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NALOXONE BLOCKS ETHANOL-MEDIATED APPETITIVE CONDITIONING AND LOCOMOTOR ACTIVATION IN ADOLESCENT RATS

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Abstract

Age-related differences in ethanol sensitivity could put adolescents at risk for developing alcohol-related problems. Little information exists, however, about adolescent sensitivity to ethanol's appetitive effects and the neurobiological mechanisms underlying ethanol reinforcement during this developmental stage. The present study assessed the role of the opioid system in adolescent rats in an appetitive second-order schedule of ethanol reinforcement and ethanol-induced locomotor stimulation. On postnatal day 32 (PD32), animals were pretreated with the general opioid antagonist naloxone (0.0, 0.75, 1.50, or 2.5 mg/kg) and then given pairings of ethanol (0.0 or 2.0 g/kg, intragastrically) with intraoral pulses of water (conditioned stimulus 1 [CS₁], first-order conditioning phase). CS₁ delivery occurred 30–45 min after ethanol administration when the effect of ethanol was assumed to be appetitive. On PD33, adolescents were exposed to CS₁ (second-order conditioning phase) while in a chamber featuring distinctive exteroceptive cues (CS₂). Preference for CS₂ was then tested. Adolescents given CS₁-ethanol pairings exhibited greater preference for CS₂ than controls, indicating ethanol-mediated reinforcement, but only when not pretreated with naloxone. Blood alcohol levels during conditioning were not altered by naloxone. Experiment 2 revealed that ethanol induced locomotor activation soon after administration, and naloxone dose-dependently suppressed this stimulating effect. The present study indicates that adolescent rats are sensitive to ethanol's reinforcing and locomotor-stimulating effects. Both effects of ethanol appear to be mediated by endogenous opioid system activation.

Keywords

adolescence; rat; ethanol; reinforcement; locomotor activation, opioid system

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1. Introduction

Until recently, research on underage drinking has been largely neglected (Witt, 2010), partially attributable to the belief that adolescent ethanol intake is a transient phenomenon that lessens as the subject engages in family and work-related duties (Witt, 2010). Epidemiological and preclinical studies, however, show that the effects of adolescent drinking can be long lasting. Adolescent ethanol consumption is highly prevalent (with alcohol initiation in approximately 60% of teens aged 15–16 years in the United States; Johnston et al., 2009), and the risk of developing alcohol abuse and dependence is significantly higher in those who begin drinking before the age of 21 (De Wit et al., 2000; Grant and Dawson, 1997).

The use of animal models has shed some light on the determinants of ethanol intake in adolescents. When compared with adult counterparts, adolescent rats are less sensitive to several effects of ethanol that presumably serve as deterrents to escalated ethanol use (Spear and Varlinskaya, 2010). These effects include ethanol-induced locomotor impairment (White et al., 2002), sedation (Silveri and Spear, 1998), and ethanol-induced hangover (Doremus et al., 2003). Adolescents are also more resistant than adults to the suppressive effects of high-dose ethanol on social behaviors (Varlinskaya and Spear, 2002). However, adolescents are more sensitive than adults to the cognitive impairments associated with ethanol intoxication (White and Swartzwelder, 2005) and show increased social facilitation after low-dose ethanol (Varlinskaya and Spear, 2002).

A few studies have suggested that adolescents may show a distinctive response pattern to ethanol's appetitive and aversive motivational effects compared with older subjects. Ethanol-mediated conditioned place preference (CPP) is readily found in mice (Cunningham et al., 2006) and in genetically selected rats (Ciccocioppo et al., 1999; Carvalho et al., 2010), but is rare in heterogeneous, non-selected adult rats (Tzschenke, 2007). The expression of CPP by ethanol in heterogeneous adult rats has been reported only after very extensive training and pre-exposure procedures (15 days or more; Bozarth, 1990; Peana et al., 2008; Biala & Kotlińska, 1999), concurrent presentation of other reinforcers (Marglin et al., 1988) or exposure to electric shock (Matsuzawa et al., 1999). In contrast, Philpot et al. (2003) observed ethanol-mediated CPP in rats after only four training trials conducted shortly after weaning on postnatal day 25 (PD25; 0.2 g/kg) and also during late adolescence on PD45 (0.5 and 1.0 g/kg). Adolescent and adult rats also appear to differ in their sensitivity to ethanol's aversive effects. Adult, but not adolescent, rats expressed conditioned taste aversion (CTA) after pairings of ethanol (1.0 and 1.5 g/kg) and a sapid stimulus (Vetter-O'Hagen et al., 2009). Adolescents expressed CTA, albeit at a higher dose. These studies suggest that adolescents may be relatively insensitive to ethanol's aversive effects and more likely than adults to acquire ethanol-mediated appetitive learning.

Another approach for assessing ethanol's hedonic effects involves a second-order conditioning CPP procedure (Molina et al., 2006, 2007; Pautassi et al., 2008) that circumvents the traditional difficulty in establishing first-order CPP. In the second-order conditioning preparation, rats are given pairings of ethanol and a conditioned taste stimulus (CS₁) infused intraorally (e.g., very small amounts of water or sucrose). In a second phase, the intraorally infused CS₁ is paired with an environment (CS₂) featuring distinctive cues (e.g., sandpaper flooring and striped wallpaper). Ethanol-mediated conditioning is indexed by measuring preference or aversion for the CS₂ environment. Fourteen-day-old rats readily exhibited appetitive second-order conditioning when the intraoral CS₁ was paired shortly (5–20 min post-administration) after intubation with 0.5 or 2.0 g/kg ethanol or during a late phase (30–45 min post-administration) of the intoxication induced by 0.5 g/kg ethanol (Molina et al., 2006, 2007). Second-order aversion, however, emerged when the CS₁ predicted the late effects induced by 2.0 g/kg ethanol (Molina et al., 2006, 2007). A subsequent study (Pautassi et al., 2008) found age-related

differences in the expression of ethanol-mediated second-order conditioning. Adolescent, but not adult, rats expressed second-order appetitive conditioning (0.5–2.0 g/kg). This outcome was unchanged if the CS₁ predicted the initial or late stage of the ethanol post-administration interval. At higher doses (3.0–3.25 g/kg), ethanol induced conditioned aversion, which was similar in adolescents and adults (Myers et al., 2009). One caveat of these studies (Myers et al., 2009; Pautassi et al., 2008) was that the CS₁ paired with ethanol (sucrose, 10% v/v) had motivational properties of its own that may have interacted with ethanol's effects in the establishment of conditioning.

Ethanol-induced psychomotor stimulation represents yet another, albeit indirect, measure of ethanol's motivational effects. Humans at risk for developing alcohol problems show greater ethanol-induced tachycardia than individuals not at risk for the disorder (Conrod et al., 1997). Interestingly, adolescent, but not adult, rats self-administer sufficient oral ethanol to produce tachycardia (Ristuccia and Spear, 2008). Heterogeneous adult rats are also insensitive to the locomotor-activating effects of ethanol and, unlike mice (Camarini et al., 2000), typically show locomotor depression after ethanol administration (Chuck et al., 2006). However, acute ethanol administration induces locomotor activation in heterogeneous infant rats (Arias et al., 2008, 2009a, b, 2010) and adult rats selectively bred to ingest large amounts of ethanol (Rodd et al., 2004). In a recent study, we observed ethanol-induced locomotor activation in adolescent rats (PD 28) after intubation with 2.5 g/kg, but not 0.5 g/kg, ethanol (Acevedo et al., 2010). Moreover, females that had exhibited heightened sensitivity to ethanol's psychomotor effects on PD28 ingested significantly larger quantities of ethanol than counterparts less sensitive to ethanol-induced locomotor activation (Acevedo et al., 2010).

The adolescents' idiosyncratic variation in reactivity to ethanol may put them at risk for alcohol-related problems (Spear and Varlinskaya, 2010). More work is needed, however, to test the sensitivity of adolescents to ethanol's hedonic effects and the neurobiological mechanisms underlying ethanol reinforcement during this stage. The opioid neurotransmitter system is known to be involved in ethanol reinforcement and intake. Administration of opioid receptor antagonists inhibits CPP induced by ethanol in adult mice (Bechtholt and Cunningham, 2005) and infant rats (Nizhnikov et al., 2009) and disrupts appetitive conditioning induced by ethanol in neonatal rats (Nizhnikov et al., 2006). The psychomotor stimulant effects of ethanol also appear to be opioid-dependent. General opioid antagonism inhibits tachycardia produced by alcohol ingestion in healthy subjects (Peterson et al., 2006) and dose-dependently reduces ethanol-induced locomotor activation in adult mice (Camarini et al., 2000) and infant rats (Arias et al., 2009b). The involvement of the endogenous opioid system in mediating ethanol's motivational effects in adolescents has yet to be explored.

The present work assessed: (i) the sensitivity of adolescent, heterogeneous rats to ethanol's reinforcing and psychomotor stimulating effects and (ii) the participation of the opioid system in these effects of ethanol. Appetitive conditioning with ethanol was tested in Experiment 1 with a second-order conditioning procedure after administration of the general opioid antagonist naloxone. In addition to the important issue of testing the role of the endogenous opioid system, water instead of sucrose was used as the CS₁ paired with ethanol's effects, which was different from our previous second-order conditioning study (Pautassi et al., 2008). This procedural refinement removes potential confounding factors associated with the use of non-neutral sapid CSs and establishes the generality of the effect. Experiment 2 tested ethanol-induced locomotor activation after various doses of naloxone. In adult rats (Linseman and Lê, 1997), naloxone can alter ethanol's pharmacokinetics. Therefore, we also measured blood alcohol levels (BALs) in adolescents following naloxone or vehicle injection.

2. Materials and Methods

2.1. General procedures

2.1.1. Subjects—A total of 175 adolescent Wistar rats (89 males and 86 females) were used. These animals were 28–30 days old at the start of the experimental procedures, had a mean body weight of 123.04 ± 1.7 g (females, 113.49 ± 1.62 g; males, 131.94 ± 2.18 g), and were born and reared in the vivarium of the Center for Development and Behavioral Neuroscience at Binghamton University. The number of animals and litter representation across experiments were the following: Experiment 1 (44 males and 41 females, representing 18 litters), Experiment 2 (45 males and 45 females, representing 11 litters). Different, naïve animals were used in each Experiment. Room temperature (22–24°C) and lighting conditions (12 h/12 h light/dark cycle, with lights on at 8:00 AM) in the vivarium were automatically controlled. Births were checked on a daily basis, and the day of parturition was considered PD0. Litters were culled to 10 animals (five males and five females, whenever possible) on PD1, and the subjects were housed in standard maternity cages with *ad libitum* access to water and food. On PD21 (weaning), the animals were transferred in same-sex groups of five subjects to clean tubs lined with pine shavings. The experimental procedures complied with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1985) and were approved by the Institutional Animal Care and Use Committee within a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

2.1.2. Surgery and cannulation procedures—In Experiment 1, the delivery of the water CS₁ during conditioning and testing was done through intraoral cannulae made of polyethylene tubing. Specifically, the free end of the cannula was slipped inside another polyethylene tube, which in turn was connected to a Gilmont syringe (Barnant Co., Barrington, IL) mounted in a rotary microsyringe infusion pump (Kashinsky, Binghamton, NY). Cannula implantation surgery was performed on PD30 under an air-sealed hood and lasted about 8 min per animal. The surgery began by individually transferring the animal to an induction chamber where it was anesthetized with 2.5% isoflurane vapor via oxygen carrier gas (55 psi). The effectiveness of the anesthesia was ensured by assessing muscle tone and pupil reflexes. The back of the neck and right cheek were then shaved, and Betaiodine antiseptic and ethanol were applied to the bare skin. The animals were then transferred to a surgery table and kept warm (32–34°C) through the use of a heating pad. To maintain anesthesia, isoflurane was provided through a cone positioned next to the nose of the animal. A slit was then made in the cheek using a 14-gauge disposable needle (Harvard Instruments, Columbus, OH). A small section of polyethylene-10 tubing (10 cm; Clay-Adams, Parsippany, NJ) was run through the needle, which was subsequently removed. A 0.5 cm flange was then made on one end of the tubing. The tubing was gently pulled through the medial internal surface of the cheek. Consequently, the flanged end of the cannula was positioned over the oral mucosae while the remaining tubing exited from the mouth. The surgery continued with the insertion of another needle in the back of the neck. This second needle was guided under the skin to exit approximately 1 cm away from the site of the tube. The tube was then run through the needle until it appeared on the top of the neck. The second needle was then retracted, and the tubing was fastened with a fast-acting adhesive (Vetbond, 3M, St. Paul, MN). The animal was then placed in an individual holding cage with free access to food and water. After surgery, the animal was treated with a topical antibiotic (Neosporin, Johnson & Johnson, New Brunswick, NJ).

2.1.3. Drug preparation and administration procedures—The 2.0 g/kg ethanol dose was achieved by intragastrically (i.g.) administering 0.015 ml/kg of 16.8% ethanol solution (190-proof Ethanol, Pharmaco, Brookfield, CT). Control animals received a similar volume of tap water rather than ethanol. To administer ethanol, a 12 cm section of polyethylene-10 tubing

(Clay-Adams) was connected to a 1 ml syringe through a 27½-gauge needle. Animals were intubated in about 5 s, and ethanol was then delivered in 3–4 s.

Naloxone (Sigma-Aldrich, St. Louis, MO) was administered via intraperitoneal (i.p.) injections derived from two main stocks. The 0.75 and 1.50 mg/kg doses were derived from a 0.1 mg/ml solution, and the 2.50 mg/kg dose was derived from a 0.2 mg/ml solution. The injection volume was 0.0075 ml/g for the lowest and intermediate doses. The injection volume was 0.0125 ml/g for the intermediate and highest doses and for the animals treated with vehicle (i.e., 0.0 mg/kg). Saline (0.9%) was used as the vehicle solution.

2.1.4. Second-order conditioning and testing (Experiment 1, PD 32–33)—The second-order conditioning procedure was divided into three phases: first-order conditioning, second-order conditioning, and testing (Fig. 1). We closely followed a procedure that was first employed in preweanling rats by Molina et al. (2006,2007) and then adapted for use in adolescent rats by Pautassi et al. (2008).

Phase 1 (first-order conditioning, PD32): Animals were removed from their individual holding cages, weighed to the nearest 0.01 g (Sartorius, Gottingen, Germany), and intraperitoneally administered varying doses of the general opioid antagonist naloxone (0.0, 0.75, 1.5, or 2.5 mg/kg). Immediately after administration, the rats were individually placed in a square-shaped chamber made of wood (sides and height, 23 cm; floor lined with cotton) for 10 min. This habituation phase sought to familiarize the adolescents with the conditioning chambers and allow a better discrimination of the CS that would be employed during conditioning. Following habituation, the animals were administered ethanol (2.0 g/kg, i.g.) or its vehicle and then returned to their holding chambers. Conditioning began 30 min after ethanol intubation. Specifically, the rats were returned to the square-shaped chambers for 15 min (i.e., 30–45 min ethanol post-intubation time) and were given a conditioned stimulus (CS₁) consisting of intraoral pulses of distilled water (9 µl per pulse; pulse duration: 5 s; interpulse interval, 55 s). Thus, animals were given a total of fifteen 5 s pulses (one pulse/min for 15 min). The water pulses were delivered by connecting the intraoral cannula to the infusion pump. The habituation and conditioning phases were conducted under dim light provided by a 25 W fluorescent lamp facing a wall. During drug administration procedures and during the waiting period prior to conditioning, the experimental room was brightly illuminated by four 25 W fluorescent lamps.

Phase 2 (second-order conditioning, PD33): Animals were placed in one section of the three-chamber apparatus employed for assessing tactile preference (see Phase 3 description below). Access to the other sections of the apparatus was physically blocked by an acrylic barrier. The chamber in which the animals were introduced (28 × 20 × 21 cm) had alternating vertical black and white strips, and the floor was lined with a piece of rough sandpaper (60 grit, Gatorgrit, Fairborn, OH). The animals remained in this chamber, hereinafter referred to as CS₂, for 5 min and were stimulated with pulsed distilled water every 55 s (volume: 9 µl, pulse duration: 5 s). Animals received a total of five water pulses. In this phase, animals were given five CS₁–CS₂ pairings with no ethanol or naloxone administration.

Phase 3 (CPP test, PD33): Thirty minutes after termination of Phase 2, animals were individually placed in a three-chamber apparatus. Each interconnected chamber (28 × 20 × 21 cm each) had distinctive visual and tactile cues. The central area had white Plexiglas walls and a white Formica floor. One of the end compartments was experienced by the animals during Phase 2 and was lined with a vertical striped pattern on the walls and sandpaper on the floor (CS₂). The other end compartment had horizontal black and white stripes on the walls and a smooth, cardboard-like floor (the reverse side of the sandpaper sheet). The test (duration, 12 min) was also conducted under dim illumination and started by gently placing the animal in

the central chamber, facing away from the doors leading to the other chambers. The acrylic barrier separating the end compartments from the central compartment was absent, thus allowing free exploration of the apparatus. The behavior of the animals was filmed and rated by an experimenter blind to the treatment of the animals. The variable recorded was time spent in each chamber of the apparatus. A subject was considered to be inside a given chamber when two paws and the head were within that compartment. After each animal was tested, the apparatus was cleaned with water, and the rough and smooth surfaces were replaced.

It has been observed that spatial location cues can modulate the expression of ethanol-mediated CPP (Cunningham et al., 2006). Therefore, the spatial position of the training and test goal chambers was counterbalanced within the experimental room. Moreover, to ensure that there was no innate bias or an enhanced salience of sandpaper versus cardboard/smooth, a pilot experiment was conducted before the procedures described in the paper. Naive adolescents showed approximately 50% predilection towards sandpaper, when tested in a 12-min, sandpaper vs. cardboard test (mean percent of preference for sandpaper = 47.57 ± 5.63). This is consistent with previous experiments conducted in our lab indicating that 13 to 15 day old pups show equal preference for sandpaper and the smooth surface (i.e., Molina et al., 2006; 2007).

2.1.5. Assessment of ethanol-induced locomotor activity after naloxone administration (Experiment 2)

—On PD 28, the male and female adolescent rats were removed from their standard housing cages and individually placed in holding chambers. They were then weighed and given i.p. injections of naloxone (0.0, 0.75, 1.5, or 2.5 mg/kg). Twenty-five minutes later, they were given intubations of ethanol (2.0 g/kg) or its vehicle. The timing between the naloxone injection and ethanol intubation was based on previous studies (Arias et al., 2009b; Camarini et al., 2000).

Locomotor activity was recorded 5–20 min after ethanol administration in a Plexiglas chamber ($42 \times 42 \times 30$ cm; VersaMax Animal Activity Monitoring System, Accuscan Instruments, Columbus, OH). This test arena, in turn, was contained within another chamber ($53 \times 58 \times 43$ cm) that provided dim light, background white noise, and ventilation. The walls of the test area were surrounded by 16 photocell beams that crossed the chamber and generated a matrix of 64 sections. The location of the subject was continuously tracked by VersaMax software, which computed a minute-by-minute measure of distance traveled (cm). To compute this measure, the system uses consecutive photocell beam interruptions by the animal.

Two untreated groups were also included, one for each sex. Animals in this condition were removed from their housing cage and transferred to a holding cage. Thirty minutes later, the animals were placed into the VersaMax Animal Activity test arena. These animals, however, did not receive intubations or injections. The purpose of this procedure was to control for potential locomotor-activating effects resulting from these manipulations.

Thirty minutes after ethanol administration, trunk blood samples were obtained from those adolescents treated with ethanol and various doses of naloxone. The samples were collected through a heparinized capillary tube, centrifuged at high speed for 15 min at 3000 rpm (Micro-Haematocrit Centrifuge, Hawksley & Sons, Sussex, England), and later analyzed for BALs using an AM5 Alcohol Analyzer (Analox Instruments, Lunenburg, MA). The aim of this procedure was to determine whether naloxone treatment altered BALs at a time point that coincided with the commencement of the conditioning procedure conducted in Experiment 1. BALs are expressed as milligrams of ethanol per deciliter of body fluid (mg/dl = mg%).

2.2. Experimental design

Experiment 1 employed a 2 (female or male) $\times$ 2 (ethanol [2.0 g/kg] or vehicle treatment) $\times$ 4 (0.0, 0.75, 1.5, or 2.5 mg/kg naloxone) factorial design. A similar design was used in Experiment 2, with the exception that two isolated, untreated control groups were also included (one for each sex). The groups derived from the factorial designs consisted of 5–6 animals. Across experiments, only one male and one female from each litter were assigned to each condition to avoid overrepresentation of litters within each specific group and thus reduce litter bias that could interfere with the treatments.

2.3. Data analysis

According to preliminary analyses, no significant main effects of sex or significant interactions involving sex were found across all of the variables under consideration. No noticeable trends or tendencies involving this factor were observed. Therefore, descriptive and inferential analyses were performed by collapsing the data across sex. The male and female untreated control groups from Experiment 2 were also combined into a single untreated condition.

The dependent variable in Experiment 1 was the percent time spent in the section of the apparatus containing the CS₂ cues previously paired with the intraoral pulses of water (CS₁). The percent time in the sandpaper-lined CS₂ compartment was compared with percent time spent in the smooth-floor end compartment and in the Formica-floor central section of the apparatus. Percent time was calculated as the following: [(total time spent in sandpaper compartment $\times$ 100) / (total time spent in sandpaper compartment + total time spent in smooth compartment + total time spent in Formica compartment)]. This variable was analyzed using a fixed factor analysis of variance (ANOVA). Ethanol dose (0.0 or 2.0 g/kg) and naloxone treatment (0.0, 0.75, 1.5, or 2.5 mg/kg) were considered the between-factors. To confirm that adolescents in a given group expressed ethanol-mediated preference, percent scores were further analyzed using a *t*-test for single means against a user-defined constant. The constant represented theoretical chance preference for the CS₂ (33%). Additionally, the lower limit for the 95% confidence interval (CI) was computed. A significant difference yielded by the *t*-test and a lower limit CI over 33% were assumed to reflect ethanol-mediated CPP.

In Experiment 2, the total distance traveled (cm) during the 15 min assessment was divided into three 5 min clusters (i.e., post-administration intervals of 5–9 min, 10–14 min, and 15–19 min; hereinafter referred to as bins 1, 2, and 3). Locomotor activity was then analyzed using a three-way mixed ANOVA. Ethanol dose (0.0 or 2.0 g/kg) and naloxone treatment (0.0, 0.75, 1.5, or 2.5 mg/kg) served as between-group factors, and bin of evaluation (bin 1, 2, or 3) served as the within-measure factor. The untreated group was included in the ANOVA as an isolated control condition, thus improving the fitness of the model. Justified by our *a priori* hypothesis, planned comparisons were conducted between the untreated and 0.0-vehicle groups, one for each bin of evaluation. The aim of these analyses was to determine whether the manipulations associated with the intubation and injection of ethanol and naloxone, respectively, could induce locomotor activation by themselves.

BALs (Experiment 2) were analyzed using a one-way ANOVA (comparative factor between groups: naloxone treatment [0.0, 0.75, 1.5, or 2.5 mg/kg]).

The locus of significant main effects or significant interactions was further analyzed using follow-up ANOVAs and the Newman-Keuls *post hoc* test. Across analyses, the alpha level was set at 0.05.

3. Results

3.1. CPP scores

Fig. 2 depicts the relative time spent in the CS₂ compartment as a function of ethanol and naloxone treatment. Rats that experienced ethanol-CS₁ pairings after administration of the naloxone vehicle (group 0.0 nal -ethanol) spent significantly more percent time in the CS₂ compartment than any other group. This heightened preference for the CS₂ compartment, indicating ethanol-mediated CPP, was inhibited in animals given naloxone (0.75, 1.5, or 2.5 mg/kg) prior to ethanol.

The ANOVA yielded a significant main effect of naloxone treatment ($F_{3,77} = 4.18, p < 0.01$) and a significant naloxone $\times$ ethanol treatment interaction ($F_{3,77} = 3.70, p < 0.05$). The *post hoc* tests indicated that percent preference for the CS₂ compartment was higher in group 0.0 nal -ethanol than in all remaining groups, which in turn were not significantly different. Visual inspection of Fig. 2 may suggest that the lower dose of the antagonist (0.75 mg/kg) induced an intermediate effect, with the 0.75 nal -ethanol group spending slightly more time in the CS₂ compartment than its respective control and than counterparts given higher doses of naloxone. This impression, however, was not confirmed by the analyses. The *post hoc* tests indicated that these groups were not significantly different.

The finding of appetitive conditioning in the 0.0 nal -ethanol group was further confirmed by the *t*-test, which indicated that mean percent preference for the CS₂ compartment in this group was significantly higher than the theoretical 33% chance ($t_{10} = 2.36, p < 0.05$). The lower limit for the CI of mean percent preference for CS₂ was also above chance.

Percent preference for CS₂ in water-treated controls was not affected by naloxone dose, confirmed by the one-way ANOVA with naloxone dose as the independent between-group factor ($F_{3,38} = 0.54, p > 0.60$).

3.2. Ethanol-induced locomotor activity after naloxone administration

Distance traveled after naloxone and ethanol administration is depicted in Fig. 3. Locomotor activity was considerably higher in the first bin of evaluation and then declined substantially in bins 2 and 3. Ethanol induced reliable locomotor activation, an effect that was more pronounced during the first bin. Pretreatment with naloxone exerted a dose-dependent, inhibitory effect on ethanol-induced activity.

The ANOVA of distance traveled yielded significant main effects of ethanol treatment and bin of evaluation ($F_{1,81} = 46.22, F_{2,162} = 862.48$, respectively; both p 's < 0.001). The ethanol treatment $\times$ bin interaction, ethanol treatment $\times$ naloxone treatment interaction, and naloxone treatment $\times$ bin interaction were also significant ($F_{2,162} = 81.99, F_{3,81} = 7.23, F_{6,162} = 3.09$; respectively; all p 's < 0.01). The ANOVA revealed a significant three-way ethanol treatment $\times$ naloxone treatment $\times$ bin interaction ($F_{6,162} = 5.97, p < 0.0001$).

To better understand these interactions, follow-up ANOVAs (ethanol treatment $\times$ naloxone treatment, with the untreated condition again included in the ANOVA as an isolated control group) were performed for each bin of evaluation. For the first bin, the ANOVA revealed a significant main effect of ethanol treatment and a significant ethanol treatment $\times$ naloxone treatment interaction. The *post hoc* tests revealed that rats given ethanol after vehicle (0.0 nal -ethanol group) exhibited significantly greater distance traveled compared with any other condition, thus exhibiting ethanol-induced locomotor activation. This locomotor-activating effect of ethanol was partially inhibited by administration of 0.75 and 1.5 mg/kg naloxone. These animals moved significantly less than the 0.0 nal -ethanol group, yet they showed significantly more locomotor activation than their respective control groups (0.75 nal -vehicle

and 1.5 nal -vehicle, respectively). At the highest dose (2.50 mg/kg), the inhibitory effect of naloxone on ethanol-induced locomotor activation was complete. The *post hoc* tests indicated similar distances traveled in the 2.50 nal -ethanol and 2.50 nal -vehicle groups.

During post-intubation time 10–14 min (bin 2), the ANOVA indicated a significant ethanol treatment $\times$ naloxone treatment interaction ($F_{3,81} = 5.28, p < 0.005$). According to these *post hoc* tests, rats given ethanol after 1.5 mg/kg naloxone administration exhibited a slight, yet significant enhancement in distance traveled compared with rats given only vehicle. Rats in the 1.5 nal -ethanol group also exhibited greater locomotor activity than all of the remaining groups. In the case of the third bin (15–19 min post-intubation), neither significant effects nor significant interactions were observed.

Locomotor activity was not affected by the injection and intubation procedures. Planned comparisons indicated the absence of significant differences in locomotor activity between untreated rats and rats given 0.0 mg/kg naloxone followed by water intubation ($F_{1,81} = 0.57, F_{1,81} = 0.01, F_{1,81} = 0.01$; all p 's > 0.40).

3.3. BALs after naloxone administration

The one-way ANOVA indicated that naloxone treatment did not alter BALs measured at 30 min after administration. Mean and standard error values for BALs (mg/%) was 141.83 ± 4.38 , 150.40 ± 7.42 , 140.61 ± 7.41 and 129.06 ± 12.0 , for groups given 0.0, 0.75, 1.5 and 2.5 mg/kg naloxone, respectively.

4. Discussion

Analyzing the determinants of ethanol affinity during adolescence is important. Ethanol intake during this developmental stage can have long-lasting effects on future abuse of the drug. Grant and Dawson (1997) found a four-fold increase in the likelihood of alcohol dependence in individuals who initiated alcohol use at age 14 compared with individuals who began drinking at age 20 or later. Adolescents exhibit a distinctive ethanol response pattern (Spear and Varlinskaya, 2010). Among the several effects of ethanol, however, its motivational consequences are those that mainly modulate the seeking and taking of the drug (Lynch and Carroll, 2001; Roma et al., 2008).

Appetitive conditioning by ethanol in adolescent rats has been reported only by a few studies (e.g., Pautassi et al., 2008; Philpot et al., 2003). According to the present study, adolescent rats are sensitive to the appetitive effects of ethanol, measured using a second-order conditioning procedure. Ethanol endowed a neutral taste CS₁ (intraoral water pulses) with appetitive reinforcing properties. Subsequent CS₁–CS₂ pairings resulted in CPP for the CS₂ compartment (i.e., rough sandpaper surface and striped walls). Specifically, animals subjected to ethanol-CS₁ pairings spent significantly more time in the CS₂ compartment than control animals exposed to intraoral water infusions after vehicle intubation. The level of CS₂ preference following CS₁–ethanol pairings was also significantly above chance. These results confirm and extend previous studies of adolescent rats expressing ethanol-mediated appetitive reinforcement (Pautassi et al., 2008). This previous study had the limitations of employing sucrose (10%) as the intraoral CS₁ paired with ethanol's effects and having animals undergo food and fluid deprivation immediately before conditioning. Unclear, therefore, was whether ethanol provided the CS₁ with reinforcing effects or simply enhanced the inherent motivational value of sucrose. In the present experiment, ethanol-mediated second-order conditioning was found in nondeprived adolescents after employing a neutral CS. Thus, by excluding potential interactions between ethanol and alternative reinforcers, the present study represents progress toward the analysis of ethanol's mediation of appetitive conditioning during adolescence. It is

still possible, however, that at least part of the effective CS₁ was the sensory stimulation associated with the intraoral water pulse. Future studies are needed to elucidate this issue.

A novel finding of the present study was that ethanol-mediated second-order appetitive conditioning was dramatically inhibited by pretreatment with a general opioid antagonist. Specifically, adolescents given 0.75, 1.5, or 2.5 mg/kg naloxone 40 min before conditioning spent significantly less time in the sandpaper-lined chamber (CS₂) than animals that only experienced vehicle injections before CS₁-ethanol pairings. CS₂ preference in these naloxone-treated adolescents was approximately the same as that observed in control animals treated with various doses of the antagonist but not receiving CS₁-ethanol pairings.

Ethanol, at a dose that was effective in mediating second-order conditioned preference, also induced clear behavioral locomotor activation soon after administration. Locomotor activity in animals treated with vehicle was similar to untreated subjects. These results indicate that ethanol's locomotor-activating effects cannot be explained by the drug simply enhancing the stimulating effects associated with the injection and intubation procedures. Naloxone was highly effective in suppressing ethanol-induced locomotor activity. Specifically, 0.75 and 1.5 mg/kg naloxone attenuated the stimulating effect of ethanol, whereas the highest dose (2.5 mg/kg) completely suppressed it. Ethanol lacked locomotor-stimulating effects during the late sampling intervals, a phenomenon perhaps attributable to the development of habituation to the test environment that otherwise competes with the stimulating effect of ethanol. In a previous study, we employed a between-subjects design that minimized the role of habituation processes and similarly found that ethanol-induced locomotor activation in adolescent rats was selective for the early phase of the post-administration interval (Acevedo et al., 2010). Notably, a small but significant interaction was found between ethanol and naloxone during the second evaluation bin, with animals receiving 1.5 mg/kg naloxone exhibiting a greater distance traveled than vehicle-treated controls.

Under the present conditions, we observed no main or interaction effects of sex on the variables under analysis. This independence of ethanol's effects on sex should be considered cautiously because the relatively low number of animals of each sex across conditions could have precluded the statistical detection of sex effects. This absence of sex-related differences in ethanol's reinforcing and locomotor effects, however, was not unexpected. Previous studies found no difference between males and females in the reinforcing (Myers et al., 2009; Pautassi et al., 2008; Philpot et al., 2003) and locomotor-activating (Acevedo et al., 2010) effects of ethanol in adolescent rats. A similar lack of sex effects was observed when measuring ethanol consumption during adolescence (García-Burgos et al., 2009; Lancaster et al., 1996), although other studies have reported that females exhibit both more (Doremus et al., 2005) and less (Vetter-O'Hagen et al., 2009) ethanol consumption (g/kg) than males.

The effects of opioid system blockade during conditioning or during the locomotor activity test did not appear to be related to nonspecific effects of the antagonist. CS₂ preference in water-treated controls given 0.75, 1.5, or 2.5 mg/kg naloxone was similar to control counterparts given only vehicle. In the second experiment, locomotor activity in adolescents treated with vehicle was also unaffected by naloxone. Previous research (Linseman and Le, 1997) suggested that naloxone can alter ethanol metabolism, which was not observed in the present work. At the doses and post-administration intervals sampled, naloxone did not alter BALs.

The present results can be better explained by naloxone blunting ethanol's motivational and psychomotor effects. Animal studies indicate that at least part of ethanol's reinforcing effect is attributable to a neurochemical cascade involving activation of the endogenous opioid system (Cunningham et al., 1995; Froehlich and Li, 1994; Froehlich et al. 1991). The stimulation of

opioid receptors by ethanol has been suggested to subsequently activate dopamine neurons in the ventral tegmental area (Xiao et al., 2007). Opioid antagonists block ethanol-induced locomotor stimulation in preweanling rats (Arias et al., 2009b) and adult mice (Pastor et al., 2005). Rodent studies also showed that general (Bechtholt and Cunningham 2005; Bienkowski et al., 1999) or specific (Nizhnikov et al., 2006, 2009) opioid antagonists, administered prior to conditioning or testing, interfere with ethanol-induced motivational learning.

Similarly, studies with human subjects who drank alcohol after blocking the opioid system found lower positive mood ratings than controls (McCaul et al., 2001; Ray and Hutchison, 2007; Volpicelli et al., 1995) and a reduction in ethanol-induced psychomotor stimulation (Drobes et al., 2004; Peterson et al., 2006). These stimulating effects of ethanol are positively associated with alcohol-induced changes in mood (McCaul et al., 2001) and have been linked to heightened risk for alcohol addiction in humans (Conrod et al., 1997, 1998; King et al., 1997).

Altogether, the results from Experiments 1 and 2 provide important information regarding the relationship between the locomotor-activating and motivational properties of ethanol. An elegant series of studies conducted in preweanling rats by Arias et al. (2009a, b, 2010) suggested that these two variables can be mechanistically related. In infants, ethanol's locomotor-activating effects are dopamine-dependent (Arias et al., 2010) and the time-course of ethanol's biphasic motivational and locomotor effects coincide (Molina et al., 2007). Ethanol-induced locomotor stimulation was also greater in younger (8-day-old) than older (15-day-old) infant rats (Arias et al., 2009a). Interestingly, younger rats usually display heightened ethanol intake (Sanders and Spear, 2007) and appear to be less sensitive than older animals to ethanol's aversive motivational effects (Arias and Chotro, 2006; Hunt et al., 1991). The present study indicates that ethanol's locomotor stimulant and positive hedonic effects during the adolescent stage of development are dependent on the integrity of the endogenous opioid system.

Overall, these data indicate that, in adolescent rats, the endogenous opioid system mediates ethanol's motivational and motor-activating effects. These results agree with previous studies conducted in younger (Nizhnikov et al., 2006, 2009; Arias et al., 2009b) and older (Pastor et al., 2005, Bechtholt and Cunningham 2005; Bienkowski et al., 1999) subjects, suggesting that the transition from infancy to adolescence and from adolescence to adulthood is more continuous than abrupt. Still unknown, however, is the impact of chronic ethanol exposure on the opioid transmitter system during this critical time in brain development. Previous studies suggest that adolescents are more sensitive than adults to the alterations caused by repeated ethanol exposure in the physiology of neurotransmitter systems (e.g., glutamatergic; Pian et al., 2010). These developmental differences could represent yet another age-related difference in ethanol sensitivity that puts adolescents at risk for developing alcohol-related problems.

Research Highlights

- Ethanol induced appetitive second-order conditioning in adolescent rats.
- Ethanol induced motor behavioral stimulation in adolescent rats.
- Administration of naloxone inhibited the appetitive conditioning.
- Naloxone dose-dependently blocked ethanol's motor activating effects.
- The opioid system mediates ethanol's reinforcing and motor activating effects.

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Figure 1.

Methods for analyzing the role of the opioid system in the acquisition of ethanol-mediated appetitive conditioning in adolescent rats. Phase 1, first-order conditioning, postnatal day 32: Animals were treated with naloxone (0.0, 0.75, 1.5, or 2.5 mg/kg), followed by a brief (10 min) habituation to the experimental context. They were then treated with ethanol (2.0 or 0.0 g/kg) and stimulated with a conditioned stimulus (CS₁) consisting of intraoral pulses of water. CS₁ delivery occurred 30–45 min following ethanol. Phase 2, second-order conditioning, postnatal day 33: Animals were briefly (5 min) stimulated with water pulses while placed in a visually and tactile distinctive chamber (CS₂). Phase 3, *preference test*, postnatal day 33: The time spent in the CS₂ chamber was recorded during a 12 min place preference test. The figure and legend were adapted with permission from Pautassi et al., 2008.

Figure 2.

Ethanol-induced second-order conditioned preference in adolescent rats, with percent time spent in the CS₂ compartment during the test as a function of naloxone and ethanol treatment. During conditioning, adolescents were given naloxone, an opioid antagonist (0.0, 0.75, 1.5, or 2.5 mg/kg, i.p.), followed by ethanol administration (2.0 or 0.0 g/kg, i.g.), and were then stimulated with intraoral pulses of water (CS₁). The following day, animals experienced CS₁–CS₂ pairings and were then tested for CS₂ preference. Asterisks (*) indicate significant differences between an ethanol-treated group and its corresponding vehicle-treated control ($p < 0.05$). Vertical bars indicate the SEM.

Figure 3.

Ethanol-induced locomotor activity after naloxone treatment. Locomotor activity (distance traveled, cm) 5–9, 10–14, and 15–19 min after ethanol administration (bins 1, 2, and 3, respectively) in adolescent rats. The animals were given naloxone (0.0, 0.75, 1.5, or 2.5 mg/kg) prior to ethanol administration (2.0 or 0.0 g/kg, i.g.). Animals in the untreated group were assessed for locomotor activity but did not receive intubations or injections. Asterisks (*) indicate significant differences between an ethanol-treated group and its corresponding vehicle-treated control ($p < 0.05$). Number signs (#) indicate significant differences -- during the first bin of evaluation -- between a given ethanol-treated group and the group administered ethanol after 0.0 mg/kg naloxone (i.e., 0.0-ethanol group) ($p < 0.05$). Vertical bars indicate the SEM.