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Ethanol withdrawal induces anxiety-like effects: Role of nitric oxide synthase in the dorsal raphe nucleus of rats

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ABSTRACT

Nitric oxide (NO) mediated transmission in the dorsal raphe nucleus (DRN) has been shown to be involved in the modulation of anxiety-like behaviors. We investigated whether inhibition of nitric oxide synthase (NOS) in the DRN would prevent anxiety-like behavior induced by ethanol withdrawal. Male Wistar rats were treated with ethanol 2–6% (v/v) for a period of 21 days. Ethanol withdrawal was induced by abrupt discontinuation of the treatment. Experiments were performed 48 h after ethanol discontinuation. Rats with a guide cannula aimed at the DRN received intra-DRN injections of the non-selective NOS inhibitor *NG*-nitro-L-arginine methyl ester (L-NAME), selective neuronal NOS (nNOS) inhibitor N(ω)-propyl-L-arginine (*NPLA*), or selective inhibitor of inducible NOS (iNOS) N-([3-(amino-methyl)phenyl] methyl) ethanimidamidedihydrochloride (1400W). Five minutes later, the animals were tested in the elevated plus maze (EPM). Plasma ethanol levels were determined by gas chromatography. There was a reduction in plasma ethanol levels 48 h after ethanol withdrawal. Rats from the ethanol withdrawal group showed decreased exploration of the open arms of the EPM with no change in the exploration of enclosed arms. Intra-DRN treatment with L-NAME (100 nmoles/0.2 μL) and 1400W (1 nmol/0.2 μL), but not NPLA (10 nmoles/0.2 μL) in the DRN attenuated the decrease in the exploration of the open arms of the EPM induced by ethanol withdrawal. The major new finding of the present study is that iNOS in the DRN plays a role in the anxiety-like behavior induced by ethanol withdrawal.

Introduction

People who are physically dependent on ethanol might experience a withdrawal syndrome after abrupt interruption of ethanol intake (McKeon, Frye, & Delanty, 2008). Ethanol withdrawal signs appear within hours of cessation of alcohol intake. The signs and symptoms include tremors, agitation, sweating, nausea, vomiting, seizures, insomnia, hallucinations, delirium, tachycardia, hypertension, and anxiety (McKeon et al., 2008). In fact, ethanol withdrawal-induced anxiety is well documented. In humans, anxiety induced by ethanol withdrawal is due to the pharmacological effects that ethanol has on brain neurotransmission in anxiety-related neural circuits (Koob et al., 1998). Despite extensive investigation of withdrawal syndrome-induced anxiety, the molecular mechanism underlying the anxiogenic effects of ethanol withdrawal has not yet been completely elucidated.

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Conflict of interest: none.

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Nitric oxide (NO) is produced from the amino acid L-arginine by a family of enzymes named NO synthases (NOS) (Moncada & Higgs, 1993). NOS exist in three isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) (Förstermann, Gath, Schwarz, Closs, & Kleinert, 1995). Both eNOS and nNOS are constitutively expressed in neurons, whereas iNOS expression is regulated by immunological or inflammatory stimuli (Förstermann et al., 1995). Under physiological conditions, nNOS accounts for the majority of the NOS activity in neurons, but under pathological conditions, iNOS may contribute to the biosynthesis of NO (Yoshida, Waeber, Huang, & Moskowitz, 1995). It is well established that NO mediates anxiety-related behaviors in rodents. In this line, aversive stimuli are described to activate NO-producing neurons (Beijamini & Guimarães, 2006; Krukoff & Khalili, 1997). Moreover, intracerebral injection of NO donors induces aversive reactions in rats (de Oliveira, Del Bel, & Guimarães, 2001). Finally, injection of NOS inhibitors in brain areas implicated in the modulation of anxiety-like behavior, such as the medial nucleus of the amygdala (MeA) (Forestiero, Manfrim, Guimarães, & de Oliveira, 2006), dorsolateral periaqueductal gray matter (DLPAG) (de Oliveira & Guimarães, 1999), and dorsal raphe nucleus (DRN) (Spiacci, Kanamaru, Guimarães, & Oliveira, 2008), induces anxiolytic-like effects in rodents.

The DRN is an important component of the brain circuit that mediates anxiety-related behaviors in rodents (Graeff, Guimarães, De Andrade, & Deakin, 1996). The enzyme nNOS is constitutively expressed in the DRN (Wang, Guan, & Nakai, 1995), and inhibition of the NO signaling pathway in this brain area by NO synthase inhibitors induces anxiolytic-like effects in rats (Spiacci et al., 2008). Importantly, a functional interaction between the DRN and the anxiety-like behavior induced by ethanol withdrawal has been proposed. In this line, it was demonstrated that 5-HT_{1A} autoreceptors in the DRN are involved in ethanol withdrawal-induced anxiety-like behavior (Overstreet, Knapp, Angel, Navarro, & Breese, 2006). More recently, it was described that excitation of *dorsal raphe* (DR) neurons following chronic ethanol exposure contributes to enhanced anxiety during ethanol withdrawal (Lowery-Gionta, Marcinkiewcz, & Kash, 2015).

NO has been described to be involved in the behavioral effects of ethanol withdrawal. Adams et al (Adams, Sewing, Chen, Meyer, & Cicero, 1995) showed that L-NAME, a non-selective NOS inhibitor, inhibited withdrawal severity by decreasing the intensity of signs of hyperactivity, tremors, and rigidity in rodents. More recently, the contribution of NO neurotransmission to withdrawal-induced anxiety has received growing attention. Bonassoli et al. (Bonassoli, Milani, & de Oliveira, 2011) showed that ethanol withdrawal activates NO-producing neurons in brain areas implicated in the modulation of anxiety-like behavior such as the paraventricular nucleus (PVN), DLPAG, and DRN. Importantly, inhibition of iNOS in the DLPAG decreased ethanol withdrawal-induced anxiety-like behavior in rats (Bonassoli, Contardi, Milani, & de Oliveira, 2013). This finding supports the involvement of the NO signaling pathway in the DLPAG in the modulation of anxiety-like behavior induced by ethanol withdrawal. However, the role of the nitrergic pathway in the DRN in such responses remains elusive.

Taken together, the above-mentioned observations indicate that the NO signaling pathway plays a role in ethanol withdrawal-induced anxiety-like effects in rodents. Since DRN is an important area that mediates anxiety-related behaviors, we hypothesized that activation of NO-producing neurons in the DRN might play a modulatory role in anxiety-like behavior induced by ethanol withdrawal. Here, we sought to investigate the effect of NOS inhibition in the DRN in the anxiogenic effect induced by ethanol withdrawal.

Materials and methods

Animals

All animal procedures were in accordance with the Guide for the Care and Use of Laboratory Animals of the National Research Council and were approved by the local Committee of Ethics on Animal Research (#07.1.992.53.2). Male Wistar rats initially weighing 230–250 g (50–60 days old) were housed in groups of two per cage under a 12/12-h light/dark cycle (lights on at 6:30 AM) at 23 ± 1 °C and given free access to food. Access to water, ethanol, or sucrose solutions was *ad libitum*, with the exception of the periods determined for withdrawal as follows.

Ethanol treatment and control groups

Rats were randomly divided into three groups: control rats received water *ad libitum* for 23 days, isocaloric rats received a solution containing an isocaloric amount of sucrose (82.6 g/L) instead of ethanol, and ethanol rats received chronic treatment with ethanol, beginning with a solution of 2% ethanol (v/v) being gradually increased after 3 days to 4% ethanol (day 4 to day 6) and then to 6% ethanol (day 7 to day 20). On day 20, ethanol solution (6%) was removed and returned the next day (day 21) for 2 h. After that, the rats received water until day 23, thereby ensuring an abstinence period of 48 h.

The same procedure was adopted for the isocaloric group. In this group, the caloric content of the sucrose liquid diet was adjusted to match that of the ethanol-treated groups as previously described (Tirapelli et al., 2008). The sucrose group was included in the study protocol to evaluate whether alterations in caloric intake following ethanol consumption might explain the effects of ethanol withdrawal on behavioral responses.

Rats from the acute group received ethanol for 2 h on day 21 and then were submitted to the same period of 48 h of abstinence, which was chosen based on previous studies (Gonzaga et al., 2015; Padovan, Batistela, Queiroz, & Tirapelli, 2010). These animals were deprived of water for 24 h. Then, animals had free access to ethanol (6%) or water for 2 h. Forty-eight hours later, rats from the acute groups were tested in the EPM as described below. Acute treatment was performed in age-matched animals as compared to the withdrawal group.

For determination of plasma ethanol levels, rats were divided into the following groups, according to the liquid diet they received and period of abstinence: control (water); acute and chronic ethanol sacrificed immediately (0 h), 24 h, or 48 h after the last dose of ethanol.

Determination of plasma ethanol levels

Rats were anesthetized with urethane (25%; 1.25 g/kg body weight; 5 mL/kg; Sigma–Aldrich, St. Louis, MO, USA) and blood (1 mL) was collected from the inferior vena cava using heparinized syringes. Samples (100 μL) were transferred into 20-mL headspace vials and analyzed on a Varian CP3380 gas chromatographer (Varian, CA, USA) as previously described (Gonzaga et al., 2015). Plasma ethanol levels were evaluated before (0 h), 24 h, or 48 h after ethanol withdrawal. Results are expressed as mg/dL.

Stereotaxic surgery

Stereotaxic surgery was performed as described previously (Almeida, Trovo, Tokumoto, Pereira, & Padovan, 2013). Briefly, animals were anesthetized with 2.5% 2,2,2-tribromoethanol (250 mg/