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Involvement of neuronal nitric oxide synthase in cross-sensitization between chronic unpredictable stress and ethanol in adolescent and adult mice

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ABSTRACT

The peculiar neurochemical profile of the adolescent brain renders it differently susceptible to several stimuli, including stress and/or drug exposure. Among several stress mediators, nitric oxide (NO) has a role in stress responses. We have demonstrated that adolescent mice are less sensitive to ethanol-induced sensitization than adult mice. The present study investigated whether chronic unpredictable stress (CUS) induces behavioral sensitization to ethanol in adolescent and adult Swiss mice, and investigated the influence of Ca^{2+}-dependent nitric oxide synthase (NOS) activity in the phenomenon. Adolescent and adult mice were exposed to repeated 1.8 g/kg ethanol or CUS and challenged with saline or ethanol. A neuronal nitric oxide synthase (nNOS) inhibitor, 7-nitroindazole (7NI), was administered along with ethanol and CUS to test its effects on behavioral sensitization. Both adolescent and adult mice displayed cross-sensitization between CUS and ethanol in adult mice, with adolescents showing a lower degree of sensitization than adults. nNOS inhibition by 7NI reduced both ethanol sensitization and cross-sensitization. All age differences in the Ca^{2+}-dependent NOS activity in the hippocampus and prefrontal cortex were in the direction of greater activity in adults than in adolescents. Adolescents showed lower sensitivity to cross-sensitization between CUS and ethanol, and the nitric oxide (NO) system seems to have a pivotal role in ethanol-induced behavioral sensitization and cross-sensitization in both adolescent and adult mice.

Introduction

Adolescence is a period of brain maturation, during which regions of the brain undergo dramatic changes. Endogenous and exogenous stimuli may elicit different responses in adolescents than those presented by adults (Gulley & Juraska, 2013), such as responses to stress and/or drug exposure. Ontogenetically, the prefrontal cortex is one of the last brain regions to mature, undergoing prominent neural changes throughout adolescence (Spear, 2000). Evidence suggests that the hippocampus may be more sensitive to the deleterious effects of ethanol during adolescence in both animals and humans (De Bellis et al., 2000; Markwiese, Acheson, Levin, Wilson, & Swartzwelder, 1998). However, this effect is not always consistent. Age-related sensitivity to ethanol-induced learning deficits seems to depend on the task and context, with adolescents showing greater or lower learning impairment, compared to adults (Hunt & Barnet, 2016; Land & Spear, 2004a, 2004b). Moreover, the causality/consequence between alcohol use during adolescence and smaller hippocampal volume in humans is not clear (Squeglia, Jacobus, & Tapert, 2014).

Repeated administration of ethanol in low doses may cause a progressive augmentation of its locomotor response, known as

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behavioral sensitization (Masur & Boerngen, 1980; see Camarini & Pautassi, 2016 for review). This phenomenon has been studied as an animal model of neuroadaptation in drug dependence (Robinson & Berridge, 2008), as well as drug-induced psychosis (Robinson & Becker, 1986; Segal & Kuczenski, 1997). Repeated stress or exposure can also induce behavioral sensitization and render an animal more sensitive to several effects of drugs of abuse – a phenomenon termed cross-sensitization (Burke & Miczek, 2014; Stewart & Badiani, 1993). Indeed, behavioral cross-sensitization is observed between stress and ethanol in both adolescent and adult rodents (for review, see Burke & Miczek, 2014). Stress exposure can raise ethanol intake and preference in both animals (Morais-Silva, Fernandes-Santos, Moreira-Silva, & Marin, 2016; Norman et al., 2015; Quadir et al., 2016) and humans (Sinha, 2001), although some studies have shown no impact of stress or a reduction in ethanol intake when rodents have access to alcohol during or immediately after stress (van Erp & Miczek, 2001; Marianno, Abrahao, & Camarini, 2017). Stress exposure also increases behavioral sensitivity to ethanol (Quadir et al., 2016). As such, cross-sensitization may help explain why stress exposure is so closely involved in alcohol relapse (Becker, 2012), and in the high rate of comorbidity between alcoholism and affective disorders (Fuehrlein et al., 2014).

Exposure to stressors enhances the vulnerability to drug/ethanol addiction, presumably via control of glucocorticoid-dependent increases in dopamine release within the nucleus accumbens (Piazza & Le Moal, 1996). In addition, ethanol activates and disrupts HPA axis and extrahypothalamic reward and stress systems (Becker, Lopez, & Doremus-Fitzwater, 2011; Burke & Miczek, 2014; Ciccocioppo et al., 2009).

Several neurotransmitter systems are implicated in ethanol-induced behavioral sensitization (Camarini & Pautassi, 2016, as review), including nitric oxide (NO). NO is a signaling molecule that modulates the release of several neurotransmitters and acts as a critical regulator of cellular plasticity (McLeod, López-Figueroa, & López-Figueroa, 2001). Among the numerous mediators of stress, NO regulates stress responses by controlling the corticotropin-releasing hormone (CRH) neurosecretory system (Prast & Philippu, 2001; Riedel, 2000), and by influencing adrenocorticotropic hormone (ACTH) activity (Rivier, 2001). The neuronal isoform of its synthetic enzyme, nitric oxide synthase (nNOS), is localized in several brain areas, including those involved in stress and addiction, such as hippocampus and prefrontal cortex (Blackshaw et al., 2003; Kubota & Kawaguchi, 1994). Nitrergic neurons and brain NOS activity reach maturity approximately after 21 postnatal days in rats (Hvizdosova, Tomasova, Bolekova, Kolesar, & Kluchova, 2014; Lizasoain, Weiner, Knowles, & Moncada, 1996). Exposure to stress increases nNOS activity in the hippocampus via glucocorticoid release (Harvey, Oosthuizen, Brand, Wegener, & Stein, 2004), and increases the number of nNOS positive neurons in the prefrontal cortex (Campos, Piorino, Ferreira, & Guimarães, 2013). Furthermore, Itzhak and Anderson (2008) showed that the gene encoding neuronal nitric oxide synthase (nNOS) is required for the development of ethanol-behavioral sensitization, particularly in adolescent mice that show higher sensitivity to ethanol sensitization.

Here, we used chronic unpredictable stress (CUS), a model that can cause a long dysregulation of the hypothalamic-pituitary-adrenal axis, to investigate: 1) whether or not adolescent Swiss mice express a differential sensitivity to stress and ethanol cross-sensitization compared to adult counterparts, and the role of nNOS in this phenomenon; and 2) the effects of repeated ethanol and repeated stress plus ethanol exposure on constitutive Ca^{2+}-dependent NOS activity in the prefrontal cortex and hippocampus of adolescent and adult Swiss mice.

Materials and methods

Subjects

Adolescent (postnatal day – PND = 28–30) and adult Swiss (PND = 65–70) male mice were provided by the Instituto de Ciências Biomédicas (Institute of Biomedical Sciences) of the Universidade de São Paulo. Groups of four animals were housed in polypropylene cages (27.5 cm length × 16.5 cm width × 13 cm height) in a room with controlled temperature (21 ± 1 °C), on a 12-h light/dark cycle (with lights turned on at 7:00 AM) and with food and water *ad libitum*, during an acclimatization period of at least 7 days. Mice in the CUS group were subjected to a 12-h food and water deprivation regimen, as described below. Each animal was used in only one experimental procedure. The experimental procedures were approved by the Ethical Committee for Animal Use (CEUA) of the Instituto de Ciências Biomédicas, Universidade de São Paulo (Protocol #134/09).

Drugs

Ethanol (20% v/v; Merck do Brasil, Rio de Janeiro, RJ, Brazil) was diluted in saline solution (0.9% NaCl in distilled water) and administered intraperitoneally (i.p.) at a dose of 1.8 g/kg. Control animals received isovolumetric saline (SAL) injections (i.p.).

Due to its low solubility in water (<50 μg/mL) (Bush & Pollack, 2000; Wangensteen et al., 2003), 7-nitroindazole (7NI; a neuronal nitric oxide synthase inhibitor, Sigma-Aldrich; São Paulo, SP, Brazil) was solubilized in dimethyl sulfoxide (DMSO; Merck do Brasil; Rio de Janeiro, RJ, Brazil), propylene glycol (Merck do Brasil; Rio de Janeiro, RJ, Brazil) and distilled water, 1:3:6, and administered i.p. in 0.1 mL/10 g body weight (Itzhak & Martin, 2000) at a final concentration of 15 mg/kg. Another set of mice received 7NI in drinking water, prepared every day in 50 mL of water, containing the equivalent of 15 mg/kg/day for each mouse (Tesser-Viscaíno et al., 2009).

Behavioral sensitization

Locomotor activity was measured to access behavioral sensitization to ethanol, CUS, and cross-sensitization. Behavioral testing was conducted in the open-field apparatus (40-cm diameter arena, surrounded by a 50-cm wall). The floor of the arena was divided into three circles, which in turn were subdivided into quadrants, totaling 19 zones: 18 quadrants and a central circle. Each mouse received an injection of SAL or ethanol (according to the experimental group) and was placed individually in the center circle of the open-field arena and observed for 10 min. The frequency of entries into each zone was recorded by a blind experimenter. Each frequency unit corresponds to the act of the mouse placing its four paws into a zone. The open-field was cleaned with a 5% alcohol solution in water, prior to the introduction of the next mouse to reduce possible odor trails left by the preceding mouse. To avoid circadian effects on the behavior of mice, the tests were performed at the same time of the day (between 9:00 AM and 11:00 PM), and representative mice from each group were tested simultaneously.

The chronic unpredictable stress (CUS) protocol

To better understand the effects of chronic stress on behavioral sensitization and nNOS activity, animals from the CUS group

underwent a chronic unpredictable stress paradigm, as previously described (Araújo, DeLucia, Scavone, & Planeta, 2003; Fitzgerald, Ortiz, Hamedani, & Nestler, 1996; Ortiz, Fitzgerald, Lane, Terwilliger, & Nestler, 1996; Quadir et al., 2016), with minor modifications.

The CUS protocol consisted of exposing the animals to different stressors at different time points, as described in Table 1.

Corticosterone serum levels

Corticosterone serum levels were measured to study the effects of ethanol and CUS, and their cross-sensitization upon the stress response. A set of five mice was randomly chosen from each group to have their blood collected from the tail vein at the same time of the day (from 9:00–11:00 AM). Corticosterone levels were measured on Day 0 (before experimental procedures) and on Day 12 (challenge day), and measurements were conducted 15 min and 180 min after the challenge injection, to evaluate possible differences among groups at the peak levels of corticosterone after ethanol injections and upon return to baseline. Corticosterone levels returned to baseline by 180 min and thus, those measurements were excluded from the graphs. Blood samples were incubated in a bath at 37 °C for 60 min and then centrifuged for 5 min at 9700 ×*g* to obtain the serum. The serum corticosterone levels were determined using an ELISA kit (Abcam; Cambridge, Massachusetts, United States), following the manufacturer's protocol.

Ca^{2+}-dependent NO synthase (NOS) activity

Ca^{2+}-dependent NOS (mainly [nNOS] + [endothelial] − [eNOS]) activity was measured to help understand the effects of ethanol and stress on NOS in the hippocampus and prefrontal cortex. The assay is based on the ability of NOS to convert [^{3}H] L-arginine into [^{3}H] L-citrulline (Bredt & Snyder, 1990). Three hours after the last injection, animals were euthanized, and the brains were rapidly removed and immediately stored in the bio-freezer at −80 °C. The hippocampus and prefrontal cortex were dissected and the samples (pooled between two mice) were homogenized in five volumes of cold incubation buffer (50 mM Tris–HCl buffer, pH 7.4) containing 1 mM phenylmethyl sulphonyl fluoride (PMSF) and 1 mM L-citrulline. The homogenates were incubated for 30 min with 1 mM NADPH, 2 mM $CaCl_2$, and 10 μM L-arginine containing 100,000 dpm of [2,3,4,5-^{3}H] L-arginine monohydrochloride at room temperature (25–27 °C).

Pharmacological controls of enzymatic activity were carried out in parallel. As previously described (Faria et al., 1997), each sample was corrected by its negative control of NOS-mediated conversion of L-arginine to L-citrulline, which was performed by the addition of 1 mM L-NAME (a NOS inhibitor) to the incubation medium. In addition, Ca^{2+}-independent NOS (i.e., iNOS) activity was assessed by adding 1 mM EGTA and omitting $CaCl_2$ from the incubation medium.

The protein content of the samples was determined by Bradford assay, and NOS activity was expressed as pmol L-citrulline produced/min per mg of protein.

Table 1
Chronic unpredictable stress protocol.

Day	Stressor
1	Damp sawdust (6:00 PM) for 12 h
2	Immobilization (10:00 AM) for 1 h
3	Cold isolation (3:00 PM) for 1 h and light on (6:00 PM) for 12 h
4	Light off (12:00 PM) for 3 h and swim stress (3:00 PM) for 5 min
5	Damp sawdust (6:00 AM) for 12 h and food/water deprivation (6:00 PM) for 12 h
6	Swim stress (2:00 PM) for 4 min and isolation (6:00 PM) for 12 h
7	Cold isolation (2:00 PM) for 15 min and light off (3:00 PM) for 2 h
8	Damp sawdust and light on (6:00 PM) for 12 h
9	Isolation and food/water deprivation (6:00 PM) for 12 h
10	Immobilization (4:00 PM) for 1 h and light on (6:00 PM) for 12 h
11	Swim stress (9:00 AM) for 4 min and immobilization (10:00 AM) for 1 h

Experimental design

The experimental design of Experiments 1 and 2 is illustrated in Table 2.

Experiment 1: Cross-sensitization between CUS and ethanol, corticosterone levels, and nNOS activity in adolescent and adult mice

Adolescent and adult mice received daily i.p. injections of SAL or 1.8 g/kg ethanol, or were exposed to CUS over the course of 11 days (Days 1–11). Then, mice were challenged with either SAL (SAL-SAL, ETOH-SAL, CUS-SAL) or 1.8 g/kg ethanol (SAL-ETOH, ETOH-ETOH, CUS-ETOH) on Day 12 (n = 10 mice/group). The locomotor activity was measured in the open-field during 10 min, immediately after the injections. Blood was sampled and brain tissue was collected as detailed above.

Experiment 2: Effects of 7NI on ethanol sensitization and cross-sensitization between ethanol and CUS

First, we tested whether 7NI could affect the locomotor activity in adolescent and adult mice (n = 28). Mice received vehicle (VEH) or 15 mg/kg 7NI i.p., 30 min before a SAL injection, for 11 days. On the 12th day, all mice were challenged with a SAL injection and the locomotor activity was measured (n = 7 mice/group) (Fig. 4A). SAL, ethanol, and CUS groups challenged with SAL in Experiment 1 were

Table 2
Experimental designs.

Age	Treatment days 1 – D11		Challenge (Day 12)
Experiment 1. Cross-sensitization between CUS and ethanol, corticosterone levels, and nNOS activity in adolescent and adult mice			
Adolescent and adult mice	SAL	(n = 10/age)	SAL
	Ethanol	(n = 10/age)	SAL
	CUS	(n = 10/age)	SAL
	SAL	(n = 10/age)	Ethanol
	Ethanol	(n = 10/age)	Ethanol
	CUS	(n = 10/age)	Ethanol
Experiment 2. Effects of 7NI on ethanol sensitization and cross-sensitization between ethanol and CUS			
Adolescent and adult mice	First injection	Second injection	
	VEH (n = 7/age)	SAL	SAL
	7NI (n = 7/age)	SAL	SAL
Adolescent and adult mice	First injection	Second treatment	Ethanol
	VEH (n = 7/age)	SAL	
	VEH (n = 7/age)	Ethanol	
	VEH (n = 7/age)	CUS	
	7NI (n = 7/age)	SAL	
	7NI (n = 7/age)	Ethanol	
	7NI[a]	CUS	

Blood samples were collected on Day 0 and on Day 12, at 15 and 180 min after SAL/ethanol challenge injections for corticosterone measurement. Mice were killed by cervical dislocation at 180 min after injection, and the brains were dissected into prefrontal cortex and hippocampus to evaluate NOS activity.
Total n = 120 mice.
Total n = 133 mice.
[a] Two CUS groups received 7NI: one group was injected with 7NI i.p. in the morning (n = 14; 7 adolescents and 7 adults), while the other group received 7NI in the drinking water (n = 21; 10 adolescents and 11 adults).

not included in Experiment 2, due to the lack of differences in the locomotor activity among them (see Fig. 1).

Adolescent and adult mice received VEH or 15 mg/kg 7NI i.p., 30 min before receiving repeated SAL or 1.8 g/kg ethanol over the course of 11 days. Due to the unpredictable characteristics and variety of stresses throughout the day experienced by animals in the CUS group, we ran three groups of CUS mice, one receiving VEH and two receiving 7NI for each age, such that one of the 7NI groups was administered i.p. injections of 7NI daily, during the morning, and the other 7NI group received 7NI in drinking water for 11 days. On Day 12, all mice were challenged with ethanol.

On Day 12, the CUS groups were challenged with 1.8 g/kg ethanol. The locomotor activity was evaluated in the open-field immediately after the challenge injection for 10 min.

Statistical analysis

The behavior and NOS activity data were analyzed by analysis of variance (ANOVA). Three-way factorial ANOVA 3 × 2 × 2, considering treatment (SAL, ethanol, CUS) × challenge injection (SAL, ethanol) × age (adolescent, adult) was performed to analyze the locomotor frequency in adolescent and adult mice submitted to ethanol or CUS treatment. Three-way repeated-measures ANOVA considering treatment (SAL, ethanol, CUS), challenge (SAL, ethanol), and time (Day 0: basal levels, 15 min and 180 min after the last injection on Day 12) was conducted to analyze the corticosterone levels in each age. To test whether 7NI administered daily, i.p. or in drinking water, would differently affect the locomotor activity of mice exposed to CUS, a two-way ANOVA was performed, considering age (adolescent, adult) and 7NI via means of administration (i.p. injection or drinking water). Analysis of the effects of 7NI on locomotor frequency were conducted in two separate ANOVAs considering SAL and ethanol challenge injections, as follows: a two-way ANOVA (age × pretreatment) compared the locomotor frequency in adolescent and adult mice pretreated with VEH or 7NI, followed by a SAL injection treatment (Day 1–11) and challenged with SAL (Day 12); a three-way ANOVA (age × pretreatment × treatment) compared the locomotor frequency in adolescent and adult mice pretreated with VEH or 7NI followed by treatment with SAL, ethanol, or CUS (Day 1–11) and challenged with ethanol (Day 12).

Bonferroni tests were used as a *post hoc* test. All data are expressed as mean ± SEM. Significance was defined at the 0.05 level.

Results

Experiment 1: Cross-sensitization between CUS and ethanol, corticosterone levels, and nNOS activity in adolescent and adult mice

The locomotor frequency in adolescent and adult mice treated daily with SAL, ethanol, or CUS, and challenged with either SAL or ethanol, is presented in Fig. 1. Both adolescent and adult mice developed ethanol-behavioral sensitization and exhibited a cross-sensitization between CUS and ethanol, as the locomotor frequency of these groups was higher than that of the respective groups receiving acute ethanol (SAL-ETOH) or SAL (SAL-SAL) [$F_{\text{treatment} \times \text{challenge}}(2,108) = 6.16$; $p < 0.01$]. However, adolescents showed a lower ethanol sensitization and cross-sensitization, compared to adults [$F_{\text{age} \times \text{treatment}}(2,108) = 3.33$, $p < 0.05$; $F_{\text{age} \times \text{challenge}}(1,108) = 41.58$, $p < 0.01$]. These data indicate cross-sensitization between stress and ethanol in adolescent and adult mice, with more robust sensitization in adults vs. adolescents.

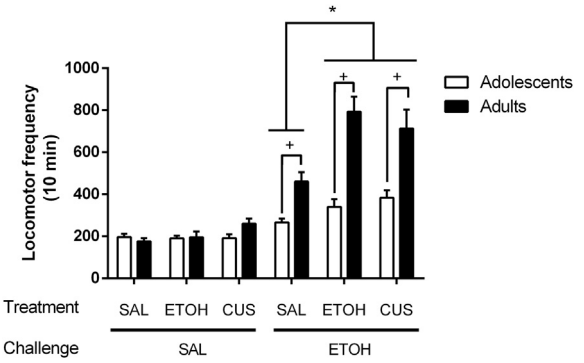

Fig. 1. Cross-sensitization between chronic unpredictable stress (CUS) and ethanol (ETOH) in adolescent and adult Swiss mice. Data are expressed as locomotor frequency (mean ± S.E.M.). Mice were exposed to saline i.p. (SAL), 1.8 g/kg ethanol i.p. (ETOH), or chronic unpredictable stress (CUS) over 11 days (Treatment) and then were challenged with saline (SAL-SAL, CUS-SAL, ETOH-SAL) or ethanol (SAL-ETOH, CUS-ETOH, ETOH-ETOH) prior to locomotor testing. $p < 0.05$, three-way ANOVA followed by Bonferroni post-tests. *ETOH-ETOH, CUS-ETOH groups showed higher locomotor frequency than SAL-ETOH groups; $^{+}$adult mice showed higher locomotor frequency than adolescent mice within the same treatment. n = 10 mice/group.

The basal corticosterone serum levels (Day 0), as well as the levels at 15 min after the challenge injections of SAL or ethanol, are shown in Fig. 2. Separate three-way ANOVAs were performed for each age. In adolescents, we found a time effect [$F_{\text{time}}(2,48) = 32.93$, $p < 0.01$] and a treatment × time interaction [$F_{\text{treatment} \times \text{time}}(4,48) = 6.86$, $p < 0.01$]. The effect of time revealed that corticosterone levels peaked at 15 min after the challenges. Analysis of the interaction by Bonferroni *post hoc* test revealed that corticosterone levels were higher at 15 min after challenge compared to basal levels in mice exposed to CUS. Adolescent mice exposed to CUS showed higher corticosterone peaks than those pretreated with SAL or ethanol groups at 15 min. In adults, the three-way ANOVA revealed a time effect [$F_{\text{time}}(2,48) = 67.5$, $p < 0.01$] and a treatment × challenge × time interaction [$F_{\text{treatment} \times \text{challenge} \times \text{time}}(4,48) = 4.75$, $p < 0.01$]. Similar to the adolescent groups, an effect of time also revealed that the corticosterone levels peaked at 15 min after the challenge injections (SAL or ethanol). Analysis of the interaction by Bonferroni test revealed that the ETOH-ETOH and CUS-ETOH groups showed greater corticosterone levels at 15 min compared to their basal levels. The ETOH-ETOH group showed higher levels of corticosterone than the SAL-SAL, ETOH-SAL, CUS-SAL, and SAL-ETOH groups at the 15-min time point. Corticosterone levels returned to baseline levels at 180 min in both age groups (time points were excluded from the graph). Considering that corticosterone levels peaked at 15 min after the challenges, a three-way ANOVA was performed to detect age differences at this time point. An age × treatment × challenge interaction [$F_{\text{age} \times \text{treatment} \times \text{challenge}}(2,48) = 3.94$, $p < 0.05$] revealed that the adult ETOH-ETOH group had higher corticosterone levels than its respective adolescent group.

Ca^{2+}-dependent NOS activity induced by CUS and ethanol

The activity of Ca^{2+}-dependent NOS in the prefrontal cortex and hippocampus of adolescent and adult mice treated with SAL, ethanol, and CUS and challenged with either SAL or ethanol is shown in Fig. 3A and B, respectively. Three-way ANOVA revealed an age × treatment × challenge interaction [$F_{\text{age} \times \text{treatment} \times \text{challenge}}(2,44) = 9.16$, $p < 0.01$]. Pair-wise comparisons showed that CUS-SAL and ETOH-ETOH adult groups exhibited higher NOS activity than their respective adolescents. However, age differences

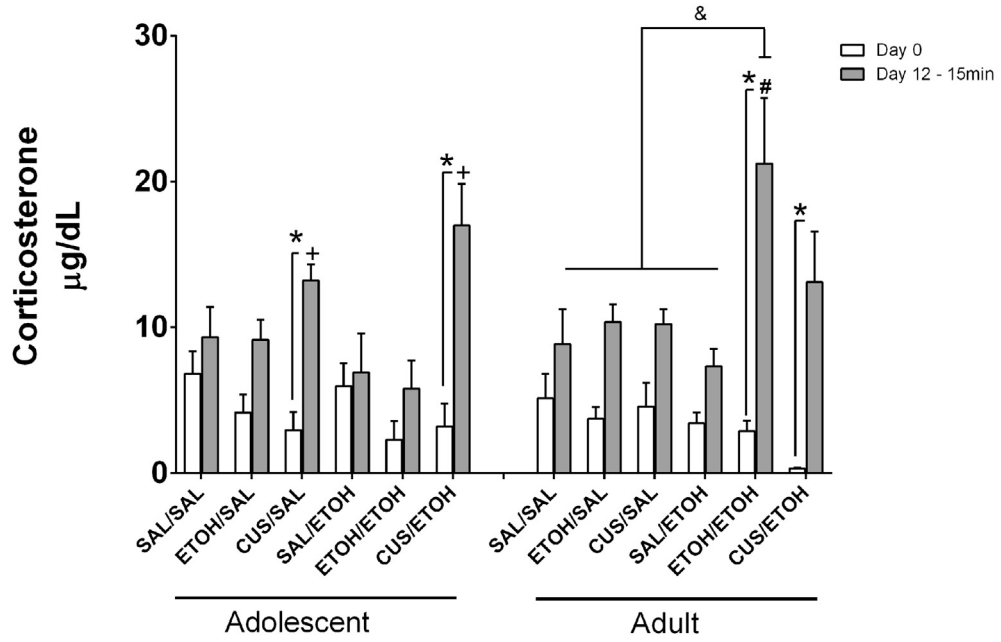

Fig. 2. Corticosterone serum levels of adolescent (ADL) and adult (AD) mice. Corticosterone was measured at three time points: Day 0: day before the start of the experiment; Day 12: 15 min and 180 min after saline (-SAL) or ethanol (-ETOH) challenge injections. Corticosterone levels are expressed in µg/dL. The results are represented in mean ± S.E.M. *corticosterone serum levels are higher than their respective basal level after challenge injections ($p < 0.01$); +corticosterone levels are higher than SAL or ETOH groups at 15 min, regardless of the challenge injection, within adolescent mice; &corticosterone levels are higher at 15 min compared to other groups, except CUS-ETOH in adults; #different from the respective ETOH/ETOH adolescent group ($p < 0.01$). n = 10 mice/group.

were not detected in CUS-ETOH (also referred to as "cross-sensitized") mice. CUS increased NOS activity compared to SAL (SAL-SAL), but only in adult mice. NOS activity was higher in ETOH-ETOH adult mice than in mice that received SAL after ethanol treatment (ETOH-SAL). In addition, only in adolescents, CUS-ETOH mice exhibited higher NOS activity than ETOH-ETOH mice.

Analysis of NOS activity in the hippocampus by a three-way ANOVA revealed an age × treatment × challenge interaction [$F_{age \times treatment \times challenge}(2,43) = 7.84$, $p < 0.01$]. Adult mice treated with SAL, ethanol, or CUS and challenged with SAL (SAL-SAL, ETOH-SAL, CUS-SAL) exhibited higher NOS activity, compared to their respective adolescent groups. Regarding the groups challenged with ethanol, adult mice acutely treated with ethanol (SAL-ETOH) showed higher NOS activity, compared to their respective saline-injected control (SAL-SAL) and to its respective adolescent group. CUS adult mice (CUS-SAL) exhibited higher NOS activity, when compared to their respective saline-injected control group (SAL-SAL) and to the CUS group challenged with ethanol (CUS-ETOH).

Experiment 2: Effects of 7NI on ethanol sensitization and cross-sensitization between ethanol and CUS

Analysis of the data from mice pretreated with vehicle or 7NI i.p. and challenged with SAL showed no effects of age [$F(1,24) = 1.85$, $p > 0.05$], pretreatment [$F(1,24) = 0.38$, $p > 0.05$], or interaction between pretreatment and age [$F(1,24) = 0.89$, $p > 0.05$]. Means ± S.E.M. of locomotor frequency are as follows: Adolescent-VEH (209 ± 17); Adolescent-7NI (236 ± 22); Adult-VEH (202 ± 11); Adult-7NI (197 ± 16), Thus, 7NI did not alter spontaneous locomotor activity in either adult or adolescent controls.

We also tested whether 7NI administered daily via i.p. injections or in drinking water would differently affect the locomotor activity of mice exposed to CUS and challenged with ethanol. Analysis of the data from adolescent and adult CUS-ETOH mice pretreated with 7NI i.p. or 7NI in drinking water did not show statistical differences of age [$F(1,31) = 1.16$, $p > 0.05$], route of administration [$F(1,31) = 1.54$, $p > 0.05$] or an age × route of administration interaction [$F(1,31) = 0.06$, $p > 0.05$]. Therefore, the effects of 7NI on locomotor frequency was long-lasting and did not depend necessarily on the immediate acute effects of 7NI, because 7NI i.p. was administered in the morning and CUS was applied at different times of the day or night. Thus, the global statistical analysis was performed using the results obtained with 7NI i.p. treatment.

The effects of pretreatment with 7NI i.p. or VEH in adolescent and adult mice treated with SAL, ethanol, and CUS, and challenged with 1.8 g/kg ethanol (on Day 12) are shown in Fig. 4. Ethanol and CUS mice that received VEH displayed higher locomotor frequency compared to SAL mice [$F_{treatment}(2,72) = 18.15$, $p < 0.01$; $F_{pretreatment \times treatment}(2,72) = 2.2$, $p < 0.05$]. These results replicate the findings of Experiment 1 and indicate the development of ethanol-induced sensitization, as well as cross-sensitization. Adult mice displayed higher ethanol-behavioral sensitization and cross-sensitization than adolescents [$F_{age}(1,72) = 56.69$, $p < 0.01$; $F_{age \times treatment}(2,72) = 4.42$, $p < 0.05$], also corroborating the data obtained from Experiment 1. Separate ANOVAs were performed for each age group. A two-way ANOVA (pretreatment × treatment) run for the adolescent group revealed that 7NI reduced the development of ethanol-behavioral sensitization and cross-sensitization with CUS, but did not alter the locomotor activity of mice receiving acute ethanol, as evidenced by the lower locomotor activity of ethanol and CUS mice pretreated with 7NI, compared with those receiving VEH [$F_{pretreatment}(1,36) = 27.47$, $p < 0.01$], [$F_{treatment}(2,36) = 7.15$, $p < 0.01$], [$F_{pretreatment \times treatment}(2,36) = 3.27$, $p < 0.05$]. A two-way ANOVA (pretreatment × treatment) run for the adult group revealed a significant effect of 7NI [$F_{pretreatment}(1,36) = 81.4$, $p < 0.01$] in all groups, i.e., the locomotor activity of mice pretreated with 7NI was lower than that observed in VEH-pretreated mice.

Discussion

The results obtained from this study confirm cross-sensitization between CUS and ethanol in adult mice (Quadir et al., 2016;

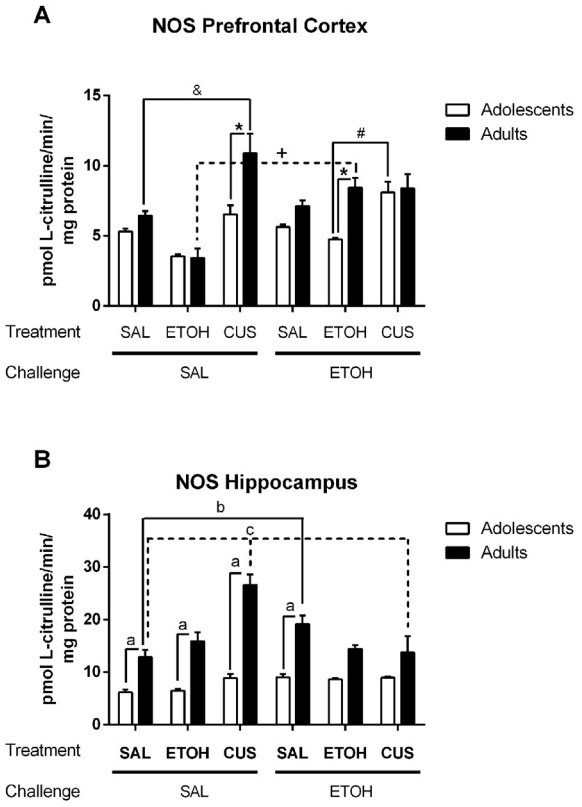

Fig. 3. Ca^{2+}-dependent NOS activity in the prefrontal cortex (**A**) and hippocampus (**B**) of adolescent and adult mice. Mice were exposed to saline i.p. (SAL), 1.8 g/kg ethanol i.p. (ETOH) or chronic unpredictable stress (CUS) during 11 days and then challenged with saline (SAL-SAL, ETOH-SAL, CUS-SAL) or ethanol (SAL-ETOH, ETOH-ETOH, CUS-ETOH). $p < 0.05$, three-way ANOVA followed by Bonferroni post-tests. (**A**) *CUS-SAL and ETOH-ETOH adult groups showed higher NOS activity than their respective adolescent groups; [&]CUS-SAL adult group showed higher NOS activity than SAL-SAL adult group; [+]ETOH-ETOH adult group showed higher NOS activity than ETOH-SAL adult group; [#]CUS-ETOH adolescent group showed higher NOS activity than ETOH-ETOH adolescent group; (**B**) [a]SAL-SAL, ETOH-SAL, CUS-SAL, and SAL-ETOH adults groups showed higher NOS activity than their respective adolescent groups; [b]SAL-ETOH adult group showed higher NOS activity than SAL-SAL adult group; [c]CUS-SAL adult group showed higher NOS activity than SAL-SAL and CUS-ETOH adult groups. n = 10 mice/group.

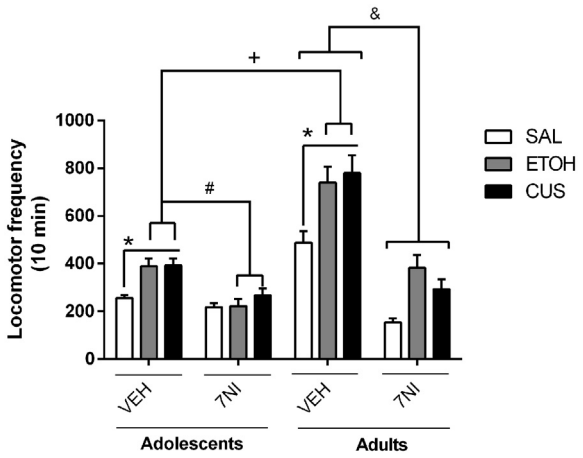

Fig. 4. Locomotor frequency (mean ± S.E.M.) of mice challenged with 1.8 g/kg ethanol on Day 12. Adolescent and adult mice were pretreated daily with 15 mg/kg 7-nitroindazole (7NI) i.p. or vehicle 30 min before saline, 1.8 g/kg ethanol, or CUS for 11 days and were challenged with 1.8 g/kg ethanol on Day 12. *ETOH-VEH and CUS-VEH groups showed higher locomotor frequency than SAL-VEH groups; [+]Locomotor frequency in ETOH-VEH and CUS-VEH adult groups was greater than in their respective adolescent groups; [#]Locomotor frequency in 7NI-ETOH and 7NI-CUS adolescent groups was lower than in their respective VEH groups; [&]locomotor frequency in 7NI-SAL, 7NI-ETOH, and 7NI-CUS adult groups was lower than in their respective VEH groups. n = 7–11 mice/age group.

Roberts, Lessov, & Phillips, 1995) and extend these findings to adolescent Swiss mice. The present study also highlights that neural adaptations in response to repeated ethanol and stressors differ between adolescents and adults. We have demonstrated that adolescents are less sensitive to ethanol-induced sensitization than adults (Carrara-Nascimento, Griffin, Pastrello, Olive, & Camarini, 2011; Carrara-Nascimento, Olive, & Camarini, 2014; Faria et al., 2008), and here we extend this lower sensitivity also to CUS-ETOH cross-sensitization. Locomotor sensitization and cross-sensitization are behavioral proxies of alcohol/stress-induced neuroadaptations, and the complexity of the brain development during adolescence may explain age differences in those behavioral responses to ethanol/stress.

Interestingly, ethanol-sensitized adult mice showed both higher sensitization and corticosterone levels, compared to adolescents. Ethanol itself activates the HPA axis, resulting in increased corticosterone levels (Thiagarajan, Mefford, & Eskay, 1989). It has previously been shown that acute ethanol injection induced higher corticosterone levels in adult mice, compared to adolescents, although these differences seemed to be sex-dependent, with males having a smaller magnitude rise than females (Willey, Anderson, Morales, Ramirez, & Spear, 2012). In our study, however, the age difference was detected only in mice repeatedly treated with ethanol, but not in cross-sensitized mice.

Studies report hyposensitivity (Faria et al., 2008; Stevenson, Besheer, & Hodge, 2008) or hypersensitivity (Hefner & Holmes, 2007) to ethanol's stimulating or sensitizing effects during adolescence, compared to adulthood. We showed that CUS-ETOH adolescents also displayed lower cross-sensitization, compared to adults. Age-related differences were also observed for different behavioral parameters, such as higher sensitivity in alcohol intake after stress in adults versus adolescents (Spanagel, Noori, & Heilig, 2014) and lower cocaine-induced CPP and corticosterone levels in adolescent mice previously exposed to social defeat stress, in contrast to higher levels in adults (Montagud-Romero et al., 2015). The ontogeny in the HPA axis should be considered as a hypothetical reason for the observed age differences in drug sensitivity, behavioral sensitization, and cross-sensitization to ethanol in adolescents, as adolescent rats are less sensitive to negative feedback control of the HPA axis after stress than adults (Sapolsky, Meaney, & McEwen, 1985). This being said, the HPA axis is functional in adolescents, as demonstrated by increases in corticosterone after saline injections in this study. However, the degree to which acute ethanol stimulates HPA and increases plasma corticosterone levels is lower in adolescents compared to adults. In adolescents, corticosterone levels increased from 4.1 ± 1.8 (basal) to 6.02 ± 2.69 (15 min) μg/dL, while in adults the change was from 3.4 ± 0.78 (basal) to 7.35 ± 1.21 (15 min) μg/dL. In fact, adolescents show higher, similar, or lower ACTH and corticosterone responses, depending on the type of stressor (Romeo, Patel, Pham, & So, 2016).

nNOS inhibition by 7NI reduced both ethanol sensitization and cross-sensitization with stress, which is in agreement with studies showing that inhibition of nNOS blocks cocaine-induced sensitization and cross-sensitization between cocaine and methamphetamine (Itzhak, 1997), methamphetamine sensitization (Inoue, Arai, Shibata, & Watanabe, 1996), and morphine sensitization (Zarrindast, Askari, Khalilzadeh, & Nouraei, 2006). The NOS system appears to have an important role in behavioral sensitization, in drugs' rewarding effects, and other aspects of addiction. It is involved in alcohol preference (Lallemand & De Witte, 1997), opioid

withdrawal syndrome (London, Kimes, & Vaupel, 1995), cocaine self-administration (Collins & Kantak, 2002), nicotine addiction (Vleeming, Rambali, & Opperhuizen, 2002) and fencamfamine addiction (Munhoz et al., 2003). The sedative effects of ethanol are also influenced by NO, since NOS inhibition increases the hypnotic effects of ethanol in rats (Adams, Meyer, Sewing, & Cicero, 1994; Ferreira, Valenzuela, & Morato, 1999). The NO system is partially modulated by NMDA (N-methyl-D-aspartate) receptors, since calcium influx through NMDA receptors activates Ca^{+2}/calmodulin-dependent nNOS (Brenman & Bredt, 1997; Garthwaite & Boulton, 1995). NMDA receptor antagonists block the development of amphetamine and cocaine sensitization (Wolf & Jeziorski, 1993; Wolf & Khansa, 1991), and we demonstrated that inhibition of NMDA receptors by MK-801 prevents ethanol-induced sensitization in adult mice (Camarini, Frussa-Filho, Monteiro, & Calil, 2000). Our data showed that a neuronal NOS inhibitor reduced both ethanol sensitization and CUS-ETOH cross-sensitization. Taking these data together, we suggest that the NMDA-NO-pathway has an important role in ethanol-induced sensitization and most likely has an important role in cross-sensitization between CUS and ethanol in both adults and adolescents.

Although 7NI had effects on both age groups, we found differences in the NOS activity in the prefrontal cortex and hippocampus. Interestingly, regardless of the treatment, all age differences in the Ca^{2+}-dependent NOS activity were in the direction of greater activity in adults than in adolescents. The higher NOS activity in adult ethanol-sensitized mice compared to the respective adolescent group is particularly interesting because of the role of nNOS in neuronal plasticity, memory, and learning (Bredt, 1999). Elevated NO levels in the hippocampus and prefrontal cortex may have an influence in learning processes (Zhang, Chen & Wang, 1998), strengthening the outcome of the behavioral sensitization. These results can be explained by protracted maturation of the brain regions, especially the prefrontal cortex, during development. Neurotransmitter systems go through significant changes during adolescence (Andersen, Rutstein, Benzo, Hostetter, & Teicher, 1997; Tarazi, Tomasini, & Baldessarini, 1998; Teicher, Andersen, & Hostetter, 1995), including the nitrergic and glutamatergic systems (Guilarte & McGlothan, 1998; Haberny et al., 2002, for review). The latter undergoes robust pruning and neuronal death during neocortical development, and as for the nitrergic neurons, studies show they are mature around PND 21 (Haberny et al., 2002; Hvizdosova et al., 2014; Lizasoain et al., 1996). Protracted development of NMDA receptors and NO systems, as also here evidenced by lower NO production in adolescents, could have contributed to the low sensitivity to behavioral sensitization in adolescents. Briefly, considering that behavioral sensitization is partially dependent on learning processes, since context-dependent behavioral sensitization is more consistent and long-lasting than unconditioned sensitization (Badiani, Oates, & Robinson, 2000; Crombag, Badiani, & Robinson, 1996; see Camarini & Pautassi, 2016 for review), lower NOS activity in the adolescent mice may have influenced their lower magnitude of behavioral sensitization compared to adults.

Herein, we also demonstrated that CUS increased Ca^{2+}-dependent NOS activity in adult but not in adolescent mice in both hippocampus and prefrontal cortex. In adults, it has been shown that stress enhances mRNA and protein levels of nNOS and nNOS activity in the hippocampus (Zhou et al., 2007) and nNOS expression in prefrontal cortex subregions (Campos et al., 2013; Vila-Verde, Marinho, Lisboa, & Guimarães, 2016). As described above, nNOS activity is closely related to activation of NMDA receptors. Acute and chronic stress increase glutamate release in the hippocampus and prefrontal cortex (Fontella et al., 2004; Moghaddam, 2003). Our findings suggest that the stress-induced activation of the NMDA-nNOS system may be disrupted in adolescents.

In conclusion, adult mice showed greater ethanol sensitization accompanied by higher corticosterone levels and Ca^{2+}-dependent NOS activity in the prefrontal cortex compared to adolescents. Moreover, cross-sensitization between CUS and ethanol were also higher in adult mice. Despite these age differences, the NO system seems to have a pivotal role in ethanol-induced behavioral sensitization and cross-sensitization between chronic stress and ethanol in both adolescent and adult mice.

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