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Exposure to chronic intermittent nicotine vapor induces nicotine dependence

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ABSTRACT

Animal models of drug exposure are important tools for the study of the neurobiological mechanisms of nicotine dependence and as preclinical models for medication development. There are few non-invasive animal models of nicotine exposure and currently there is no known animal model of second-hand exposure to nicotine. We hypothesized that chronic administration of nicotine vapors would produce blood levels of nicotine in rodents that are clinically relevant to those observed in human smoking and that rodents exposed to nicotine vapors would develop dependence to nicotine. We developed a system that vaporizes nicotine in the air in a stable, reliable and consistent manner. Intermittent exposure to nicotine vapor (0.2 mg/m^3) for 8 or 14 h per day for 7 days produced a concentration of nicotine in the blood of 22 ng/mL. Sixteen hours after removal from nicotine vapors, rats showed significant somatic withdrawal signs precipitated by mecamylamine (1.5 mg/kg). These results provide a new rodent model of nicotine dependence using vapor administration that produces consistent levels of nicotine in the blood that are relevant for both heavy smoking and second-hand smoking, using a non-invasive technique that mimics the intermittent aspect and route of administration in humans.

1. Introduction

An increasing amount of nicotine addiction research is undertaken each year, justified by the estimated 3 million yearly deaths from the consequences of nicotine addiction (Maskos et al., 2005). Animal models of drug exposure are important tools for the study of the neurobiological mechanisms of nicotine dependence and as preclinical models for medication development. The most commonly used methods for examining the effects of chronic nicotine administration are osmotic minipumps, repeated subcutaneous injections, intravenous self-administration and cigarette smoke exposure (Koob and Le Moal, 2006). Although these methods are extremely useful and have demonstrated robust predictive validity for tobacco addiction, they also have important limitations. Exposure to nicotine using osmotic minipumps cannot mimic the intermittent aspect of nicotine exposure during cigarette smoking and repeated injections and self-administration cannot mimic the route of human exposure and are labor intensive and relatively invasive techniques. In addition, there is currently no known animal model to study the effect of exposure to very low levels of nicotine in the air, such as those observed after second-hand exposure to cigarette smoke. Although tobacco smoke exposure is a highly relevant and validated technique to model the effect of second hand smoking (Seymour et al., 1997), this technique cannot discriminate the effects of nicotine from the effects of the approximately 4000 additional compounds known to be present in cigarette smoke (Wynder and Hoffmann, 1979).

The purpose of the present study was to validate a rodent model of nicotine administration that produces consistent levels of nicotine in the blood that are relevant for both heavy smoking and second-hand smoking, using a non-invasive and high throughput technique that could mimic both the intermittent aspect and route of administration in humans. To this end, we developed a system based on previous nicotine inhalation research (Waldum et al., 1996) that allowed us to vaporize nicotine in the air in a stable, reliable and consistent manner. We hypothesized that the administration of nicotine vapors would produce blood levels of nicotine that are clinically relevant to those observed in second-hand smoking (<5 ng/mL; Argacha et al., 2008) and heavy smoking (10–50 ng/mL; Matta et al., 2007). We further hypothesized that rodents exposed to nicotine vapors would develop dependence to nicotine, defined here as the manifestation of a somatic withdrawal syndrome similar to that observed in other studies of rodent nicotine exposure (Epping-Jordan et al., 1998; Grieder et al., 2010; Malin et al., 1992).

2. Methods

2.1. Animals

Male Wistar rats (Charles River) weighed 200–250 g at the time of testing. Animals were housed in groups of 3 or 4 in Plexiglas cages in

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humidity- and temperature-controlled (22 °C) rooms on a 12 h light/dark cycle with lights off from 10:00 AM to 10:00 PM. Each rat was handled for 2–3 days before training by placing groups of animals on a table top and repetitively handling each animal during a 5 min period. Animals had *ad libitum* access to food and water throughout the course of the studies. All procedures were conducted in strict adherence to the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996).

2.2. Nicotine vapor chambers

Four standard rat cages were contained within separate, sealed Plexiglas chambers into which nicotine vapor (or untreated air for control chambers) was independently introduced. Nicotine vapor was created by bubbling medical quality air at a flow rate of 10 or 20 l per min [LPM] through a gas-washing bottle containing a solution of pure nicotine (free base, Sigma Aldrich). Nicotine vapors are produced by the natural phenomena of evaporation that is maximized by the bubbling of air with a constant air flow. The highly concentrated nicotine vapors were then passed through a drop-catch bottle (Waldum et al., 1996) and further diluted by the addition of 60 LPM of clean air in a 2000 mL Erlenmeyer vacuum flask at room temperature. The final nicotine–air mixture was homogeneously distributed between the different chambers at a flow rate of 15 LPM. Concentrations of nicotine vapor were adjusted by varying the flow rate at which nicotine was bubbled. Identical chambers with controlled untreated air were used for control animals.

2.3. Induction of nicotine dependence

Animals were exposed to nicotine vapor for 2, 8 or 14 h per day for 1 or 7 days. Control animals were exposed to untreated air in vapor chambers.

2.4. Blood nicotine analysis

Blood was collected during the last hour of the last day of vapor exposure and 2, 4, 6, 8, 16 and 24 h after the cessation of vapor exposure. Tail blood was collected by cutting the tip of the tail (2 mm) off with a clean razor blade. Blood from each animal (0.2–0.5 mL) was collected in Eppendorf tubes containing evaporated heparin and kept on ice. After centrifugation, plasma was collected into fresh Eppendorf tubes and analyzed by the liquid chromatography mass spectrometry mass spectrometry (LC-MS/MS) method (O'Dell et al., 2006).

2.5. Somatic signs of withdrawal

Somatic signs of withdrawal were assessed following administration of mecamylamine (1.5 mg/kg, s.c.) to precipitate withdrawal or vehicle (0.9% saline) for spontaneous withdrawal. Rats were placed in a clean cage and observed for 10 min at 30 min and 16 h after removal from the nicotine vapor chambers. The somatic signs that were observed and recorded were blinks, gasps, writhes, head shakes, ptosis, teeth chattering, and yawns (Malin et al., 1992). Multiple successive counts of any sign required a distinct pause between episodes. The total number of somatic signs in the observation period was defined as the sum of the number of occurrences of all of the aforementioned signs.

2.6. Statistical analysis

Results were analyzed with Statistica software using a two-way repeated measures analysis of variance (ANOVA) or paired Student's *t*-tests. Posthoc Duncan's tests were performed where appropriate. *p* values of less than 0.05 were considered to be significant.

3. Results

To evaluate the stability of the concentration of nicotine vapor in air, nicotine was bubbled using a gas-washing bottle at a flow rate of 20 LPM and the levels of nicotine in the air produced by the gas-washing bottle were monitored during 4 subsequent hours using the ethanol-trapping technique and UV spectrophotometry at 260 nm (Waldum et al., 1996). The concentration of nicotine in the vapor chamber air over 4 h was stable and constant, with an average concentration of 1.09 ± 0.02 mg/m^3 (1 h = 1.07 mg/m^3; 2 h = 1.07 mg/m^3; 3 h = 1.14 mg/m^3; 4 h = 1.07 mg/m^3). These concentrations are similar to values reported in a previous study using a similar technique (Waldum et al., 1996).

To investigate whether exposure to nicotine vapor could produce significant blood levels of nicotine and to examine the pharmacokinetic profile of blood nicotine levels after vapor exposure, rats were exposed to 2 or 8 h of nicotine vapor for one day. Blood samples were obtained before nicotine vapor exposure (baseline) and at 0, 2, 4, 6, 8, 16 and 24 h after exposure. The blood nicotine levels obtained after 2 h of exposure (63 ± 13 ng/mL, Fig. 1) were similar to the blood levels observed in heavy smokers throughout the day (Matta et al., 2007), whereas the concentration of nicotine in the blood that was achieved after 8 h of exposure (139 ng/mL ± 21) was approximately 3 times higher than nicotine blood levels observed in regular smokers. A two-way repeated measures ANOVA showed a significant effect of time ($F_{5,20} = 27.5$, $p < 0.05$) and group ($F_{1,4} = 63.4$, $p < 0.05$) and a group × time interaction ($F_{5,20} = 5.6$, $p < 0.05$; Fig. 1). The amount of nicotine in the blood progressively declined and was no longer significant in either group 24 h after exposure ($p > 0.05$).

We next hypothesized that a better model of human nicotine consumption and blood levels would be produced by chronic exposure to nicotine at a lower air nicotine level in the vapor chambers. We chronically exposed animals (8 h per day for 7 days) to lower air nicotine levels (0.2 mg/m^3) than in the previous experiment. The concentration of nicotine in the vapor chamber was adjusted by decreasing the airflow rate from 20 LPM to 10 LPM and by diluting the nicotine vapor by adding 60 LPM of clean air. Accordingly, the concentration of nicotine in the air decreased from 1.1 mg/m^3 to 0.2 mg/m^3. This value represents the concentration of nicotine that the rats would inhale. We hypothesized that this 5-fold decrease in the nicotine air level would allow us to obtain blood nicotine levels that are relevant to those observed in smokers and that we could maintain these levels for longer periods of time. The blood concentration of nicotine was measured at the conclusion of the seventh day of exposure and 16 h after removal from the chambers. Intermittent exposure to nicotine vapors for 8 h per day for 7 days produced an average plasma nicotine concentration of 22.05 ± 3.8 ng/

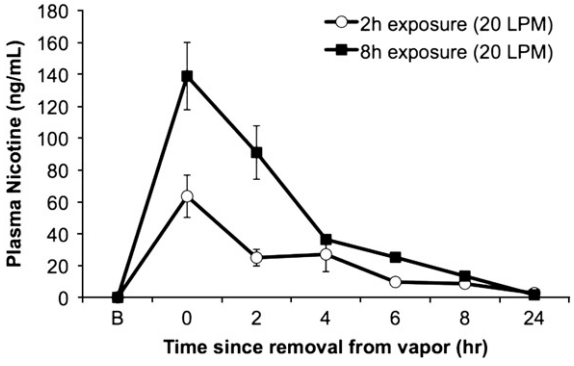

Fig. 1. Rats were exposed to 2 or 8 h of nicotine vapor for one day. Blood samples were obtained before nicotine vapor exposure (baseline, B) and at 0, 2, 4, 6, 8 and 24 h after exposure. The plasma nicotine concentration reached significant blood levels during exposure (*$p < 0.05$) and progressively declined over the next 24 h. Data represents means +/− SEM.

mL. Sixteen hours after removal from chronic nicotine vapors, the plasma nicotine concentration was 3.49 ± 0.62 ng/mL.

To examine whether chronic intermittent exposure to nicotine vapor would produce somatic withdrawal, we exposed rats to 8 and 14 h of regular air or nicotine vapors (0.2 mg/m^3) for 7 days and administered saline or the nicotinic receptor antagonist mecamylamine (1.5 mg/kg) to precipitate withdrawal. Somatic signs of nicotine withdrawal were observed 30 min and 16 h after removal from nicotine vapors for the group exposed to 8 h of vapor per day. A two-way repeated measures ANOVA showed a significant effect of treatment ($F_{1,12} = 15.2, p<0.05$) and of time ($F_{2,12} = 5.9, p<0.05$) and a treatment × time interaction ($F_{2,12} = 5.5$, $p<0.05$; Fig. 2A). Rats exposed to nicotine vapor for 8 h per day and pretreated with saline did not show a significant somatic withdrawal syndrome at 30 min or 16 h following removal from vapors in comparison to control rats exposed to regular air ($p>0.05$). Rats exposed to nicotine vapors for 8 h per day and pretreated with mecamylamine showed significant somatic signs of withdrawal after 30 min ($p<0.05$) and 16 h of precipitated withdrawal ($p<0.05$) in comparison to controls. To reduce the number of animals used and because the largest somatic withdrawal syndrome was observed at 16 h after removal from nicotine vapor in the group exposed to 8 h of vapor per day, an additional group of rats exposed to 14 h of vapor per day was observed for somatic withdrawal 16 h after removal from vapors. A two-way repeated measures ANOVA showed a significant effect of treatment ($F_{1,36} = 14.5$, $p<0.05$) and of time ($F_{1,36} = 25.3$, $p<0.05$) and a treatment × time interaction ($F_{1,36} = 21.6$, $p<0.05$; Fig. 2B). Rats exposed to nicotine vapors for 14 h per day and pretreated with saline did not demonstrate a significant somatic withdrawal syndrome ($p>0.05$). However, rats exposed to nicotine vapors for 14 h per day and pretreated with mecamylamine showed significant somatic signs of withdrawal ($p<0.05$). Rats exposed to regular air and pretreated with saline or mecamylamine did not show significant signs of somatic withdrawal ($p>0.05$), demonstrating that mecamylamine alone does not produce somatic withdrawal signs. These results suggest that intermittent exposure to both 8 and 14 h per day of nicotine vapors can induce nicotine dependence, defined here as the emergence of a somatic abstinence syndrome after cessation of nicotine exposure.

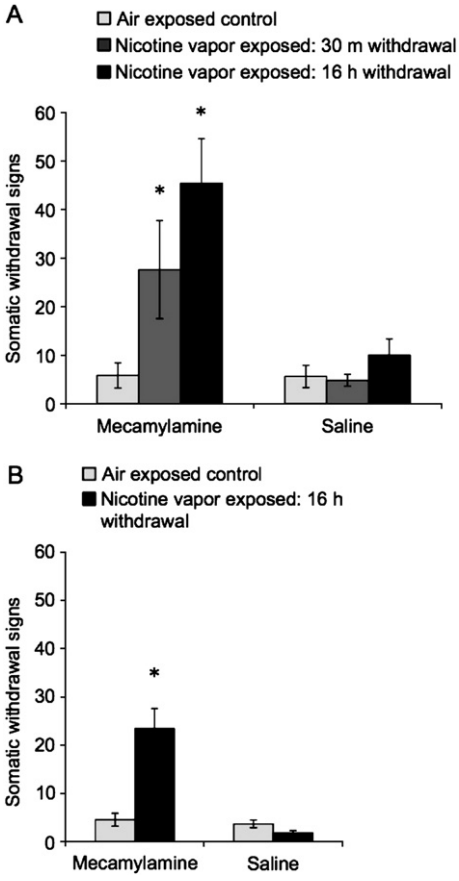

Fig. 2. Somatic withdrawal signs after chronic exposure to nicotine vapors. (A) Rats were exposed to untreated air (control) or nicotine vapor for 8 h per day for 7 days and given a single injection of mecamylamine (1.5 mg/kg) to precipitate withdrawal or saline on the last day of exposure. Rats treated with mecamylamine and exposed to nicotine vapors showed significant somatic withdrawal signs after both 30 min and 16 h of withdrawal in comparison to control rats treated with regular air (*$p<0.05$). Rats treated with saline did not demonstrate significant somatic withdrawal signs. (B) Rats were exposed to untreated air (control) or nicotine vapor for 14 h per day for 7 days and given a single injection of mecamylamine (1.5 mg/kg) to precipitate withdrawal or saline on the last day of exposure. Rats treated with mecamylamine and exposed to nicotine vapors showed significant somatic withdrawal signs after 16 h of withdrawal in comparison to control rats treated with regular air (*$p<0.05$). Rats treated with saline did not demonstrate significant somatic withdrawal signs. Data represents means +/− SEM.

4. Discussion

Currently there is a gap in animal models of nicotine exposure such that the most commonly used methods for examining the effects of chronic nicotine administration are invasive and cannot mimic intermittent nicotine exposure or exposure to very low levels of nicotine in the air as in second-hand exposure. Here we have provided a new rodent model of nicotine vapor administration that produces consistent levels of nicotine in the blood that are relevant for both heavy smoking and second-hand smoking. Nicotine vapor administration is a non-invasive, high-throughput technique that mimics the intermittent aspect and route of administration in humans. The nicotine vapor chamber system described here allows the vaporization of nicotine in the air in a stable, reliable and consistent manner. The administration of nicotine vapor to rats produced plasma nicotine concentrations that are clinically relevant to those observed in human smoking. Finally, rodents that were intermittently exposed to nicotine vapors for one week developed dependence to nicotine, as evidenced by the demonstration of a precipitated somatic abstinence syndrome.

Vaporization of nicotine during cigarette smoking is obtained by increasing the temperature of nicotine up to its flash point (temperature at which nicotine vapor burns: 124 °C; Windholz et al., 1983). In our model, vapor-phase nicotine is obtained by evaporation of nicotine at room temperature (Waldum et al., 1996). The vaporization of nicotine without the use of heat is possible because nicotine (free base) has a relatively high volatility at room temperature compared to other drugs of abuse (nicotine: 2.6×10^{-2} Torr, cocaine: 9.8×10^{-6} Torr, Δ9-THC: 1×10^{-7} Torr, and heroin: 5.7×10^{-8} Torr; Meng et al., 1997). The constant bubbling of nicotine (free base) with medical graded air increased the surface of air/nicotine exchange and limited the saturation of air with nicotine to ultimately increase the vaporization of nicotine. In addition to the vapor-phase nicotine in the chambers, it is possible that some of the nicotine vapor re-condensed into small droplets, however, the use of a drop catching bottle considerably limited this phenomenon. Nicotine at high concentrations is considered a hazardous substance that may lead to serious health complications such as skin irritation, infertility and cancer (Waldum et al., 1996; Weisberg, 1985; Wynder and Hoffmann, 1979). However, exposure to nicotine vapor for 24 h per day for 2 years at a concentration 2–3 times higher than that used in the present study did not lead to an increase in mortality, atherosclerosis or tumors in rats, suggesting that this method of nicotine exposure is safe both for the animals and the experimenter (Waldum et al., 1996).

Inhalation is a very potent route of drug administration, and is characterized by fast absorption from the nasal mucosa and the extensive lung capillaries (Meng et al., 1997). The inhalation of tobacco smoke results in an elevation of arterial blood nicotine concentration (Meng et al., 1997; Rose et al., 1999). Previous studies using inhalation

of nicotine have focused on smoke inhalation (Ohnishi et al., 2007), however the drawback of this technique is that it is not possible to know the specific effect of nicotine separate from the other 4000 plus compounds that are inhaled from tobacco smoke (Wynder and Hoffmann, 1979). The nicotine vapor model described here allows for the specific determination of the effect of nicotine vapor independent of the other constituents of cigarette smoke.

We observed that exposure to nicotine vapors (0.2–1.1 mg/m^3) for 2 to 14 h would produce blood nicotine concentrations relevant to those found in moderate to heavy smokers (Matta et al., 2007), confirming a previous report (Waldum et al., 1996). After chronic (7 days) intermittent (8 h per day) exposure to 0.2 mg/m^3 of nicotine vapors, the blood concentrations of nicotine averaged 22 ng/mL, a value that resembles the plasma nicotine concentration of a regular smoker throughout the day (Matta et al., 2007). These results demonstrate that the concentration of nicotine vapor in the air can be easily modified to produce the desired level of nicotine exposure, which may mimic blood nicotine levels observed in humans after heavy, moderate, or second-hand smoking.

Chronic intermittent exposure to nicotine vapor produced dependence to nicotine, as measured by precipitated somatic withdrawal signs, both 30 min and 16 h after cessation of nicotine exposure. These results are similar to those reported in previous studies using osmotic minipumps (Epping-Jordan et al., 1998; Grieder et al., 2010; Malin et al, 1992; Watkins et al., 2000) or intravenous self-administration (Paterson and Markou, 2004). The lower number of somatic withdrawal signs in the 14 h compared to the 8 h exposure group may be explained by the fact that different cohorts of rats were scored by different experimenters. However, it is important to note that both groups exhibited a robust increase in somatic signs of withdrawal after mecamylamine injection. The 16 h time point falls within the period of peak nicotine withdrawal in the rat (Epping-Jordan et al., 1998; Grieder et al., 2010). Interestingly, we did not observe a spontaneous somatic withdrawal syndrome after 8 or 14 h of daily intermittent exposure. These results are similar to a previous study showing that intermittent exposure to nicotine self-administration for 1 h per day for 5 days per week does not produce spontaneous somatic withdrawal, whereas unlimited access for 7 days per week produced robust spontaneous somatic withdrawal (Paterson and Markou, 2004). It is therefore possible that rats exposed intermittently to nicotine habituate to the repeated experience of spontaneous withdrawal at the conclusion of each day. An alternative explanation is that the low nicotine blood levels observed at this time point (3.49 ng/mL) may have been sufficient to prevent the expression of spontaneous withdrawal. This hypothesis is supported by the fact that the nicotinic receptor antagonist mecamylamine precipitated somatic signs of withdrawal, probably by preventing the action of circulating nicotine in the brain (Malin et al., 1994). It is then possible that spontaneous withdrawal would be observed in rats treated with nicotine vapors after a longer period of withdrawal when blood nicotine concentrations were further decreased.

Taken together, these results demonstrate that chronic intermittent nicotine vapor inhalation induces significant blood nicotine levels and produces physical dependence. The concentration of nicotine in vapor chamber air can be adjusted to produce blood levels of nicotine that are relevant to heavy, regular or second-hand smoking. Administration of chronic nicotine through vapors does not require surgery, is high-throughput and is less stressful to the animal than repeated injections, minipumps or intravenous infusions. Nicotine vapor administration provides a method for the investigation of the effect of acute or chronic nicotine inhalation and provides a new animal model for the investigation of the neurobiology of nicotine dependence.

Conflict of interest

Maury Cole is an owner of La Jolla Alcohol Research Inc, a company focused on the development of inhalation chambers. No La Jolla Alcohol Research Inc funds were used in this work. Olivier George, Taryn Grieder and George Koob declare that no financial support has been received from any individual or corporate entity for research or professional service, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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