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Protocol Article

Experimental protocol for detecting higher alcohol consumers from a conventional rat line based on basal anxiety

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A B S T R A C T

Predisposition for a high alcohol intake and the impact of alcohol-abstinence-relapse may be reliable experimentally performed in conventional adult rat lines if animals received juvenile exposure to alcohol (e.g., by forced consumption) and selecting those individuals with high basal anxiety levels during juvenile periods. Importantly, a forced alcohol consumption phase must be followed by an imposed withdrawal period to form an exposure-abstinence cycle (at least two cycles are required) which allow to obtain animals with notorious alcohol relapses. The easier way to test alcohol relapses is through voluntary ethanol intake models. On the other hand, the anxiety classification may be performance by classical paradigms such as an elevated plus maze test, defensive burying behavior test or any other. Here, we provide a step-by-step protocol description to detect higher alcohol consumers animals from male Wistar rats. This protocol should be especially useful for those interested in studying the participation of specific brain nucleus [e.g., periaqueductal gray (PAG)] and/or the neurotransmitters involved [e.g., neuropeptide Y (NPY)] in the alcohol intake phenomena if it is combined with stereotaxic surgery. However, every administration route of treatments or experimental design is appropriate; the limit is the own imagination, and the resources.

A R T I C L E I N F O
Method name: Anxiety-based alcohol susceptibility detection method
Keywords: Drug intake, Alcohol, Relapse, Anxiety, Elevated plus-maze, Defensive burying
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Specifications table

Subject area:	Neuroscience
More specific subject area:	Behavioral neuroscience
Protocol name:	Detecting higher alcohol consumers from conventional rat lines based on basal anxiety
Reagents/tools:	1. Elevated plus maze apparatus, Panlab (Mod: LE840A). 2. Glass tubes of 25 × 200 mm with respective mouthpiece for diet liquid access. 3. Ethanol solutions. Typically, 2–10% ethanol for the forced alcohol consumption (FAC) periods and 5%, 10%, and 20% for the voluntary alcohol consumption/relapse assessment. 4. (Optional). If the project involves i.c.v. administration: stereotaxic frame (e.g., World Precision Instruments, Sarasota, FL, USA. Mod: 502,650, Stoelting Co, David Kopf, or Harvard Apparatus, etc.). A Rat Brain Atlas for Stereotaxic Coordinates (e.g., [9]. 5. Guide cannulas. They can be manufactured from hypodermic needle (23 gauge) with an inserted stylet made it from dental needle (31 gauge). 6. Microinjection syringes (e.g., 1 µL) (e.g., Hamilton Co, Reno, NV, USA). 7. Microinjection Syringe Pump (e.g., World Precision Instruments, Sarasota, FL, USA. Mod: UMP3T-2). 8. Defensive burying behavior test apparatus (e.g., Intellibio Innovation, Nancy, France. Mod: A-1802-00024). 9. Video Recorder system. It is preferable an automatic behavior analyzer software for revising and analyzing the behavioral tests (e.g., defensive burying behavior test; DBB). 10. Vibratome or Cryostat for the brain histology. 11. Staining the coronal brain sections with Cresyl-Violet, Hematoxylin-Eosin, or Methyl-Blue (e.g., available at Sigma Aldrich). 12. Stereoscopic microscope for visualizing the histological samples and verifying the microinjection site. 13. Statistical software.
Experimental design:	1. Classify the male Wistar rats in low (LA) and high anxiety (HA) by using the elevated plus maze test (EPM), DBB or the specific test according to the kind of anxiety under study. 2. Performance a FAC protocol during the juvenile age for 30 days followed by a forced withdrawal period of 8 days to obtain an exposure-withdrawal cycle. Repeat the cycle, at least once. 3. (Optional). If you require i.c.v. administration , proceed to implant the cannulas by stereotaxic surgery according to a rat brain stereotaxic atlas. 4. At this step, you can analyze miscellaneous behavioral, molecular, and biochemical parameters among high-alcohol consumers and comparing them with low-alcohol consumers and the link with juvenile levels of anxiety. 5. Test the voluntary alcohol consumption (VAC) in your groups. You should expect a higher consumption in the HA group. 6. (Optional). Report of blood alcohol concentrations (BACs; mg/dL or mg %), and its correlation with the alcohol intake. 7. Analyze your data by a multifactorial approach (e.g., three-way ANOVA).
Trial registration:	Not applicable.
Ethics:	The protocol is in accordance with the procedures established by the NIH in the Guide for the Care and Use of Laboratory Animals in the USA (NIH Publications No. 8023, revised 1978), and by the Mexican Guidelines for Animal Care (NOM-062-ZOO-1999).
Value of the protocol:	1. It allows developing a reliable method for obtaining higher alcohol consumer animals from male Wistar rats. 2. It easily evaluates the link on anxiety and alcoholism and the potential participation of numerous neurotransmitters, receptors, and brain nuclei. 3. It can be carried out in every neuroscience laboratory, with basic equipment and training, and may help to save money and resources.

Description of protocol

Rationale:

- The anxiety-based alcohol susceptibility detection method allows to obtain male Wistar rats susceptible for higher alcohol-intake which are useful for studying the alcoholism

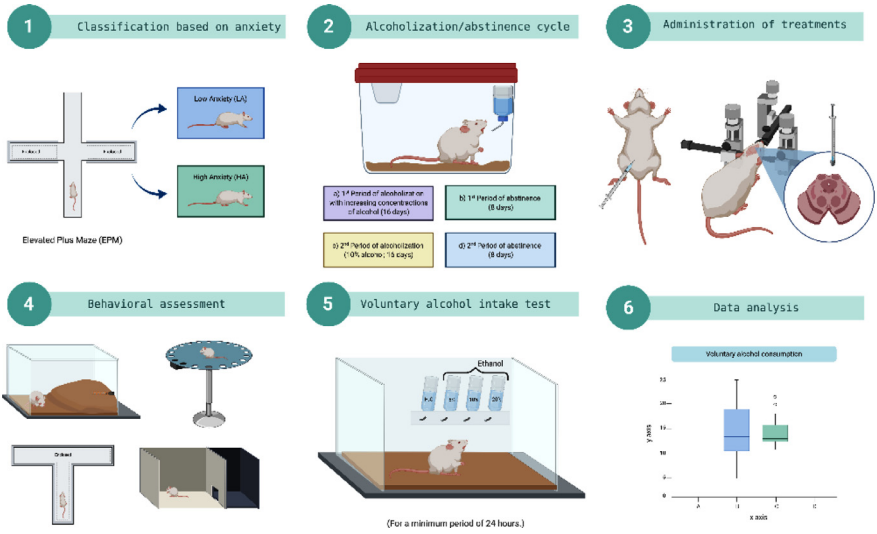

Fig. 1. Graphical description of the protocol.

pathophysiological phenomena (e.g., alcohol intake, withdrawal, and relapse) easily from conventional rat lines by classifying them according to its juvenile basal anxiety levels.

- Animals with high basal anxiety levels (HA) are susceptible for a higher alcohol consumption during its mature life [3,13]. But a previous alcohol exposition during the juvenile life is mandatory to guarantee the alcohol preference during the mature life of animals.
- This protocol is compatible with i.c.v. administration, but also admit systemic or any other pathway if the neurotransmission systems involved is trying to be pharmacologically studied.

Step by step description of the protocol:

1. Animals. For this protocol, male Wistar rats are needed. Note: although the present protocol was validated only in male Wistar rats it is possible that similar results may be achieved by using other common linages and/or female rats. Clearly, the above notion remains to be probed.
2. The elevated plus maze (EPM) test allows classifying the level of "primary, innate or basal" anxiety in rats [6,7]. It is based on the observation of spontaneous activity of rodents when they are placed in a novel and aversive environment produced by the height, lighting, and open spaces. In rat populations, proactive coping behavior is distributed in a U-shaped curve, meaning that only a small portion of the population will display an intermediate coping style and the remaining individuals are characterized as either clearly proactive or clearly passive coping [12,15]. The EPM consisted of two open arms (50 $\times$ 10 cm) and two enclosed arms (50 $\times$ 10 $\times$ 50 cm), raised 50 cm from the floor, located in a room with a dim white light. Assessing time spent in the closed and open arms, as well as the number of entries (i.e., when all paws of the rats were into the arm) in a period-time (usually 5 min) test. The percentage of time spent on the open arms (time on the open arms/time on open + time on closed arms X 100) is a validated index of anxiety (Fig. 1).
3. Expose male Wistar rats to alcohol intake in a prolonged period during its juvenile age. Forced alcohol consumption after weaning (e.g., at post-natal day 21; PND-21, during 30-day) is an easy way for the above. For this, slow increases from a very low alcohol concentration to a higher one may be followed. Importantly, it is required to incorporate withdrawal periods (e.g., a prolonged period of alcohol restriction). Moreover, a repetition of the forced alcohol/withdrawal cycle is recommended. This step is especially critical for the development of clear alcohol relapse in adult individuals. Long-term forced alcohol use with repeated episodes of alcohol

deprivation leads to high alcohol consumption and aids in the installation of addiction [5,8]. In this protocol, animals received alcohol as the only source of fluid in diet (i.e., a FAC) for sixteen days as follows: four days with 2% ethanol (v/v), four days with 5% ethanol (v/v) and eight days with 10% ethanol (v/v). After the first FAC period, rats were exposed to an alcohol-withdrawal period for eight days. Immediately after, the FAC-withdrawal cycle was repeated (Fig. 2). Admittedly, forced alcohol consumption may not be the closest model for the mainly voluntary alcohol intake in humans. Nevertheless, it should be highly probable that distinct protocols of voluntary alcohol intake also work. Clearly, those would require to be validated.

4. As an optional step, stereotaxic surgery, implantation of osmotic pumps or any systemic administration may be included. Especially, for assessing the participation of a neurotransmitter/receptor in a brain area, implantation of cannulas or electrodes may be a performance by stereotaxic surgery first described in the mid-1940s, this technique has evolved for the well-being of the rodent [4].
5. Analyze miscellaneous aspects of pathophysiology, pharmacology, molecular biology, genetic, and its co-relationship of anxiety juvenile basal levels with adult alcohol intake, withdrawal, and/or relapse.
6. Measure the voluntary alcohol consumption in all your groups. For example: in rats housed individually, you can bring access to four standard glass tubes (70 mL, 25 × 200 mm) equipped with a glass mouthpiece containing a terminal hole (diameter = 1 mm) to allow fluid intake by licking with minimum spillage. The glass tubes must be previously mounted on the front of the cage, and each will be filled with different solutions: fresh water, 5% v/v ethanol-water, 10% v/v ethanol-water, and 20% v/v ethanol-water. In our example, ethanol concentrations were chosen based on those employed by Spanagel and Hölter [11] and Mendoza-Ruiz et al. [8] [8, 11]. The four tubes should be randomly rearranged daily to avoid position preference. Each glass tube is weighed to quantify the amount of alcohol consumed per solution. Alcohol intake is calculated in grams of absolute alcohol per kg of body weight (g/kg BW) for 24 h. Alcohol preference is calculated as the total ethanol intake (5% + 0% + 20%) (g) divided by the total fluid intake (g) and expressed as %/24 h. Voluntary alcohol consumption might be considered indicative of alcohol relapse in those animals previously exposed to FAC. It must be verified that tubes lack of any spillage. To this, an empty housing cage with bottles can be used as a spillage control and damaged ones must be replaced.
7 Performance histologic analysis according to your aims. For example: if your treatments were via i.c.v. injections, only data from animals with a verified location of the cannula must be considered for the study.
8. (Optional). Analyze blood alcohol concentrations (BACs; mg/dL or mg %). This step should be considered as it allows to discuss and study metabolic aspects of alcoholic intake in higher drinkers. There are several options to measure BAC, for example: via the Aldehyde Dehydrogenase Activity Assay Kit (Cayman® Ann Arbor, Mi. USA), with the samples analyzed with BIOTEK® (Winooski, Vt. USA), MOD. SYNE GYM X microplate reader.
9. Data analysis. Use the preferred software for the appropriate statistical test.

Validation of the protocol

In Vázquez-León and coworkers [14], male Wistar rats were used, 24 h after the classification of the animals by their level of anxiety, the alcohol intake protocol began which consisted of two periods of forced alcohol intake were applied during 16-day. The first period was carried as follows: 4-day of 2% ethanol (v/v), 4-day of 5% ethanol (v/v) and 8-day of 10% ethanol (v/v) (modified from [1]. The rats classified as high anxiety (HA) consumed more alcohol voluntarily ($P < 0.05$) (g/kg/24 h) than low anxiety (LA) rats (Fig. 2A). At the end of the first period, the rats were imposed to an 8-day withdrawal period. Immediately after, it started with the second period of forced alcohol intake for 16-day of 10% (v/v) alcohol intake. Notoriously, a higher alcohol intake in the HA vs. the LA groups remained ($P < 0.05$) (Fig. 2B).

Juvenile, adolescent, and young adult rats at PND 22–28, 30–34, and around PND 60 respectively consume high concentrations of ethanol (15 and 30% v/v) that are rejected by adults of similarly rat

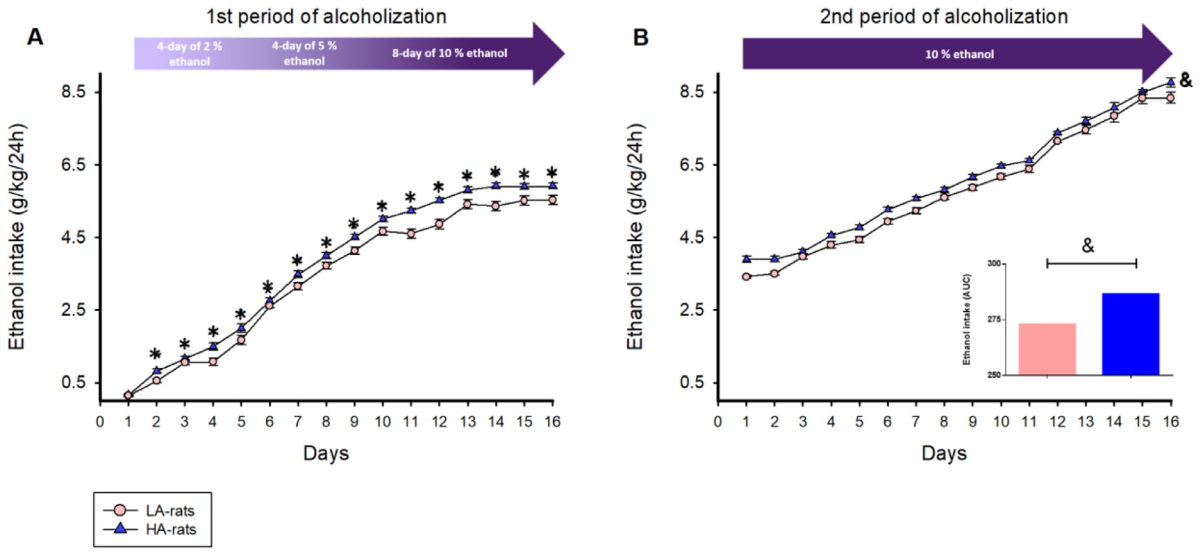

Fig. 2. Alcohol intake protocol. Daily ethanol consumption of HA and LA classified rats, $n = 48$ per group. A two-way ANOVA was performed and comparisons were analyzed by a Student-Newman-Keuls *post-hoc* test. A) 1st period of FAC for 16 days with increasing concentrations of ethanol, $^{*}P < 0.05$ HA vs LA between days; B) After a period of abstinence of 8 days the second period of FAC begins with 10% of ethanol. Additionally, the area under the curve (AUC) is shown, $^{\&}P < 0.05$ HA vs LA. Data taken from Vázquez-León et al. [14].

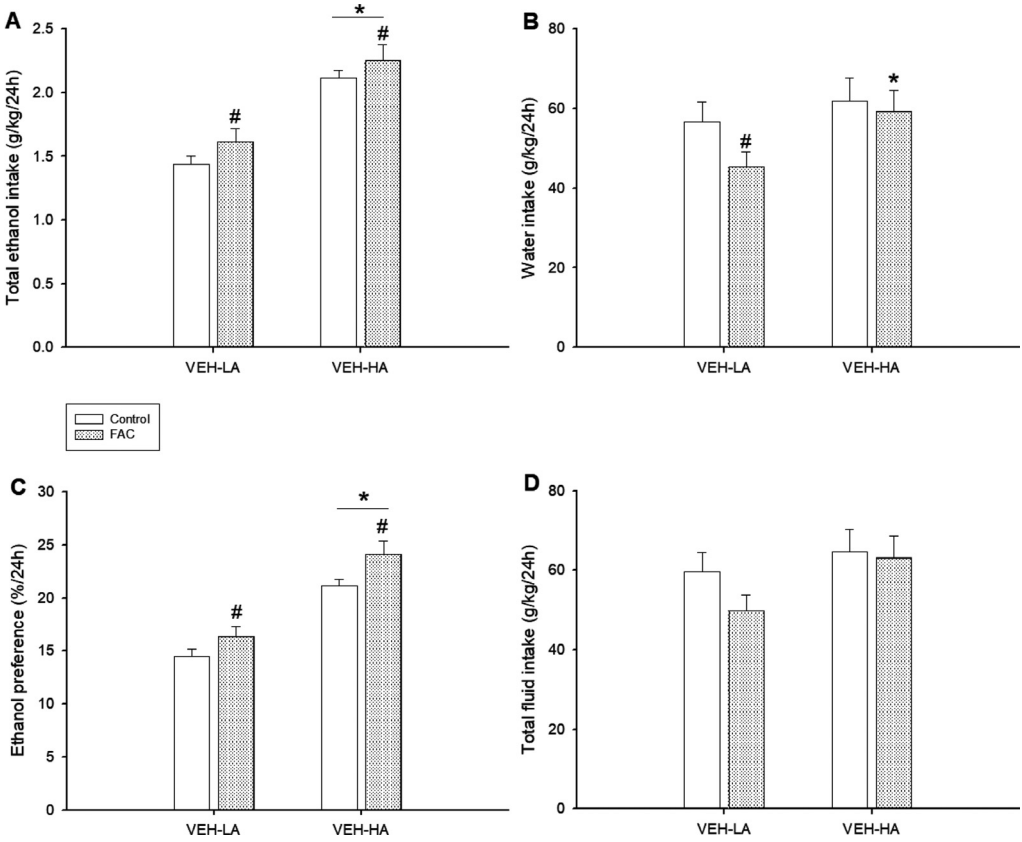

Fig. 3. Effects of anxiety levels (LA or HA) in alcohol-naïve (control) or forced alcohol consumption (FAC) on alcohol and water intake. A one-way ANOVA was performed and comparisons were analyzed by a Student-Newman-Keuls *post-hoc* test. $^{*}P < 0.05$ HA vs. LA, $^{\#}P < 0.05$ FAC vs. Control in LA or HA groups, respectively. Data are expressed as the mean ± SEM ($n = 8$ per group). Data taken from Vázquez-León et al. [14].

strains [8,13]. With this alcohol intake protocol, at the end of the second FAC period, our animals maintained an alcohol consumption of approximately 8.0–8.5 g / kg / 24 h [14].

Fig. 3 shows the effect of anxiety levels (LA or HA) on alcohol and water consumption during 24 h in rats subjected to FAC and alcohol-naïve (Control) rats (n=8 each). FAC produced a higher (P<0.05) alcohol intake and alcohol preference in both LA and HA rats; the alcohol intake was higher (P<0.05) in HA animals than in LA ones (Figs. 3A and 3C).

The FAC-LA group intake less water than the alcohol-naïve-LA group ($P < 0.05$); whereas the FAC-HA group intake more water ($P < 0.05$) than the FAC-LA group (Fig. 3C). However, no differences were detected in total fluid intake between groups (Fig. 3D). Hence, changes in alcohol intake are not due to differences in total fluid intake. Lastly, the alcohol consumption reached by HA-Wistar rats in the present protocol is moderate if compared to the reported by using alcohol-preferring animals (e.g., Sardinian rats) [10] and to other protocols such as the intermittent access to 20% ethanol in a 2-bottle choice procedure in Long-Evans rats [2]. Thus, it would be interesting to test the present protocol in rats with more alcohol natural preference. Remarkably, even in a partially alcohol-resistant strain (i.e., Wistar rats), the present protocol may differentiate those individuals with higher alcohol-intake predisposition and study the neurotransmitters involved [14].

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Fig. 1 and graphical abstract were created with BioRender.com.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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