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Nicotine vapor method to induce nicotine dependence in rodents

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Abstract

Nicotine, the main addictive component of tobacco, induces potentiation of brain stimulation reward, increases locomotor activity and induces conditioned place preference. Nicotine cessation produces a withdrawal syndrome that can be relieved by nicotine replacement therapy. In the last decade, the market for electronic cigarettes has flourished, especially among adolescents. The nicotine vaporizer or electronic nicotine delivery system is a battery-operated device that simulates the experience of tobacco smoking without inhaling smoke. The device is designed to be an alternative for conventional cigarettes that emits vaporized nicotine that is inhaled by the user. This report describes a procedure to vaporize nicotine in the air to produce blood nicotine levels in rodents that are clinically relevant to those that are observed in humans and produce dependence. We also describe how to construct the apparatus to deliver nicotine vapor in a stable, reliable, and consistent manner and how to analyze air for nicotine content.

Keywords

Nicotine; Vapor; Dependence; Withdrawal

Introduction

Preclinical models of nicotine dependence are extensively used to determine the pharmacological effects of nicotine, unveil the neurobiological mechanisms that underlie nicotine addiction, and facilitate medication development. For the last century, tobacco smoking has largely dominated the tobacco market; snuff and chewing tobacco represent relatively unpopular alternatives to nicotine self-administration (Cepeda-Benito, 1993; Tomar, 2002). The recent rise of electronic cigarette (e-cig) use in the last decade has radically changed the tobacco market and epidemiology of nicotine use. For example, e-cig use has already surpassed traditional cigarette use in teenagers and young adults (Demissie et al., 2017; Westling et al., 2017). Such a rapid and dramatic change in the way people self-administer nicotine is both a challenge and unique opportunity for preclinical researchers to develop models of nicotine use and nicotine dependence with better face validity. The most important challenges with regard to modeling e-cig use in animals are the difficulty in achieving a stable level of nicotine vapor exposure and the potential health risks to the experimenter through accidental exposure to nicotine vapor. A key outcome of the

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development of better animal models of e-cig use is the possibility of testing the causal relationship between nicotine vapor exposure and health risks, including the potentially higher vulnerability to develop tobacco use disorders. Indeed, e-cigs are often promoted as safer alternatives to traditional cigarettes, but little is actually known about the health risks associated with these devices. It is also unclear whether e-cigs may be effective smoking-cessation aids or whether they actually perpetuate nicotine addiction and thus interfere with quitting.

In this protocol, we describe a novel approach to produce nicotine vapor safely, easily, and reliably. We also illustrate a simple method for detecting the nicotine concentration in the air and blood. The behavioral approaches to measure affective symptoms of withdrawal, such as conditioned place aversion, hyperalgesia, anxiety-like behavior, and intracranial self-stimulation, can be performed in these animals at different time points of nicotine vapor exposure. This method is noninvasive for rats and has better face validity than existing techniques to study the neurobiological and psychological changes that are associated with e-cig use.

Basic Protocol

There are currently three general methods that are used to expose laboratory animals to nicotine: (1) injections (subcutaneous or intravenous), (2) transdermal delivery (nicotine patch), and (3) nicotine vapor inhalation. The overall goal of these methods is to administer nicotine so that nicotine levels in blood are relevant to first-, second-, or third-hand exposure. Nicotine concentrations vary greatly between single and repeated exposures, which are often used to investigate the pharmacological effect of nicotine with prolonged treatment (several weeks) to model the development of nicotine addiction. Injections and transdermal delivery are the easiest approaches for preclinical researchers, but they lack face validity because they employ a different route of administration than e-cigs and traditional cigarettes (i.e., pulmonary route).

The nicotine vapor system that we describe below solves this problem by giving rats access to nicotine vapor in small chambers that can be individually controlled, thus providing a flexible approach that can accommodate various delivery and dosage regimens in the same cohort of rodents. The nicotine vapor system allows access to the animals while they are exposed to nicotine, which also permits the administration of test compounds and/or sampling of biological fluids without disturbing the nicotine environment. The size and portability of the vapor chambers allow them to be implemented in any laboratory space. The method is not labor-intensive for research personnel and only requires minimum training. It can also be used for prolonged periods of time (weeks to months) without stressful manipulations (e.g., injections or pump implantation). Furthermore, it can produce high blood levels of nicotine that lead to dependence (as defined below). This system can be used in both rats and mice, with the only difference that the size of the chambers should be adequate for each species. Finally, this model is a major refinement of existing animal models of nicotine dependence that are easily approved by Institutional Animal Care and Use Committees because it allows investigators to reduce the number of animals to be used and reduce experimental pain and distress by avoiding surgery and postoperative pain.

Nicotine Vapor Machine/Components

1. Standard rat cages (18 inch length × 11 inch height × 11 inch width; Allentown).
2. Sealed Plexiglas chambers (25 inch length × 16 inch height × 21.5 inch width; LABEX).
3. Pure nicotine free-base ([−]-nicotine, ≥ 99% purity; Sigma-Aldrich).
4. Pyrex gas-washing bottle (250 ml, Sigma Aldrich).
5. Drop-catch bottle (Sigma Aldrich).
6. 2000 ml Erlenmeyer vacuum flask (Fisher Scientific).

Induction of Nicotine Dependence

1. Fill the gas-washing bottle with 100 ml of nicotine free-base.
2. Connect the gas-washing bottle to an air supply with the air flow rate set to 10 or 20 L/min.

   *NOTE: Nicotine vapor is produced by bubbling air with a constant airflow to produce the evaporation of nicotine. The nicotine vapor passes through a drop-catch bottle (*Waldum et al., 1996*) and then is diluted by the introduction of 60 L/min of clean air in a 2000 ml Erlenmeyer flask at room temperature. The nicotine-air mixture is uniformly distributed between the chambers using a constant flow rate (5–20 L/min). The concentration of nicotine vapor can be adjusted by modifying the flow rate at which nicotine is bubbled. Air controls are treated in a similar way, with the only difference that the air that enters the cages does not contain nicotine*.
3. Place the animals (n = 1 to 2) in the cages, and place two cages in the chambers to expose the animals to nicotine or air vapor. No acclimation period is required.

   Bubble nicotine at 2–20 L/min. This depends on the concentration of nicotine that the experimenter chooses, the body weight of the animals, and the strain. It is usually set at 5 L/min for a 300 g Wistar rat, providing 7 mg/m^3 of nicotine.
4. Expose the animals to nicotine air for 14–21 h/day for 7–21 consecutive days to produce dependence. Intermittent schedules that include every-other-day exposure and exposure during weekdays only also lead to dependence.
5. Measure somatic signs of dependence as below.

Analysis of Nicotine Content in the Test Chamber

Reagents

1. Helium, purified.
2. Hydrogen, prepurified.
3. Air, filtered. Ethyl acetate (Sigma-Aldrich).

4. Triethylamine (Sigma-Aldrich).
5. Desorbing solution: add triethylamine (0.01%) in ethyl acetate (100%).
6. Quinoline secondary stock solution (100 μg/ml), obtained from the dilution of 10.0 ml of quinoline primary stock solution to 100 ml with desorbing solution.
7. Quinoline primary stock solution (1.0 mg/ml), obtained from the dilution of 100 mg quinoline to 100 ml with desorbing solution.
8. Nicotine primary stock solution (1.0 mg/ml), obtained from the dilution of 100 mg nicotine in 100 ml of desorbing solution.
9. Nicotine secondary stock solution (10 μg/ml), obtained from the dilution of 1.0 ml of nicotine primary stock solution in 100 ml of desorbing solution.

Equipment

1. Sampling: Glass tube (SKC, catalog no. 226-93 or equivalent), 70 mm length and 7 mm outer diameter.
2. Personal sampling pump, 0.1–1.0 L/min.
3. Gas chromatograph with a nitrogen-phosphorous detector, an integrator, and an Rtx-5 capillary column.
4. Ultrasonic bath.
5. Vials, autosampler with PTFE-lined caps.
6. Microliter syringes (10 μl).

Special Precautions

Nicotine is classified as a neurotoxin and possible teratogen (Merck and Co; Whitehouse Station, 1996). When the operator prepares the nicotine solution and tests the chambers, it is recommended to avoid nicotine vapor inhalation, skin contact, and ingestion of high nicotine concentrations. Quinoline is classified as moderately toxic, a severe eye irritant, and possible carcinogenic (Merck and Co; Whitehouse Station, 1996). The experimenter should avoid skin contact (readily adsorbed), inhalation, and ingestion. Ethyl acetate is flammable and a fire hazard. Triethylamine is an eye, skin, and respiratory irritant. It is highly recommended to wear appropriate protective clothing (i.e., nitrile gloves, solid scrub jacket, safety mask) and work with these compounds in a well-ventilated hood.

Sampling

1. Each personal sampling pump should be calibrated with an in-line sampler.
2. Immediately before sampling, break the ends of the tubes and attach the open ends of the tubes to the sampling pump with elastic tubing.
3. Sample at 1.0 L/min for 1 h.
4. At the end of sampling, the tubes must be capped with plastic caps and stored in a cold and dark place.

Sample Preparation

1. The front and back sorbent sections of the sampler (glass wool included) should be placed in separate vials.
2. Add the desorbing solution (1 ml) to each vial.
3. Add the quinoline secondary stock solution (10–50 μl) to both the calibration standards and sample vials and cap vials. The level of nicotine that will be in the samples should be estimated, and a similar amount of quinoline should be added.
4. Desorb nicotine by placing the vials in an ultrasonic bath (30 min).

Calibration and Quality Control

1. Calibration
   - **1.1** Add nicotine stock solution standards (at least 6) to desorbing solution (1.0 ml) to cover the range of interest.
   - **1.2** Add quinoline stock solution standards (at least 6) equal to the concentration of nicotine.
   - **1.3** Seal vials with caps.
   - **1.4** Analyze samples and blanks.
   - **1.5** Chart the (ratio of nicotine/quinoline areas *vs.* nicotine (μg n).
2. Calculate desorption efficiency (DE)
   - **2.1** Discard the back sorbent section of the sampler.
   - **2.2** Add a known volume of calibration stock solution onto the front sorbent section of each sampler.
   - **2.3** Wait several minutes for equilibration, cap the ends of the tubes, and keep overnight in the dark.
   - **2.4** Desorb the samplers and analyze with remaining vials.
   - **2.5** Chart DE *vs.* nicotine recovered (μg).

Measurement

1. Inject samples (1 μl) using an autosampler.
2. Measure peak areas and calculate the ratio of nicotine to quinoline.
3. Calculate (corrected for DE) the mass (μg) of nicotine in the sample front section (Wf) and back section (Wb) and in the media blank front section (Bf) and back section (Bb). If Wb > Wf/10, then repeat sampling because it may indicate sample loss.
4. Calculate the concentration (C) of nicotine in the air volume sampled (V):

$$C = \frac{Wf + Wb - Bf - Bb}{V}, mg/m^3$$

Evaluation of Method

The average DE for nicotine is usually ~0.9. The instrumental limit of detection is often ~0.01 μg/sample. Nicotine is usually stable at 5°C for at least 2 weeks if it is kept in the dark. The levels of environmental tobacco smoke (ETS) range from 1 to 100 $\mu g/m^3$ (ASTM, 1990).

Analysis of Nicotine Content in Blood

Materials

1. 100 μl aliquot of plasma.
2. 1.5 ml Eppendorf microcentrifuge tubes.
3. 10 μl of 2-phenylimidazole (1 μM).
4. 20 μl of 20% NaOH.
5. 400 μl of dichloromethane (DCM).
6. Vortex mixer.
7. Centrifuge set at 1500 × *g*.
8. Liquid chromatography mass spectroscopy (LC-MS)

Measurement

1. Hold the rat firmly on a table using a towel, a cone, or restraint apparatus and cut the tip of the tail using a razor blade.
2. Collect blood (0.2–0.5 ml) in Eppendorf tubes that are coated with heparin, and place the tubes on ice.
3. Centrifuge and collect plasma into Eppendorf tubes and analyze using LC-MS/MS (O'Dell et al., 2006).
4. Add plasma (100 μl) to a 1.5 ml Eppendorf microcentrifuge tube.
5. Add 10 μl of 2-phenylimidazole (1 μM).
6. Add 20 μl of NaOH (20%) and vortex well for 10 s, followed by the addition of 400 μl of DCM and an additional 10 s of vortexing.
7. Centrifuge at 1500 × *g* for 10 min, and discard the upper layer.
8. Add 10 μl of HCL (6 M) to the DCM layer.
9. Vortex the samples as above, centrifuge for 10 min at 1500 × *g*, and remove the aqueous layer and any precipitate.

10. Transfer the bottom DCM layer to a new microcentrifuge tube and evaporate using nitrogen.
11. Store the samples at −70°C once fully lyophilized.
12. Determine the levels of nicotine/cotinine using LC-MS.
13. Reconstitute the samples with 25 µl of the liquid chromatography mobile phase that contains ethyl-nor-cotinine (0.25 pmol).
14. After homogenization, inject 1 µl into the LC-MS using a capillary autosampler maintained at 5°C.
15. Separate nicotine, cotinine, and the internal standards using a 1×150 mm polyhydroxyethyl-A column (5 µm spheres, 100 Å pore size; Poly LC) and an isocratic mobile phase that consists of ammonium formate (20 mM) in acetonitrile 72% (v/v) with formic acid 0.1% (v/v) delivered at 20 µl/min using a capillary liquid chromatography pump.
16. Deliver the eluent from the column to the MS using a microelectrospray interface (with a nebulization pressure of 13 psi and nitrogen [300°C at 7 L/min]).
17. Quantify nicotine, cotinine, and the internal standards using ion monitoring at the following mass-to-charge (m/z) ratios: nicotine = 163.1, cotinine = 177.1, ethyl-nor-cotinine = 191.2, 2-phenylimidazole = 145.2.
18. Calibration curves are require three standard concentrations, measured in triplicate.

    If measurements are not as expected, double check that the standard curve is within the correct range of concentrations and that all of the solutions are freshly made.

Induction of Nicotine Dependence

Animals are exposed to nicotine vapor for 14–24 h/day for 7–21 consecutive days. Control animals are exposed to untreated air in vapor chambers.

The monitoring of daily body weights and somatic signs of withdrawal after 24/48 h of nicotine cessation is necessary.

Somatic Signs of Withdrawal

Nicotine dependence is defined by the emergence of a nicotine abstinence syndrome after the cessation of chronic nicotine exposure. Such an abstinence syndrome is observed in humans and rodents (Epping-Jordan et al., 1998; Hildebrand et al., 1997; Malin et al., 1994; Malin et al., 1993; Malin et al., 1992; Shiffman and Jarvik, 1976) and is associated with both somatic and motivational components. In rats, the somatic signs of nicotine withdrawal include ptosis, facial fasciculation, abdominal constrictions, and hyperalgesia. The motivational components include craving (increase in progressive-ratio responding) and anxiety-like behavior.

Materials

1. Plastic transparent cylindrical container (30 cm × 29 cm).
2. Video recording device.

Procedure

Score spontaneous withdrawal signs 24 h after the last nicotine administration. To measure somatic withdrawal signs, place the rats in the plastic opaque cylindrical container where they can move around freely. Observe each rat for 30 min in the plastic transparent cylindrical container while video recording behavior to observe:

1. Abdominal constrictions, including gasps, writhes, and cheek tremors.
2. Facial fasciculations, including cheek tremors, chews, teeth chattering, ptosis, and eye blinks.
3. Miscellaneous signs, including shakes, head-shakes, escape attempts, licks, scratches, and yawns.

Important—Animals must be habituated to the room and the cages for 1–3 days before testing (Watkins et al., 2000) in order to avoid misreading exploratory behaviors for the new environment, as withdrawal scores. A pause between episodes is required to count multiple and successive counts of any sign. If ptosis is continuously present, then it is counted only once every minute. The total number of somatic signs is the sum of individual occurrences of each sign.

Advantages Compared with Other Methods

Overall, the nicotine vapor system is well adapted for studies in which biological parameters are only measured at the end of a particular treatment period, once the animals are removed from the environment, and for experimental protocols that require the development of nicotine dependence. This nicotine inhalation chamber provides the following advantages: flexibility in terms of exposing animals to different levels and/or schedules of nicotine; the possibility of combining the delivery of nicotine and alcohol in a two-bottle choice paradigm; relatively low cost; relatively modest space requirements coupled with ease of use in any type of laboratory space. Another advantage of this model is that nicotine vaporization is obtained without the use of heat. Nicotine (free base) has relatively high volatility at room temperature (2.6×10^{-2} Torr), but this makes it easier and cheaper to obtain nicotine vaporization compared with other drugs. One can then use the desired nicotine concentration based on the animal's body weight, strain, or gender.

Summary

- Rodents can be exposed to nicotine without requiring injections or surgery, procedures that are stressful as well as labor-intensive.
- The system is versatile: (1) Nicotine doses can be adjusted on an individual basis as a function of weight and subject-sex. (2) The investigator has full control over

the regimen of nicotine delivery, including repeated intermittent exposure. This represents a very significant advantage over any other currently available method. In contrast, subcutaneous pumps, for example, provide continuous delivery, and self-administration in saline solution is controlled by the animal, not by the investigator, in terms of levels and duration of intake. (3) Nicotine can be delivered for prolonged periods of time and at high levels, which are critical parameters to induce dependence. (4) Investigators can sample biological fluids, such as blood, and administer other test compounds while the animals are exposed to nicotine.

Commentary

Background Information

This novel animal model is a noninvasive, high-throughput technique that simulates the intermittent aspect, duration, and route of administration of e-cig exposure in humans. The nicotine vapor system produces reliable and stable levels of nicotine vapor in the air with minimum equipment requirements. Previous reports have shown that this approach can lead to blood nicotine levels that are clinically relevant to human smoking (Gilpin et al., 2014).

Critical Parameters

Special attention should be paid to the animals' health. The animals will show significant loss of body weight if the concentration of nicotine is too high, but this does not necessarily indicate that the animals are sick because nicotine is a potent appetite suppressant. Monitoring body weight daily or every other day is required.

Troubleshooting

(1) Having color-coded tubes for "in" and "out" vapor flow may help troubleshoot the apparatus if there are any disconnections of the tubes from the nicotine-containing flask to the chambers. (2) If the experimenter notices condensation in the walls of the airtight containers where rodents are exposed, then this may suggest that nicotine air is going in but not out of the chamber. The vacuum flask may need to be changed. Check to see if there is any clogged tube that blocks air flow.

Statistical Analyses

The researchers must demonstrate that vaporizing nicotine into airtight chambers where rodents are exposed leads to blood nicotine levels that are similar to those found in heavy smokers (i.e., 20–70 ng/ml; (Gourlay and Benowitz, 1997; Henningfield et al., 1993). Lower levels of nicotine can be achieved and maintained if required by investigators who study the effects of low exposure to the drug. All samples should be analyzed in duplicate, and statistical analyses should be performed using one-way analysis of variance followed by Fisher's Least Significant Difference *post hoc* test. For individual means comparisons, Student's paired or unpaired *t*-test should be used. Values of $p < 0.05$ should be considered statistically significant.

Understanding Results

Time Considerations

The duration and dose of nicotine to which the animals are exposed depend on the scientific question (first-, second-, third-hand exposure). This will depend on the experimental design. Some researchers may be interested in the induction of nicotine dependence. This may require 7–21 consecutive days of intermittent nicotine vapor exposure. Others may be interested in daily *vs.* weekly exposure to nicotine vapor. The experimental design can be modified based on the hypothesis under test.

Maintenance

The tubes that are connected to the bottles need to be changed between experiments and animal cohorts. Nicotine may deposit on the inner walls of the tubes, resulting in a change of the nicotine concentration. Nicotine is light-sensitive, and the solution of pure nicotine should be contained in a brown gas-washing bottle or in a covered compartment of the nicotine vapor machine.

Anticipated results

The nicotine content in the chambers should be approximately 7 mg/m^3 when using a flow rate of 10 L/min. The body weight of the animals that are exposed to 14 h of nicotine per day for 7 consecutive days should be 30–40% lower than air-exposed animals. These animals are more sensitive to pain compared with air-exposed rats and exhibit robust somatic signs of withdrawal (George et al., 2010; Grieder et al., 2010) and higher motivation to self-administer nicotine during early withdrawal (Gilpin et al., 2014).

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Significance Statement

We describe a novel approach to produce nicotine vapor safely and easily. We also illustrate a simple method for detecting the nicotine concentration in the air and blood. Behavioral approaches to measure affective symptoms of withdrawal, such as conditioned place aversion, hyperalgesia, anxiety-like behavior, and intracranial self-stimulation, can be performed in these animals at different time points of nicotine vapor exposure. This method is noninvasive for rats and has better face validity than existing techniques to study the neurobiological and psychological changes associated with electronic cigarette use.

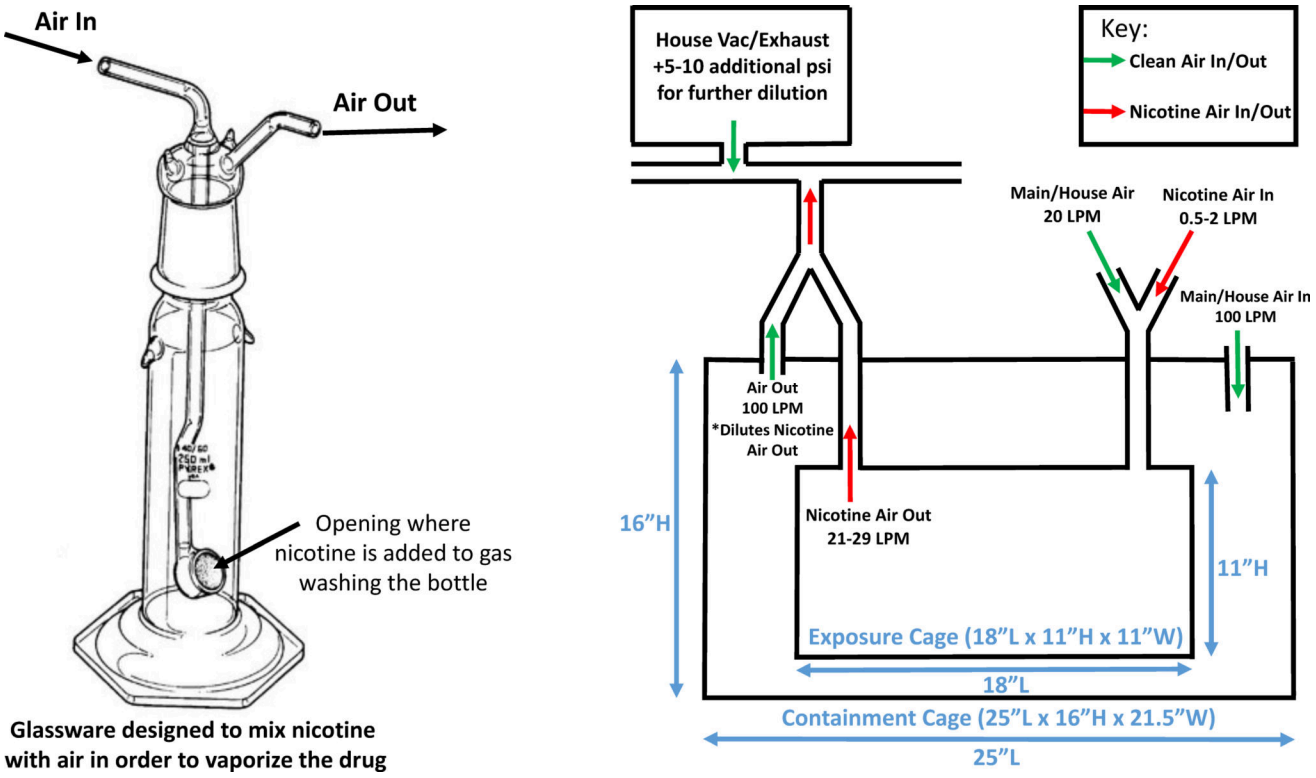

Figure 1.

Left panel: schematic of a gas-washing bottle designed to mix nicotine and air. Right panel: Schematic view of 'in' and 'out' air flows.

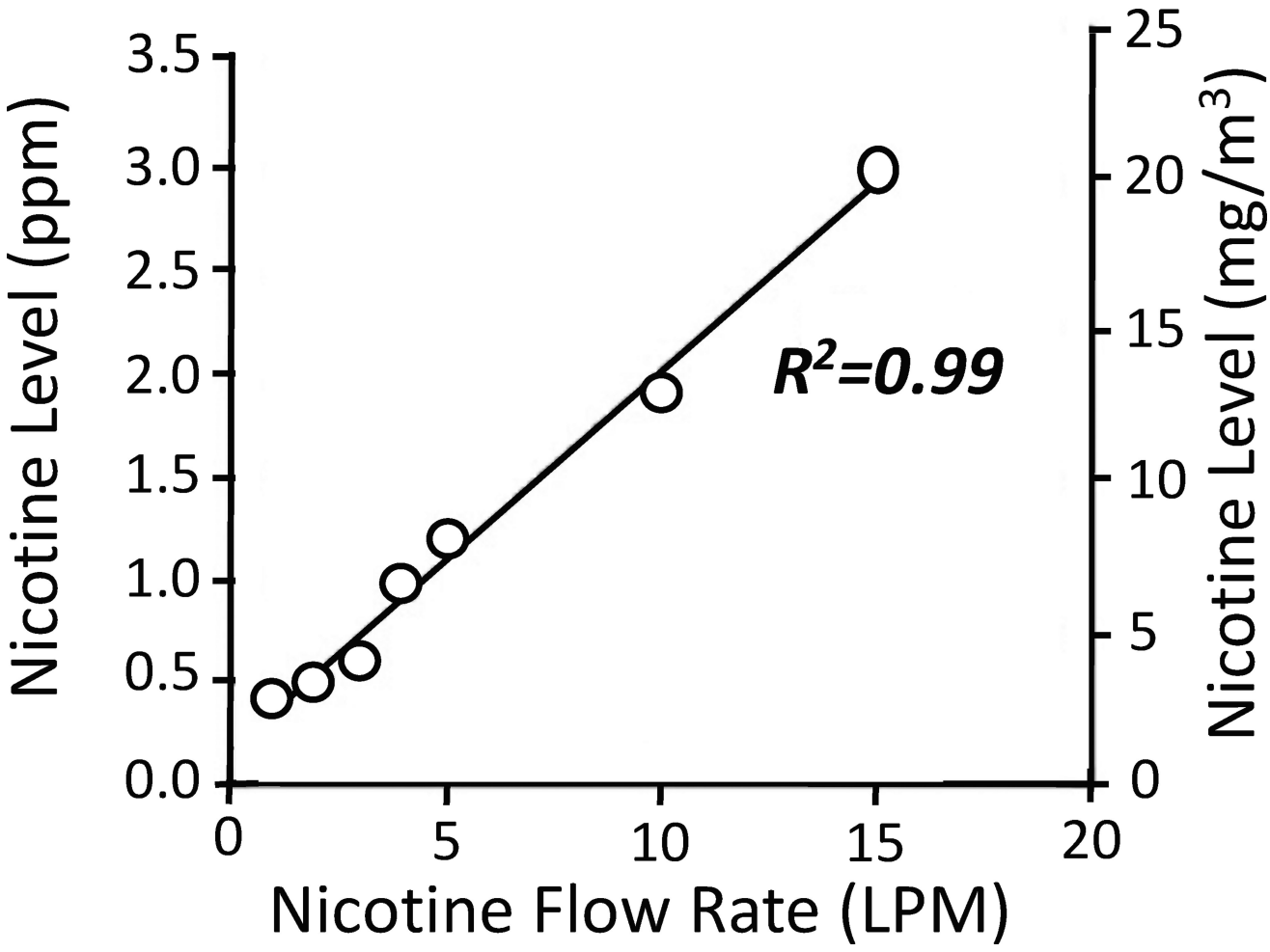

Figure 2.

Presents the air nicotine concentrations as a function of nicotine flow rate. (From Gilpin NW, Whitaker AM, Baynes B, Abdel AY, Weil MT, George O. Nicotine vapor inhalation escalates nicotine self-administration. Addict Biol 2014;19(4):587-92. doi: 10.1111/adb.12021. With permission from the authors.)

Table 1

Mean ± S.E.M of individual somatic signs of spontaneous and precipitated nicotine withdrawal. Mecamylamine (1.0 mg/kg, s.c) was injected 30 min prior the withdrawal scoring. Student's *t*-test analyses were used.

	Spontaneous (24 hr Nicotine Free)			**Precipitated (Mecamylamine s.c)**		
	Air	**Nicotine**	***Df = 10***	**Air**	**Nicotine**	***Df = 10***
Teeth Chat	1.2 ± 0.4	3.3 ± 0.5 (**)	$t = 3.15$; $p < 0.01$	2.2 ± 1.3	10.5 ± 1.9 (**)	$t = 3.6$; $p < 0.01$
Blinks	0.3 ± 0.2	0.5 ± 0.5	$t = 0.3$; NS	7.7 ± 3.7	7.3 ± 2.4	$t = 0.07$; NS
Head Shakes	1.5 ± 0.6	2 ± 0.3	$t = 0.65$; NS	3.0 ± 1.7	9.0 ± 5.1	$t = 1.119$; NS
Paw tremors	1.5 ± 0.5	4.3 ± 0.5 (**)	$t = 4.029$; $p < 0.01$	1.7 ± 0.9	1.3 ± 0.8	$t = 0.28$; NS
Abdominal	0.2 ± 0.17	1.3 ± 0.3 (**)	$t = 3.13$; $p < 0.01$	2.0 ± 0.8	9.7 ± 1.4 (**)	$t = 4.7$; $p < 0.01$
Yawn	0.3 ± 0.3	0.8 ± 0.4	$t = 0.85$; NS	0.2 ± 0.2	0.3 ± 0.3	$t = 0.44$; NS
Tremors	0.7 ± 0.3	2.7 ± 0.2 (**)	$t = 5.071$; $p < 0.01$	0.8 ± 0.5	2.8 ± 0.2 (**)	$t = 3956$; $p < 0.01$
Gait	0.8 ± 0.4	2.3 ± 0.3 (*)	$U = 5.5$; $p < 0.05$	0.7 ± 0.3	2.2 ± 0.5(*)	$U = 5.5$; $p < 0.05$
Pilo erection	0.3 ± 0.2	2.3 ± 0.3 (**)	$U = 1$; $p < 0.01$	0.7 ± 0.2	2.0 ± 0.3 (**)	$U = 2$; $p < 0.01$
Ptosis	0.3 ± 0.3	0.7 ± 0.4	$U = 15$; NS	0.7 ± 0.5	2.3 ± 0.5	$U = 7$; NS

Significance was indicated by

* $p<0.05$ and

** $p<0.01$.

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