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Effects of the Kappa Opioid Receptor Antagonist, Nor-Binaltorphimine, on Ethanol Intake: Impact of Age and Sex

Melissa Morales, Rachel I. Anderson, Linda P. Spear, and Elena I. Varlinskaya

Center for Development and Behavioral, Neuroscience, Department of Psychology, Binghamton University, P.O. Box 6000, Binghamton, New York 13902-6000

Melissa Morales: mmorale2@binghamton.edu

Abstract

The kappa opioid receptor (KOR) antagonist, nor-binaltorphimine (nor-BNI), was used to investigate the role of the KOR system in mediating ethanol intake. On P25 (adolescent) or P67 (adult) male and female rats were individually housed and given ad libitum access to food and water. The experimental procedure was initiated on P28 or P70: animals were given 30 min/day access to a 10% ethanol/supersaccharin solution every other day (3 baseline exposures). On the day after the final baseline test, rats were injected with nor-BNI (0, 2.5, 5, 10 mg/kg), with testing initiated 24 hr later (30-min access every other day, 3 test exposures). Nor-BNI (10 mg/kg) increased ethanol intake in adult males, whereas the same dose decreased intake in adult females, suggesting pronounced sex differences in KOR-associated mediation of ethanol intake in adulthood. There was no impact of nor-BNI in adolescent animals of either sex, suggesting that the KOR may play less of a role in modulating ethanol intake during adolescence.

Keywords

adolescence; ethanol intake; Nor-BNI; sex differences; rat; kappa opioid receptor

INTRODUCTION

Adolescence is a transitional period from immaturity to maturity associated with hormonal, neural, and behavioral changes that are highly conserved across a variety of mammalian species (Spear, 2000, 2010). It is also a developmental period during which alcohol use is typically initiated in humans (Johnston, O'Malley, Bachman, & Schulenberg, 2013). While adolescents may consume alcohol less frequently than adults do, they drink more alcohol per occasion, with this adolescent-typical enhancement of ethanol intake evident in both humans (Chen & Kandel, 1995) and laboratory rodents, including rats (Doremus, Brunell, Rajendran, & Spear, 2005; Maldonado, Finkbeiner, Alipour, & Kirstein, 2008; Vetter, Doremus-Fitzwater, & Spear, 2007), mice (Tambour, Brown, & Crabbe, 2008) and rodents selectively bred for high alcohol consumption (Dhaher, McConnell, Rodd, McBride, & Bell, 2012). In humans, early initiation of drinking plays a substantial role in alcohol-related

problems later in life (Grant & Dawson, 1998). Individuals who engage in even episodic heavy drinking at an early age are more likely to become alcohol-dependent (Bonomo, Bowes, Coffey, Carlin, & Patton, 2004; Grant, Stinson, & Harford, 2001; Hingson, Heeren, & Winter, 2006) and to experience a number of long-lasting adverse psychosocial consequences (Wells, Horwood, & Fergusson, 2004). Given that ethical restrictions prevent the use of human adolescents in alcohol research, the use of animal models for investigating the behavioral effects and the neural mechanisms underlying elevated ethanol intake during this stage of development has proven to be valuable (see Spear, 2000, 2010, 2011; Witt, 2010).

Animal research suggests that adolescent and adult rats respond differently to ethanol—with adolescents being particularly insensitive to the adverse effects associated with ethanol, such as conditioned taste aversion, social inhibition, and withdrawal-induced anxiety (Anderson, Varlinskaya, & Spear, 2010; Doremus, Brunell, Varlinskaya, & Spear, 2003; Morales, Varlinskaya, & Spear, 2011; Varlinskaya & Spear, 2006). Although adolescents demonstrate a relative insensitivity to some of the adverse effects associated with ethanol, they also demonstrate an increased sensitivity to the positive (e.g., socially facilitating) effects of ethanol (Varlinskaya & Spear, 2002, 2004, 2006). It is possible that the decreased sensitivity to the aversive—but increased sensitivity to the positive—effects of ethanol in adolescents relative to adults may be driving the higher ethanol consumption observed during adolescence. In other words, adolescents may be consuming greater amounts of ethanol because, unlike adults, they are not receiving the cues associated with ethanol that would terminate drinking.

Although developmental research has focused on several neural systems upon which ethanol exerts its actions (e.g., GABA, glutamate—see Eckardt et al., 1998, for review; Moy, Duncan, Knapp, & Breese, 1998; Pascual, Boix, Felipo, & Guerri, 2009; Pian, Criado, Milner, & Ehlers, 2010; Primus & Kellogg, 1990; Ramirez, Varlinskaya, & Spear, 2011; Silveri & Spear, 2002), ontogenetic studies on the involvement of the opioid systems in mediating ethanol effects are lacking in adolescent and adult animals (however, see Sable, Bell, Rodd, & McBride, 2006; Varlinskaya & Spear, 2009). Opioid receptors belong to the superfamily of 7-transmembrane G protein-coupled receptors and consist of three main classes of opioid receptors: mu (μ), delta (δ), and kappa (κ) (MOR, DOR, and KOR, respectively), each preferentially binding to different endogenous ligands. For example, β -endorphins bind with high affinity to MOR and DOR; enkephalins have highest affinity for DOR; and dynorphins (DYN) bind with highest affinity to kappa opioid receptor (KOR) (Herz, 1997; Raynor et al., 1996). Opioid receptors are activated both by their endogenously produced ligands as well as exogenously administered drugs and play a role in analgesia, depression, sedation, tolerance, and addiction (Bodnar, 2010).

Several lines of evidence suggest that positively reinforcing and stimulatory effects of ethanol are associated with activation of MOR and possibly DOR, whereas aversive and dysphoric consequences of ethanol administration are associated with the DYN/ KOR system (Herz, 1997). For example, male rats demonstrated a conditioned place preference for MOR agonists while an aversion was seen following conditioning with KOR agonists (Mucha & Herz, 1985). Similar findings have been obtained from self-administration

studies: laboratory rodents will self-administer MOR and DOR agonists (Devine & Wise, 1994; Tang & Collins, 1985), whereas they will not self-administer drugs that activate the KOR (Tang & Collins, 1985). Although less well investigated, other evidence as well supports the suggestion that the DYN/KOR system may play a role either in the aversive properties of ethanol or in ethanol-induced negative reinforcement (Lindholm, Werme, Brene, & Franck, 2001). For instance, ethanol-preferring strains of rats and mice have lower levels of dynorphins or prodynorphin mRNA in the nucleus accumbens than ethanol nonpreferring strains (Jamensky & Gianoulakis, 1997; Nylander, Hyttia, Forsander, & Terenius, 1994). The dysphoric effects of KOR activation may be related to a decrease in dopamine release in the nucleus accumbens and caudate putamen—an effect in opposition to the enhanced dopamine release observed following MOR activation (Di Chiara & Imperato, 1988a).

Given the positively reinforcing effects of MOR activation, it is not surprising that pharmacological blockade of the MOR with selective (e.g., β -funaltrex-amine, CTOP) and nonselective (e.g., naltrexone) antagonists effectively reduces ethanol intake, effects seen across a variety of species and selected lines as well as under a variety of experimental conditions (Oswald & Wand, 2004; Sable et al., 2006; although see Herz, 1997). In contrast, enhanced activity of the DYN/KOR system would be expected to limit ethanol intake, with pharmacological blockade of KOR therefore expected to elevate levels of ethanol consumption. However, pharmacological KOR manipulations have been shown to produce mixed effects on ethanol intake and self-administration. In a study by Walker and Koob (2008), ethanol-dependent male rats demonstrated a decrease in operant ethanol self-administration on days in which rats received a selective KOR antagonist, norbinaltorphimine (nor-BNI), administered intracerebroventricularly relative to saline days, whereas no effect was evident in nondependent animals (Walker & Koob, 2008). Systemic administration of nor-BNI produced the same effect in ethanol-dependent male rats (Walker, Zorrilla, & Koob, 2011). In yet another study examining nor-BNI's ability to regulate ethanol intake in male Long-Evans rats, no change in operant self-administration was observed, although an elevation in dopamine release was seen toward the end of the consumption period (Doyon, Howard, Shippenberg, & Gonzales, 2006). In contrast, a study by Mitchell, Liang, and Fields (2005) revealed that a single injection of nor-BNI (10 mg/kg) was able to increase ethanol consumption in nondependent male rats relative to their own baseline (pretreatment) and to saline-treated controls. Procedural differences such as number of injections (e.g., multiple vs. a single injection), route of administration (e.g., intracerebroventricular vs. subcutaneous), prior ethanol history (ethanol-dependent vs. nondependent), or mode of ethanol administration (e.g., operant self-administration vs. oral consumption) may account for these discrepant findings.

Given the diversity of findings, more research is needed to examine effects of nor-BNI on ethanol intake. Studies conducted to date have focused only on adult male rodents, and hence assessment of nor-BNI effects on ethanol intake should be extended to other important populations as well (e.g., adolescents). To explore whether the endogenous DYN/KOR system may contribute to the differential ethanol drinking patterns seen between adolescents and adults, the current experiments assessed the effects of pharmacological blockade of the KOR on ethanol intake in relatively ethanol-naïve adolescent and adult

Sprague–Dawley rats. These age comparisons were conducted in both males and females, given the lack of prior work examining ethanol intake following antagonism of the KOR system in females. Although there are ample studies examining sex differences in the role of the opioid receptor systems for other dependent measures (e.g., pain sensitivity) (see Rasakham & Liu-Chen, 2011 for references), females have been little studied after KOR manipulations in the alcohol field. This is surprising given evidence for women to experience different drinking problems than men (e.g., NIAAA, 2004), with reliable sex differences in ethanol intake typically seen in adult rodents as well (Doremus et al., 2005; Vetter-O'Hagen, Varlinskaya, & Spear, 2009), although these sex differences do appear to depend on the rat lines studied (Dhaher et al., 2012).

MATERIALS AND METHODS

Subjects

A total of 192 male and female Sprague–Dawley rats derived from our breeding facility were used in the current experiments. On postnatal day (P) 1, all litters were culled to 8–10 pups with a sex ratio of six males and four females kept whenever possible. Pups were weaned on P21, pair housed with same-sex littermates, and given ad libitum access to food (Purina Lab chow, Lowell, MA) and water in a temperature-controlled vivarium with a 14:10 hr light–dark cycle (lights on at 0700 hr). At all times animals were treated in accordance with guidelines for animal care established by the National Institutes of Health under protocols approved by the Binghamton University Institutional Animal Care and Use Committee.

Experimental Design

Experiments 1 and 2 were designed identically except that males were used in the former whereas females were used in the latter. The experimental design for Experiments 1 and 2 was a 2 age (adolescent, adult) $\times$ 4 nor-BNI dose (0, 2.5, 5.0, 10.0 mg/kg) factorial with 10 animals placed into each of the 8 groups. No more than one animal from a litter was placed into an experimental group, thus reducing the possibility of litter effects (Holson & Pearce, 1992). Experiment 3 was conducted to determine if the observed differences in consumption of the 10% ethanol/supersaccharin (SS) solution seen in Experiments 1 and 2 were ethanol-specific and not related to general increases or decreases in fluid consumption. Both males and females were examined for consumption of the SS solution alone in this experiment using a two nor-BNI dose (0, 10.0 mg/kg) factorial, with eight animals placed into each experimental group.

Intake Procedure

Starting on P25 or P67, adolescent and adult rats were singly housed in a standard clear plastic tub and were given 3 days to acclimate to their new home environment before the onset of testing. Although we have previously utilized a pair-housing procedure for measuring ethanol intake (Vetter-O'Hagen & Spear, 2011), rats in the present series of experiments were isolate-housed to facilitate the ethanol intake procedure. However, housing conditions are important to consider given that the opioid systems are heavily involved in stress and social behaviors (see Bodnar, 2010 for review; Trezza, Damsteegt,

Achterberg, & Vanderschuren, 2011). For example, there is evidence to suggest that rats respond differently in a variety of behavioral tests (e.g., conditioned place preference, tail-flick test) depending on whether they were housed individually or in an enriched environment (Smith, Bryant, & McClean, 2003).

Beginning on P28 or P70 (experimental day 1), animals were given intake tests every other day, with each test consisting of 30 min access in the home cage to one bottle containing a 10% ethanol/SS solution (3% sucrose and .125% saccharin in 10% ethanol)—modified from the “supersac” intake procedure (Ji, Gilpin, Richardson, Rivier, & Koob, 2008) and used previously in our laboratory (Broadwater, Varlinskaya, & Spear, 2011). Intake sessions conducted on experimental days 1, 3, and 5 served as baseline 1, 2, and 3, respectively. On experimental day 6, animals were weighed and injected subcutaneously with one of four doses of the selective KOR antagonist, nor-binaltorphimine (nor-BNI) (Tocris, Minneapolis, MN): 0 (.9% saline vehicle), 2.5, 5.0, 10.0 mg/kg/ml. They were then left undisturbed for 24 hr until intake testing on experimental day 7. Nor-BNI was injected once, 24 hr before the first test day intake session (i.e., experimental day 7), given evidence that nor-BNI has long-lasting (up to several weeks) antagonistic effects on the KOR (see Metcalf & Coop, 2005 for references), although it may have MOR antagonistic effects within the first few hours postadministration (Broadbear, Negus, Butelman, de Costa, & Woods, 1994). Intake tests conducted on experimental days 7, 9, and 11 served as tests 1, 2, and 3, respectively (see Fig. 1 for pictorial representation of intake procedure). At all times except during intake sessions, rats were given unlimited access to tap water; thus, they were never water deprived.

Blood Ethanol Determination

Immediately following the last intake session (experimental day 11), tail blood samples were collected for determination of blood ethanol concentrations (BECs). Tail blood was collected into a heparinized tube and stored at -80°C until analysis of BECs. Samples were assessed for BECs via headspace gas chromatography using a Hewlett Packard (HP) 5890 series II Gas Chromatograph (GC) (Wilmington, DE). Blood samples were thawed and 25- μl aliquots were placed in airtight vials. Vials were placed in a HP 7694E Auto-Sampler, which heated each individual vial for 8 min, and then extracted and injected a 1.0 ml sample of the gas headspace into the GC. Ethanol concentrations in each sample were determined using HP Chemstation software which compares the peak area under the curve in each sample with those of standard curves derived from reference standard solutions.

Data Analysis

Prior to analysis, all daily intake data were checked for outliers (2 standard deviations above or below the mean), with data points meeting this criteria not included in the analyses. Across the experiments, single day intake data from six males (two adolescent, four adult) from Experiment 1 and seven females (five adolescent, two adult) from Experiment 2 were so eliminated (i.e., $<2\%$ of the data points) and replaced with the group mean for that day. The data set was also subjected to Levene's test for homogeneity of variance prior to analysis. Data from Experiments 1 and 3 did not violate this assumption on any day. However, data in Experiment 2 violated the assumption on 2 out of the 3 test days, with a

log transformation yielding the best outcome for improving homogeneity of variance. Analysis of these transformed data yielded the same results as for the nontransformed data; therefore, results and graphs are depicted using nontransformed data for all experiments. Data were analyzed using a mixed model analysis of variance (ANOVA), with age, body weight gain across the experimental procedure, ethanol intake on baseline days (g/kg) and percent of baseline intake [(post-nor-BNI/baseline day 3) $\times$ 100] being the factors of interest. Test day intakes were converted to percent of baseline intake given notable (and expected) age differences in baseline ethanol consumption (e.g., Figs. 2 and 4). In order to avoid inflating the possibility of type II errors on tests with at least 3 factors (Carmer & Swanson, 1973), Fisher's planned pairwise comparison test was used to explore significant effects and interactions. Pearson R correlations were conducted to compare ethanol intake on the final test day (i.e., test day 3) and BECs. Significance was set at $p < .05$, and all data are expressed as mean $\pm$ standard error (M $\pm$ SEM).

RESULTS

Experiment 1: Effects of Nor-BNI on Ethanol Intake in Males

Body Weight Gain—In males, the ANOVA of body weight gain across the experimental procedure revealed only a main effect of age [$F(1, 72) = 458.79, p < .0001$]. As expected, adolescents (91.02 ± 1.29 g) gained significantly more weight than adults (34.92 ± 2.26 g).

Baseline Ethanol Intake—The ANOVA of baseline intake across baseline 1, 2, and 3 revealed main effects of age [$F(1, 78) = 97.20, p < .001$] and day [$F(2, 156) = 6.47, p < .01$]. Adolescents consumed significantly more ethanol on a g/kg basis than adults did, and rats consumed more ethanol on the last baseline day than on baseline days 1 and 2 regardless of age (Fig. 2).

Test Day Ethanol Intake—Analysis of ethanol intake on test days 1, 2, and 3 indexed via percent of baseline intake revealed a significant test day $\times$ age $\times$ nor-BNI dose interaction [$F(6, 124) = 2.21, p < .05$]. On the final test day, adults given the highest dose of nor-BNI (10 mg/kg) consumed significantly more ethanol than saline-injected counterparts relative to intake from the last baseline day, with no differences in intake emerging in adolescents (Fig. 3). Analysis of BECs collected immediately after the final intake session revealed no significant main effects or interactions; although, there was a trend ($p = .07$) for adolescent (11.84 ± 2.75 mg/dl) males to have significantly lower BECs than adults (20.79 ± 3.79 mg/dl). Positive correlations between ethanol intake on test day 3 and BECs emerged in both adolescent and adult males ($r = .67$ and $.82$, respectively).

Experiment 2: Effects of Nor-BNI on Ethanol Intake in Females

Body Weight Gain—As expected, analysis of body weight gain in females revealed that adolescents (60.79 ± 1.15 g) gained significantly more weight throughout the experimental procedure relative to adults (18.14 ± 1.18 g) [$F(1, 72) = 656.28, p < .0001$].

Baseline Ethanol Intake—In females, a significant age $\times$ day interaction of baseline ethanol intake emerged [$F(2, 156) = 4.57, p < .0001$]. On each baseline day, adolescent

females consumed more ethanol on a g/kg basis than their adult counterparts. Intake of the adult females increased significantly from day 1. In contrast, intake of the adolescent females declined slightly across days, with a significant decline in intake observed by the last day relative to the first baseline day (Fig. 4).

Test Day Ethanol Intake—Analysis of ethanol intake on test days 1, 2, and 3 demonstrated a significant age $\times$ nor-BNI dose interaction [$F(3, 72) = 2.66, p = .05$]. Adult females administered 10 mg/kg nor-BNI consumed significantly less ethanol than saline-treated controls across all test days. In contrast, no significant nor-BNI effects emerged in adolescent females, although there was a trend ($p = .07$) for adolescent females given 5 mg/kg nor-BNI to consume significantly more ethanol than their saline-treated counterparts (Fig. 5). This analysis also revealed a significant main effect of day [$F(2, 144) = 7.55, p < .001$], with ethanol intake on test days 2 (127.54 ± 6.66) and 3 (143.14 ± 10.69) being significantly higher than test day 1 (111.06 ± 7.34). Analysis of BECs after the last test revealed a significant main effect of age [$F(1, 52) = 5.36, p < .05$], with adolescent females (17.67 ± 4.51 mg/dl) having lower BECs than their adult counterparts (33.58 ± 5.38 mg/dl). BECs were positively correlated with ethanol intake in adolescent females ($r = 0.63$), whereas only a trend emerged in adult females ($r = .33$).

Experiment 3: Effect of nor-BNI on Consumption of "Supersac" in Adult Males and Females

Given that the opioid systems are implicated in responsiveness to sweet taste (Slawecki, Hodge, & Samson, 1997), it was necessary to confirm that changes in consumption of sweetened with supersaccharin ethanol solution (increase and decrease in adult males and females, respectively) observed following 10 mg/kg nor-BNI was not due to sweet taste and was indeed, ethanol-specific. To accomplish this, a total of 32 adult animals (16 males and 16 females) followed the same experimental procedure as described above, with animals given access only to a supersaccharin solution containing no ethanol, and injected with either 0 or 10 mg/kg nor-BNI on experimental day 6 ($n = 8/\text{group}$).

In males, the ANOVA of percent of baseline intake revealed no significant differences in intake between saline ($122.57 \pm 17.53\%$) and nor-BNI ($151.80 \pm 17.71\%$) exposed animals. Similarly, in females, analysis of percent of baseline intake revealed no main effect of dose, with females given saline ($122.61 \pm 9.11\%$) and 10 mg/kg nor-BNI ($113.69 \pm 9.43\%$) consuming roughly equivalent amounts of the solution as a % of baseline intake.

DISCUSSION

The present experiments support a role for the DYN/ KOR system in modulating ethanol consumption in adult male and female rats. While adult intake changed as a result of pharmacological blockade of the KOR by the selective antagonist nor-BNI, the pattern observed was opposite in males and females. Systemic administration of nor-BNI (10 mg/kg) increased ethanol consumption in adult males on the last test day (Exp. 1); however, at the same dose, adult females consumed significantly less ethanol across the three test days (Exp. 2). The ability of pharmacological blockade of the KOR to impact ethanol consumption also varied notably with age, with adolescents of either sex not demonstrating

significant changes in ethanol intake, regardless of nor-BNI dose. These age-related differences in sensitivity to nor-BNI support the notion that the DYN/KOR system may be less involved in modulation of ethanol intake in adolescent animals relative to their adult counterparts. Despite concerns that drugs targeting the KOR system may impact general consum-matory behaviors (Bodnar, 2010), nor-BNI animals did not differ from saline controls in consumption of supersaccharin, thereby demonstrating that the nor-BNI-induced changes in consumption were ethanol-specific under the present (i.e., limited-access to a sweetened ethanol solution) test conditions.

Although a modest effect was observed following only the 10 mg/kg nor-BNI dose tested, it should be noted that this is a dose that has consistently been shown to be effective in rodents (Mitchell et al., 2005; Thompson et al., 2000). A cumulative dose–response study by Walker et al. (2011) observed behavioral effects on ethanol intake after 15 and 20 mg/kg but not the lower doses examined. Likewise, a dose–response curve in mice revealed that only after higher nor-BNI doses administered i.c.v. was the drug able to block DAMGO-induced antinociception in a tail-flick test (Horan, de Costa, Rice, & Porreca, 1991). Thus, it may be that the lower doses used in the current study were simply insufficient to antagonize KOR to the extent necessary to produce pronounced alterations in ethanol intake. Nevertheless, as expected, a single injection of nor-BNI increased ethanol intake in adult males—an effect reported previously by Mitchell et al. (2005) in a two-bottle continuous access paradigm. This increase in ethanol consumption may be related, at least in part, to a nor-BNI-associated reduction in the presumably aversive, intake-inhibiting effects of ethanol mediated via the endogenous DYN/KOR system (e.g., Shippenberg & Herz, 1986), since kappa agonists generally decrease free-access ethanol intake in rats (Lindholm et al., 2001). It is possible that adult males are extremely sensitive to the aversive properties of endogenous ligands for KOR, and, therefore, ethanol-induced activation of the DYN/KOR system may play a substantial role in inhibitory control of ethanol intake in these animals. Indeed, some experimental evidence suggests that this system may play a protective role in alcohol abuse by antagonizing the rewarding effects of ethanol (Wee & Koob, 2010), an effect perhaps mediated via KOR modulation of the dopamine system (see Bruijnzeel, 2009 for references). Given evidence for the DYN/KOR system to inhibit dopamine release in brain regions associated with reward (e.g., nucleus accumbens) (Di Chiara & Imperato, 1988a; Spanagel, Herz, & Shippenberg, 1992), to the extent that ethanol causes a release of dopamine in reward-related brain regions (Di Chiara & Imperato, 1988b), pharmacological blockade of the KOR should increase dopamine release and facilitate drinking by increasing the rewarding value of ethanol (however, see Bono, Balducci, Richelmi, Koob, & Pulvirenti, 1996; Russell, McBride, Lumeng, Li, & Murphy, 1996). Along these lines, if KORs mediate tonic inhibition over dopamine release (see Jamensky & Gianoulakis, 1997), then removing (or blocking) KOR function should lead to increases in alcohol consumption—findings supported by adult males in the present study (although see Kovacs et al., 2005). Alternatively, if the DYN/KOR system is involved in mediating the aversive properties of ethanol, with animals sensitive to these aversive properties showing low ethanol consumption, then decreasing the aversiveness of ethanol via pharmacological blockade of the KOR should increase ethanol intake. This is supported by data from a study in which Logrip et al. (2008) showed that nor-BNI was able to block the reduction in ethanol intake

observed after Tat-RACK1 activation (which increases BDNF expression, in turn increasing prodynorphin and subsequently decreasing ethanol intake). Furthermore, findings from a recent experiment conducted in our laboratory revealed that nor-BNI attenuated the ethanol-induced conditioned taste aversion seen in stressed adult males relative to saline-treated animals (Anderson, Agoglia, Morales, Varlinskaya, & Spear, 2012).

While the present results and those of Mitchell et al. (2005) found an increase in ethanol intake in adult male rats following nor-BNI, in some prior work, the opposite has been reported—that is, a decrease in ethanol consumption in nor-BNI-treated rats (Walker & Koob, 2008; Walker et al., 2011), suggesting that various factors may impact DYN/KOR modulation of ethanol intake. For instance, variations in genes that encode for DYN and the KOR have been shown to be associated with a greater risk for alcohol use disorders (Xuei et al., 2006). In rodents, factors such as prior ethanol exposure has been shown to alter the DYN/ KOR system, which may lead to differences in ethanol consumption (Lindholm, Ploj, Franck, & Nylander, 2000; Rosin, Lindholm, Franck, & Georgieva, 1999). For example, 13–14 days of ethanol exposure was found to increase DYN B concentration in the nucleus accumbens and periaqueductal gray area (Lindholm et al., 2000) while decreasing KOR mRNA levels (Rosin et al., 1999), perhaps as a compensatory reaction to the increases in DYN levels that occur after ethanol exposure (Lindholm et al., 2000). Thus, the DYN/KOR system could mediate ethanol intake differently in animals with distinct ethanol histories, or during withdrawal. If the dysphoria associated with ethanol withdrawal is related to elevated DYN activity (Przewlocka, Turchan, Lason, & Przewlocki, 1997), a KOR antagonist would be expected to dampen this negative state, perhaps reducing the impetus for consuming ethanol for its negative (presumably anxiolytic) reinforcing properties (Walker et al., 2011), and producing an opposite pattern of effects from those observed here and by Mitchell et al. (2005).

Among females, the KOR antagonist produced a decrease in ethanol consumption, regardless of test day. Stage of estrous cycle was not monitored in this experiment, and stage of estrous cycle has been shown to influence drug intake in females (Fattore, Altea, & Fratta, 2008) and analgesic responses to morphine administration (see Craft, 2008). Although decreases in ethanol intake were reliable on all three test days following nor-BNI, it is nonetheless possible that efficacy of antagonism of the KOR could have been influenced by fluctuations in reproductive hormones. Unfortunately, studies examining the effects of KOR manipulation on ethanol intake have focused predominantly on males, so little is known about how these manipulations may affect females. Yet, males and females differ in ethanol consumption (Doremus et al., 2005; Vetter-O'Hagen et al., 2009), and opioid research on antinociception have yielded some sex differences (see Rasakham & Liu-Chen, 2011). In one of the few drug studies looking at sex differences in drug responding following activation of the KOR, female rhesus monkeys given bremazocine, a KOR agonist, reduced their responding for phencyclidine to a greater extent than did males (Cosgrove & Carroll, 2004). Therefore, males and females appear to respond differently to manipulation of the KOR in regards to drug-related behaviors, consistent with the results of the present experiments.

The notable sex differences in nor-BNI effects on ethanol consumption seen in the adults in these experiments could be related in part to sex differences in responsiveness to stressful situations. Animals in the present studies were isolate-housed, a housing manipulation known to induce stress and anxiety in rodents (Palanza, 2001), with notable sex differences observed in its effects on anxiety-related behavior (Starkey, Normington, & Bridges, 2007). Isolate-housed female gerbils showed lower levels of active social interaction relative to group-housed females in a social interaction test, whereas isolate-housed males demonstrated higher levels of active interaction relative to group-housed males. Females are not always more affected, however, in that isolate-housed males demonstrated higher levels of anxiety on the elevated plus maze than group-housed males—a housing difference not seen in females (Starkey et al., 2007). Thus, while the data are limited and the findings to date are mixed, there is evidence for notable sex-related differences in the impact of isolate housing on anxiety-related measures. Investigation of sex differences in sensitivity to isolation and other stressors may benefit from study of the KOR system—work of particular importance given that females are at greater risk for most anxiety disorders (McLean & Anderson, 2009).

A common finding in the adult rodent literature is for females to consume significantly greater quantities of ethanol relative to males (Doremus et al., 2005; Vetter-O'Hagen et al., 2009; Yoneyama, Crabbe, Ford, Murillo, & Finn, 2008). Given notable sex differences in anxiety responses (Starkey et al., 2007) and evidence for both ethanol and nor-BNI to reverse stress-induced anxiety-like behaviors in rodents (Bruchas, Land, Lemos, & Chavkin, 2009; Varlinskaya & Spear, 2010), it may be the case that females in the present study consumed more ethanol relative to males for its anxiolytic properties (however, see Lopez, Doremus-Fitzwater, & Becker, 2011; Doremus et al., 2005) in order to counteract the anxiety induced by their housing in isolation. As a consequence, they may have consumed less ethanol following nor-BNI because the drug was effective in relieving their anxiety, and hence ethanol was no longer needed for anxiety reduction. Indeed, anxiolytic effects of nor-BNI have been demonstrated in a number of studies. For example, nor-BNI was shown to increase time spent in open arms on the elevated plus maze in rats (Knoll, Meloni, Thomas, Carroll, & Carlezon, 2007). Similarly, blockade of the KOR with nor-BNI in wild-type mice increased exploratory behavior in the open field and elevated plus maze tests (Wittmann et al., 2009). To the extent that social deprivation is stressful for adult females, they may consume ethanol for relief from anxiety; however, with administration of the anxiolytic compound, nor-BNI, ethanol as an anxiolytic is no longer needed to relieve their negative affective state (thereby resulting in decreases in post-nor-BNI intake).

Given that procedural/environmental variables can impact ethanol consumption in rodents (Doremus et al., 2005), parameters were kept as identical as possible for Experiments 1 (males) and 2 (females), except for the timing of testing within the calendar year. Nevertheless, it is possible that some uncontrolled differences between the two studies could have influenced ethanol intake patterns, and subsequently the effects of nor-BNI on ethanol intake on the test days. It is also possible, as discussed earlier, that differences in baseline ethanol consumption among adult males and females may have contributed to the opposite effects of nor-BNI on ethanol intake on the test days. Even though adolescents of both sexes consumed more ethanol than their adult counterparts, there were sex differences in the

pattern of ethanol consumed among adolescents. For instance, baseline intake patterns in adolescents were opposite in nature, with adolescent males increasing their intake across days whereas adolescent females decreased their consumption by the final baseline day. We have not previously observed a similar sex difference in pair-housed adolescents, or in isolate-housed adolescents given continuous access to ethanol, with adolescent males and females generally displaying similar patterns of ethanol intake across days under these circumstances (Doremus et al., 2005; Vetter-O'Hagen et al., 2009). It is not clear why adolescent females consumed less ethanol on the final baseline day; one possibility is that these young females may have consumed such high levels of ethanol on baseline day 1 that they developed a partial aversion to ethanol and therefore drank less on subsequent days, reflecting their dysphoric state. Regardless, while adults in the current experiments were affected by antagonism of the KOR system, adolescents were not, suggesting that the DYN/KOR system may play less of a role in modulating ethanol intake during adolescence. A decreased sensitivity of the KOR system in adolescent animals could contribute to the higher levels of ethanol consumption observed at this age than at maturity, a finding reported previously (e.g., Doremus et al., 2005) and replicated again on baseline intake days among both males and females in Experiments 1 and 2. It is also possible that attenuated activity of the DYN/KOR system may be associated with the reduced sensitivity of adolescents to ethanol's aversive effects (Vetter-O'Hagen et al., 2009) and possibly also to the accentuated sensitivity reported to the reinforcing effects of ethanol seen during adolescence (Pautassi, Myers, Spear, Molina, & Spear, 2008). Although KORs develop early in ontogeny, being detectable prenatally (Winzer-Serhan, Chen, & Leslie, 2003) and involved in functions related to early postnatal development and suckling behavior (Petrov, Nizhnikov, Varlinskaya, & Spear, 2006), research on levels of KOR expression across ontogeny and through adolescence are lacking.

Although there were no interactions of age with nor-BNI dose when analyzing the BEC data, there was a trend ($p = .07$) for adult males (Exp. 1) to exhibit higher BECs than adolescents, with this age effect reaching significance in females (Exp. 2). Age differences in ethanol metabolism may have contributed to the differences in BECs observed. Although findings are mixed, there is support for younger animals to metabolize ethanol at a faster rate than adults (Brasser & Spear, 2002) following an acute ethanol challenge. Additionally, previous research from our laboratory showed a trend for adolescents (P36) to metabolize an ethanol injection challenge faster than adults (e.g., Silveri & Spear, 2000), with this effect seen only with blood and not brain ethanol levels. It is likely that the observed differences in ethanol consumption patterns among adolescent and adult rats (Dhaher et al., 2012) may have driven the differences in BECs observed. For example, if adolescents consumed ethanol earlier in the session, they would have also peaked earlier and thus would have lower ethanol levels at time of blood collection. Indeed, there are increasing examples of dissociations between intake and subsequent BECs, and evidence linking the importance of intake patterns and timing for influencing post-session BECs (Bell et al., 2006).

Although the present study provides evidence for age-related differences in sensitivity to manipulation of the KOR system, more research is needed to explore the neurobiological basis of this insensitivity. While multiple systems are likely involved, the present data support a potentially important role for ontogenetic changes in the DYN/KOR system in

adolescent-typical insensitivities to the aversive effects of ethanol and possibly other drugs of abuse.

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	Baseline 1		Baseline 2		Baseline 3		Test 1		Test 2		Test 3
Days	1	2	3	4	5	6	7	8	9	10	11
Rehousing & Acclimation	30 min SS+ 10% EtOH		30 min SS+ 10% EtOH		30 min SS+ 10% EtOH	Inj.	30 min SS+ 10% EtOH		30 min SS+ 10% EtOH		30 min SS+ 10% EtOH
		off		off		off		off		off	
P25 – P27	P28	P29	P30	P31	P32	P33	P34	P35	P36	P37	P38
P67 – P69	P70	P71	P72	P73	P74	P75	P76	P77	P78	P79	P80

FIGURE 1.

Pictorial representation of the intake procedure used.

FIGURE 2.

Baseline intake (g/kg) of the EtOH/SS solution in males. Across all baseline days, adolescents consumed more EtOH/SS than adults did (*). EtOH/SS intake was significantly higher on the last baseline day compared to baseline days 1 and 2 (#). $p < .05$.

FIGURE 3.

Percent (%) of baseline intake of EtOH/SS on each test day in adolescent and adult male rats. Adult males given 10 mg/kg nor-BNI exhibited significantly greater ethanol intake on test day 3 relative to saline-treated adult males on that day (+). $p < .05$.

FIGURE 4.

Baseline intake (g/kg) of the EtOH/SS solution in females. Across all baseline days, adolescent females consumed more EtOH/SS than adult females (*). Intake in adult females increased significantly on days 2 and 3, compared to day 1 (#). In adolescent females, an opposite pattern emerged with intake declining to reach significance on the last day relative to day 1 (\$). $p < .05$

FIGURE 5.

Percent (%) of baseline intake of EtOH/SS on each test day in adolescent and adult female rats. Across all test days, adult females significantly reduced their intake after 10 mg/kg nor-BNI (+) with no effects observed in adolescent females. $p < .05$.