparison among the reinstatements induced by different stimuli**

Data obtained during the experimental conditions that most effectively reinstated nicotine-seeking behaviour (associated cues, priming of 0.18 mg/kg nicotine and exposure to intermittent stress 0.22 mA footshock for 2 s) were compared. \(\chi^2\) comparisons were used to analyse the percentage of reinstatement and one-way ANOVA to analyse the number of responses on the active and inactive holes. \(\chi^2\) showed that the percentage of mice that reached the reinstatement criterion was significantly higher with the presentation of environmental cues (90%) than with the priming injection of nicotine (30%) \(\chi^2=50.68, p<0.001\) or the brief presentation of intermittent footshock (50%) \(\chi^2=21.33, p<0.001\).

One-way ANOVA of the response on the active hole showed a main effect of group \(F_{2,32}=9.10, p<0.01\). Subsequent post-hoc analysis (Newman–Keuls) showed that the responses of animals exposed to the associated cues were significantly higher than those receiving nicotine priming \((p<0.001)\) or electric footshock \((p<0.01)\). No significant differences were shown by one-way ANOVA when comparing the number of responses on the inactive holes in these experimental conditions. Therefore, the exposure to the associated environmental cues was the most effective stimulus in reinstating nicotine-seeking behaviour in mice.

**Discussion**

In this study, we have validated for the first time a reliable model of reinstatement of nicotine-seeking behaviour after nicotine self-administration extinction in C57BL/6 mice by the exposure to the three stimuli known to elicit relapse in humans: nicotine-associated cues, nicotine-priming, and environmental acute stress. The reinstatement of nicotine-seeking behaviour was more effective after the exposure to nicotine-associated cues (90%) than after brief presentation of stress (electric footshock at 0.22 mA) (50%) or nicotine-priming injection (0.18 mg/kg s.c.) (30%).

Operant models of drug-seeking behaviour have been well developed in rats (Di Ciano et al., 2008; Gerrits et al., 2005). However, few studies have been able to develop a reliable model of reinstatement of drug-seeking behaviour in mice using psychostimulants such as cocaine (Fuchs et al., 2003; Highfield et al., 2002; Soria et al., 2008) or methamphetamine (Yan et al., 2006, 2007), which are the most effective drugs for self-administration in mice. To our knowledge, only one study has reported the reinstatement of nicotine-seeking behaviour in mice after extinction of nicotine self-administration by using footshock stress with a higher intensity than that used in the present study (Bilkei-Gorzo et al., 2008). It is important to emphasize that mice were not subjected to a food deprivation regimen and were not previously trained to self-administer food in our experimental conditions; two experimental procedures currently used to magnify nicotine-seeking behaviour. Learning the contingency for lever pressing and food reward under a food deprivation state can modify the instrumental performance elicited thereafter (Balleine and Dickinson, 2006). Such a possible confounding factor is not present in this study and the operant behaviour evaluated should then primarily arise from the motivational state of mice to obtain the reward produced by the contingent administration of nicotine.

Nicotine-reinforcing effects have been previously seen in several studies in mice (Bilkei-Gorzo et al., 2008; Picciotto et al., 1998; Stolerman et al., 1999), although nicotine potency is around 4-fold greater in rats probably due to pharmacokinetic factors related to its faster metabolization in mice (Stolerman et al., 1999). The criteria for acquisition of self-administration behaviour should be defined less strictly in mice than in other species, such as rats or primates, due to the well-known greater variability of the behavioural responses of mice in these paradigms (Thomsen and Caine, 2007). Thus, mice require longer training to obtain a stable behaviour in operant paradigms than other species and the adaptation of the mouse operant response to the changes of the different variables of the paradigm, i.e. reinforcement schedule or drug dose, was less expeditious (Thomsen and Caine, 2007). These behavioural results are in agreement with the high variability of nicotine pharmacokinetic parameters in mice (Stolerman et al., 1999).

Accordingly with these data, a minimum of four active responses per session with 80% stability and 65% discrimination over three consecutive sessions was required to reach acquisition criteria in the present
study. In spite of these acquisition criteria, mice already showed a preference for the active hole (8.14 ± 0.77) over the inactive hole (4.04 ± 0.38) on day 1 of nicotine self-administration training, and the responses on the active hole progressively increased over the training sessions. Stable active responses in the nicotine-trained groups were not due to a result of exploring the environment since these responses did not occur in mice trained to self-administer saline and produce illumination of the light. Thus, the percentage of acquisition in the saline group was only 22.22% and the mean number of active nose-pokes was 10.13 ± 1.33 in a previous study from our laboratory (Trigo et al., 2007). A similar operant response to obtain saline and illumination of the light has also been obtained in a more recent study performed in our laboratory: mean number of active nose-pokes during acquisition was 7.17 ± 2.5 with 28% acquisition. Mouse responding in the active hole was contingent on delivery of nicotine and associated cues. Thus, discrimination between the active and inactive holes occurred from day 1 of self-administration training indicating that the response on the active hole was maintained by the reinforcer. Furthermore, an ‘extinction burst’ behaviour (Cooper et al., 1987) was seen in mice during day 1 of extinction. This prototypical initial transient increase of responding in the active manipulanda has been specifically associated with operant responding for different drugs of abuse (Peltier et al., 2001; Shalev et al., 2001; Soria et al., 2008), and shows that in the present study mice had developed a reliable operant nicotine self-administration behaviour. This behaviour reflects a ‘craving-like’ state at the beginning of the extinction training that was directly related in this study to the previous history of drug consumption. In addition, the progressive increase in the inactive nose-poking responses during extinction training with the consequent reduction in discrimination also indicates that active responding was previously maintained by nicotine contingency. Indeed, this result demonstrates that the salience of unconditioned (nicotine) and conditioned (environmental cues) stimuli previously associated with active nose-poking response was extinguished, and this particular criterion has already been considered valid for extinction in previous studies (Bilkei-Gorzo et al., 2008). A stringent criterion for extinction was used in the present work, i.e. <30% of previous active responses during three consecutive sessions of the stable phase of self-administration training, similar to that used by our group in a previous study (Soria et al., 2008). Other studies in mice have reported a different criterion for methamphetamine extinction consisting of a reduction of responses to the 25% acquisition responding or <15 nose-pokes in 2 d during daily 3-h sessions (Yan et al., 2007). The extinction criteria used in other previous studies in rats are rather heterogeneous and differ from a decrease of 50% (Lesage et al., 2004) to 20% of the level of response observed during the last 3 d of nicotine self-administration (Liu et al., 2006).

In the present study, mice required an average of 22 d to reach the extinction criterion. Previous studies have reported that C57BL/6 mice are particularly resistant to extinguish a previously acquired operant behaviour. Thus, this strain required from 18 d to 20 d of training to extinguish operant self-administration for psychostimulants using similar conditions to the present work (Fuchs et al., 2003; Yan et al., 2007). The time required to extinguish nicotine self-administration in previous studies in rats was usually shorter than in mice, although it depends on the stringency of the extinction criteria used (Lesage et al., 2004; Liu et al., 2006). Thus, other studies in rats have also reported the requirement of a training period from 20 d to 30 d to reach nicotine extinction under experimental conditions similar to the present study (Cohen et al., 2005).

The percentage of mice reaching the extinction criterion was 38% in the present study. A first possible explanation for this low percentage of extinction might be the severity of the extinction criterion used in order to avoid false positives. In addiction, contextual cues such as the operant box itself could have acquired motivational salience related to nicotine administration (Shaham et al., 1997). These cues could also account for the low percentage of extinction criterion since the extinction training was performed in the same environment as the acquisition sessions.

The subtle reinforcing effects of nicotine in humans (Caggiula et al., 2001; Chiamulera, 2005) and rodents (Caggiula et al., 2001) are in contrast to the strong abuse liability of this drug. Several authors have outlined that the intrinsic effects of nicotine are not sufficient to account for the rates of tobacco consumption in humans or nicotine self-administration in animals. Indeed, a critical role of the environmental stimuli previously associated with drug consumption has been attributed to explain the high rate of nicotine relapse (Caggiula et al., 2001, 2002; Chiamulera, 2005; Liu et al., 2006). Interestingly, the exposure to environmental stimuli paired with nicotine administration was the most effective condition in reinstating nicotine-seeking behaviour in our study. This result supports the critical role played by nicotine-paired cues in sustaining nicotine self-administration after prolonged periods of abstinence and in maintaining...
smoking behaviour in humans. The mechanisms underlying the interaction between nicotine and environmental cues remain to be clarified. Nicotine rewarding effects associated with environmental stimuli could enhance the salience of previously neutral stimuli, making them more desired, and able to reinstate drug-seeking behaviour because they acquire conditioned reinforcing properties (Caggiula et al., 2001; Goldberg et al., 1981; Rose and Levin, 1991). On the other hand, nicotine improves cognitive skills (Levin et al., 2006) and attention is shifted towards smoking-related cues in smokers compared to non-smokers (Mogg and Bradley, 2002). Therefore, a bias of attention preferentially focused towards environmental cues related to nicotine administration in spite of other stimuli seems to be important for the high rate of reinstatement induced by the exposure to associated cues.

The non-contingent injection of nicotine priming only induced reinstatement in 30% of mice that have extinguished nicotine self-administration. The dose (0.18 mg/kg) and route of administration (subcutaneous) used were chosen based on a previous study showing the specific rewarding effects in the conditioned place preference and the enhancement of dopamine release in the nucleus accumbens induced under these experimental conditions in C57BL/6 mice (Berrendero et al., 2005). Due to the short duration of jugular catheter patency in mice (Thomsen and Caine, 2007), the intravenous route for nicotine priming cannot be used under the present experimental conditions. Similar doses of nicotine (0.10, 0.15 and 0.30 mg/kg) and route of administration (subcutaneous) were also previously used to reinstate extinguished nicotine-seeking behaviour in rats (Dravolina et al., 2007; Shaham et al., 1997). In our experimental conditions, the subcutaneous administration of a higher priming dose of nicotine, i.e. corresponding to the individual mean intake during the 3-d acquisition of nicotine self-administration criteria, did not reinstate nicotine-seeking behaviour (data not shown). In agreement, a bell-shaped dose–response curve of nicotine has been reported in the mouse place-conditioning paradigm where doses >0.18 mg/kg (free base) did not produce rewarding effects (Berrendero et al., 2005). The long duration required in mice to obtain reliable extinction of nicotine self-administration could impair the reinstatement of nicotine-seeking behaviour. Thus, an inverse relationship has been reported between the number of extinction sessions and the percentage of relapse reached by drug priming (Highfield et al., 2002; Yan et al., 2006). However, other authors have also reported the difficult to elicit relapse to drug-seeking behaviour in mice after non-contingent drug priming (Fuchs et al., 2003; Highfield et al., 2002; Yan et al., 2006, 2007).

Stress exposure is also an experimental stimulus widely used to reinstate nicotine-seeking behaviour in rodents (Bilkei-Gorzo et al., 2008; Buczek et al., 1999). In the present study, the exposure of mice to brief intermittent electric footshock stress induced a reliable nicotine-seeking behaviour in 50% of mice that have extinguished self-administration behaviour. The exposure to a higher intensity of stress (0.5 mA) failed to reinstate nicotine-seeking behaviour. This result is in agreement with that previously obtained under similar experimental conditions (Bilkei-Gorzo et al., 2008). In this previous study, the exposure to 0.5 mA electric footshock did not reinstate nicotine-seeking behaviour in a general population of C57BL/6 mice. In contrast, this intensity of footshock specifically reinstated nicotine-seeking behaviour in mice that were selected for a spontaneous high-stress response on a mixed C57BL/6 and C3H/J genetic background (Bilkei-Gorzo et al., 2008).

In conclusion, the present study validates for the first time a reliable operant model of reinstatement of nicotine-seeking behaviour in mice induced by the three main stimuli causing nicotine relapse in humans: environmental associated cues, nicotine priming and stress exposure. The exposure to nicotine-associated cues was the most effective stimulus to reinstate nicotine-seeking behaviour in mice. The validation of this behavioural model in mice has major relevance since it opens for the first time the possibility of using genetically modified mice to investigate the biochemical and genetic mechanisms underlying nicotine addiction. This new methodological tool will be useful in improving the knowledge of the neurobiological bases of nicotine addiction and will therefore facilitate future development of innovative treatments to prevent nicotine relapse, which still represents the main drawback in the current treatment of nicotine addiction.

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Statement of Interest
None.

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