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Neuropeptide Y (NPY) -induced reductions in alcohol intake during continuous access and following alcohol deprivation are not altered by restraint stress in alcohol-preferring (P) rats

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ABSTRACT

Administration of neuropeptide Y (NPY) reduces anxiety-like behavior and alcohol intake in alcohol-preferring rats. The present experiment examined whether the effects of NPY on alcohol drinking are modulated by stress exposure during continuous access or following ethanol deprivation. Female P rats underwent 6 weeks of continuous access to 15% v/v ethanol and water prior to intracerebroventricular (ICV) cannula implantation. Deprived rats underwent two cycles of 5 days of ethanol exposure followed by 2 days of ethanol deprivation, while non-deprived rats had uninterrupted access to ethanol. Stressed rats in both ethanol access groups were exposed to restraint stress for 1 h 4–6 h after ethanol was removed from the deprived group in both cycles. ICV infusions of 5.0 μg NPY or aCSF were administered 48 h following the deprivation/stress procedure, after which ethanol was returned. Rats showed increased ethanol intake following ethanol deprivation compared to non-deprived controls. Food and water intake were increased, while ethanol intake was decreased, in rats infused with NPY. Stress did not increase ethanol intake or alter the response to NPY. Although no stress effects were found, the present experiment replicates previous findings regarding the effectiveness of NPY in reducing ethanol consumption. Future studies aimed at determining the extent to which stress may affect relapse to ethanol drinking and response to NPY would benefit from implementing different stress paradigms and varying the pattern of ethanol access.

1. Introduction

The course of alcoholism often follows a pattern of alcohol drinking punctuated by periods of abstinence and relapse. The cyclic nature of alcoholism is particularly detrimental as withdrawal symptoms are enhanced following multiple deprivation periods (Ballenger and Post, 1978; Schuckit et al., 1995) and include augmented levels of both anxiety and craving (Duka et al., 2002; Jasova et al., 2007; Malcolm et al., 2000; Roelofs, 1985). Increases in anxiety and craving are likely to interact with other factors, such as exposure to stress and genetic predisposition to excessive alcohol drinking, and lead to relapse (Volkow and Li, 2004). Since ethanol abstinence represents a particularly sensitive period in the etiology of alcoholism, treatment strategies aimed to reduce the impact of these factors are needed.

Many of the characteristics of relapse can be effectively modeled in animals. Increased ethanol consumption and preference following a period of abstinence is indicative of an alcohol deprivation effect, or ADE (Sinclair and Senter, 1967). In animals, presence of an ADE has been posited to be a model of relapse and implicates craving-like behavior (Heyser et al., 1997; Sinclair & Li, 1989; Sinclair and Senter, 1967; Spanagel and Zieglgänsberger, 1997). Repeated alcohol deprivations have been shown to enhance the ADE in terms of the quantity (Hölter et al., 2000; Rodd-Henricks et al., 2000b; Spanagel and Hölter, 1999) and duration (Rodd et al., 2003; Rodd-Henricks et al., 2000a, Overstreet et al., 2007) of this effect. Analogous to findings from studies using detoxified human alcoholics as subjects, the repeated ADE may be at least partially mediated by the anxiogenic effect of ethanol withdrawal. For example, increased anxiety-like behavior following multiple alcohol deprivations compared to a single deprivation has been shown in both the elevated plus-maze (Hölter et al., 1998) and the social interaction test (Breese et al., 2004; Overstreet et al., 2002, 2005, 2007; Wills et al., 2009). Specifically, outbred rats exposed to ethanol in a liquid diet and alcohol-preferring (P) rats given 6 weeks of continuous ethanol access show elevated anxiety-like behavior 5–6 h after the removal of ethanol (Breese et al., 2004; Kampov-Polevoy et al., 2000; Knapp et al., 1998; Moy et al., 1997, 2000; Overstreet et al., 2002, 2007). Based on these findings, a "kindling"/stress model of alcohol abuse has been set forth by Breese et al. (2005) in which neuroadaptation to chronic intermittent ethanol exposure leads to enhanced stress reactivity and ethanol drinking.

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While alcohol withdrawal can be characterized by the presence of endogenous stress, exposure to exogenous stressors can also lead to elevated ethanol intake (Pohorecky, 1990, 1991; Sinha, 2001). Stress has been shown to be a significant contributing factor to relapse in alcoholics (Brown et al., 1995; Cooper et al., 1992; Dawson et al., 2005; Horton, 1943; Pohorecky, 1991; Sillaber and Henniger, 2004; Sinha, 2001; Volpicelli et al., 1999), but the ability of stress to increase alcohol drinking in animals is not consistent. During the acquisition and maintenance of alcohol drinking, stress has been shown to increase voluntary alcohol intake during or subsequent to stress exposure (Anisman and Waller, 1974; Bond, 1978; Caplan and Puglisi, 1986; Casey, 1960; Chester et al., 2004, 2006, 2008; Cicero et al., 1968; Croft et al., 2005; Kinney and Schmidt, 1979; Matthews et al., 2008; Mills et al., 1977, 1978; Nash and Maickel, 1985, 1988; Ng Cheong Ton et al., 1983; Powell et al., 1966; Rockman et al., 1986, 1987; Volpicelli et al., 1990; von Wright et al., 1971), but also to decrease (Brunell and Spear, 2005; Champagne and Kirouac, 1987; Sprague and Maickel, 1994; Weisinger et al., 1989) or to have no effect (Fidler and LoLordo, 1996) on ethanol drinking. There is also evidence to suggest that while under the acute effects of stress, ethanol drinking is unchanged or even reduced, but post-stress elevations in drinking are seen (Casey, 1960; Chester et al., 2006; Kinney & Schmidt, 1979; Lynch et al., 1999; Mills & Bean, 1978; Nash & Maickel, 1985; Roman et al., 2005; van Erp & Miczek, 2001). Studies examining the interaction between stress and ethanol deprivation have been equally conflicting. For example, when compared to control rats that showed an ADE, rats that received footshock stress failed to demonstrate elevated post-deprivation ethanol intake (Dayas et al., 2004). However, following repeated weekly cycles of ethanol deprivation, rats that underwent restraint, footshock, or social defeat stress showed an augmented ADE compared to unstressed controls (Breese et al., 2004; Funk et al., 2004; Overstreet et al., 2007).

One neuromodulatory system that has been implicated in both the stress and alcohol literature is neuropeptide Y (NPY). NPY been shown to reduce anxiety in several animal models (Heilig et al., 1993; Wettstein et al., 1995; Heilig et al., 1989), and alcohol consumption in selectively bred alcohol-preferring (P) and high alcohol drinking (HAD) rats given free-choice access (Badia-Elder et al., 2001, 2003; Gilpin et al., 2008a, 2005, 2003; Pandey et al., 2005; Zhang et al., 2010) and nonselected rats exposed to ethanol vapor inhalation, liquid diet, or chronic intermittent access (Gilpin et al., 2008a,c; Thorsell et al., 2005a,b). Altered endogenous NPY levels in the limbic areas of the brains of P rats (Ehlers et al., 1998; Hwang et al., 1999; Suzuki et al., 2004) may contribute to the propensity of the P rat to show greater anxiety-like behavior (Pandey et al., 2005; Salimov et al., 1996; Stewart et al., 1993) and also to drink more alcohol than their non-preferring (NP) counterparts. These behavioral characteristics of the P rat are exemplified in studies that show that this line is sensitive to the effects of stress (Breese et al., 2004; Chester et al., 2004; Overstreet et al., 2007; Vengeliene et al., 2003) and alcohol deprivation (Bell et al., 2008; McKinzie et al., 1998; Rodd et al., 2003; Rodd-Henricks et al., 2000b; Sinclair and Li, 1989) on subsequent ethanol intake. Further, the ability of NPY infusion to blunt the ADE in P rats is present after a single (Gilpin et al., 2003) and augmented after repeated (Gilpin et al., 2005) deprivation cycles. However, the effect of NPY on stress-related ethanol intake has yet to be determined.

The aim of the present experiment was twofold. First, it was of interest to determine the extent to which repeated cycles of alcohol deprivation and restraint stress, separately or in combination, contribute to increases in ethanol intake in P rats. It was predicted that, in line with the findings of Breese et al. (2004) and Overstreet et al. (2007), exposure to restraint stress during the deprivation period would augment ethanol intake in P rats to a greater degree than ethanol deprivation alone; e.g., stress would enhance the ADE. However, the present study also included a stress-exposed group of rats maintained on continuous access to ethanol; as such, it was predicted that stress alone would also increase ethanol drinking in P rats. Second, as NPY attenuates anxiety-like behavior (Heilig et al., 1989), ethanol intake (Badia-Elder et al., 2001), and yohimbine-induced reinstatement of ethanol seeking (Cippitelli et al., 2010), it was also of interest to determine whether NPY infusion would attenuate stress- and/or alcohol deprivation-induced increases in ethanol intake. The suppressive effects of NPY on ethanol drinking were predicted to be enhanced following repeated cycles of alcohol deprivation and/or stress perhaps due to global dysregulation of brain NPY systems (e.g., Gilpin et al., 2005).

2. Methods

2.1. Subjects

Subjects were 92 experimentally-naïve adult female P rats (59th–60th generation of selective breeding) aged 12–16 weeks upon arrival and weighing 291.6 (±2.84) g at the beginning of the alcohol deprivation/stress procedure obtained from the Alcohol Research Center, Indiana University School of Medicine. Rats were individually housed in plastic tub-style cages in a vivarium maintained on a reverse 12:12 h light/dark cycle (lights off at 1400 h) with food (Lab Diet 5001, PMI Nutrition International, Inc., Brentwood, MO) and water available ad libitum throughout the experiment. The protocol for the present experiment was approved by the Indiana University-Purdue University Indianapolis School of Science Institutional Animal Care and Use Committee and was conducted in accordance with NIH guidelines (Institute of Laboratory Animal Resources, 1996).

2.2. Chronic ethanol exposure

Rats were given continuous, free-choice access to 15% (v/v) ethanol and water in their home cages for a period of 6 weeks. This length of exposure is sufficient to induce dependence in P rats (Kampov-Polevoy et al., 2000), to elicit an ADE (Gilpin et al., 2003, 2005, 2008b), and to produce anxiety-like behavior after ethanol is removed (Kampov-Polevoy et al., 2000). The position of the ethanol and water bottles was alternated daily in order to control for side preference. Drinking measures taken during the last 6 days of this period were used to determine group assignments in order to match subjects based on ethanol intake and body weight.

2.3. Stereotaxic surgery

Surgical implantation of intracerebroventricular cannulae was conducted using aseptic procedures as described previously (Badia-Elder et al., 2001). Briefly, rats were anesthetized via inhalation of isoflurane (IsoFlo, Abbott laboratories, North Chicago, IL) before and during surgery (3% at 0.8–1.0 L/min). Rats were placed in a Stoelting stereotaxic instrument and a ~2 cm saggital incision was made in the midline, exposing the skull surface. A single hole was drilled through the skull aimed at either the left or right lateral ventricle using coordinates adapted from Paxinos and Watson (1998); from bregma, AP −1.0, ML ±1.5, DV −4.0. A 22-gauge guide cannula (all micro-injection cannulae components, Plastics One Inc, Roanoke, VA) was implanted and anchored using 4 stainless steel screws inserted around the implantation site around which a resin restorative (*Sun-Schein*, Henry Schein Inc, Melville, NY) and cranioplastic cement were applied. Stylets cut to the same length as the guide cannula remained therein at all times except during infusions. Injection cannula (28-gauge) extended 1.0 mm beyond the tip of the guide cannula when inserted. Rats had no access to ethanol for 24 h following surgery and were monitored for 7 recovery days to ensure that normal behaviors, such as mobility, feeding, and drinking were regained. No complications were evident in these animals post-operatively. During this time, sham infusions were performed

in order to habituate the rats to the infusion procedure, which included handling and exposure to the sound of the pump.

2.4. Infusion parameters

NPY (Porcine, American Peptide Company, Sunnyvale, CA) was dissolved in artificial cerebrospinal fluid [aCSF; Plasma-Lyte (Electrolyte) Solution, Baxter, Deerfield, IL]. The NPY dose used (5 μg; equivalent to 1.18 nmol) is the minimal dose that significantly reduced ethanol drinking in P rats in previous studies (Badia-Elder et al., 2001; Gilpin et al., 2005), thus producing a sub-maximal effect that would be modifiable by other factors such as stress and ethanol deprivation. NPY or aCSF was infused in a volume of 5 μl via polyethylene tubing (PE 50) attached to a 25 μl Hamilton syringe. A Harvard 33 microinfusion pump set at a rate of 2.5 μl/min delivered either NPY or aCSF over the course of 2 min, with the injection cannula remaining in the guide cannula for an additional minute to ensure adequate diffusion of the solution. Immediately following infusions rats were placed in clean cages with free access to food, water, and ethanol and returned to the vivarium.

2.5. Procedure

Matched for baseline ethanol (g/kg) intake, rats were assigned to one of four groups: intermittent ethanol access plus stress (INT/STRESS), intermittent access without stress (INT/NO STRESS), continuous ethanol access plus stress (CONT/STRESS), and continuous access without stress (CONT/NO STRESS). Each group was further divided into the NPY and the aCSF treatment groups. Following the 6-week chronic drinking period, all rats underwent ICV cannulation surgery. After recovery from surgery, rats in the intermittent access groups were exposed to 5 days of ethanol exposure and 2 days of ethanol deprivation per week for 3 weeks. For these animals, ethanol was removed at the beginning of the dark cycle (1400 h). Rats in the continuous access groups were treated in an identical manner except that ethanol was never removed. Between 1800–2000 h (i.e., 4–6 h after ethanol was removed in the intermittent access groups), rats in the stress groups were exposed to restraint stress for 1 h. Restraints were plastic tubes 22.3 cm in length and 6.4 cm in diameter with a nose hole in one end and an adjustable plastic ring at the other end to secure the rat in place. Infusions occurred 48 h after ethanol was removed from rats in the intermittent access group, and ethanol was replaced immediately following infusion (Fig. 1). Food, ethanol, and water consumption was measured at 2 and 24 h post-infusion.

2.6. Behavioral verification of cannula patency

Behavioral verification of cannula patency was used for inclusion criteria in the data analysis. Since 5 μg NPY has been shown to robustly increase food consumption 2 h following infusion (Levine & Morley, 1984), the criterion for NPY-infused rats was set at 1 standard deviation above the mean of food intake in aCSF-infused rats at this timepoint. That is, a rat infused with NPY must have food intake that exceeded 3 g/food/2 h (see Table 1). Use of this behavioral verification resulted in the exclusion of 4 rats from the analysis.

2.7. Data analysis

Food intake (g), water intake (ml), ethanol intake (EtOH g/body weight kg), and ethanol preference (EtOH g/total fluid intake g) measures taken at 2 and 24 h post-infusion were subjected to separate four- or three-way mixed factorial analyses of variance (ANOVAs) as described below. Bonferroni post hoc analyses were performed where appropriate. In all cases, the significance level was set at $p<0.05$.

3. Results

A total of 68 rats were included in the analyses, and the numbers in each experimental group were as follows: INT/STRESS/aCSF, n = 11; INT/STRESS/NPY, n = 8; INT/NO STRESS/aCSF, n = 9; INT/NO STRESS/NPY, n = 7; CONT/STRESS/aCSF, n = 10; CONT/STRESS/NPY, n = 6; CONT/NO STRESS/aCSF, n = 7; CONT/NO STRESS/NPY, n = 10. As several animals were sacrificed due to headcap loss by the end of the experiment and were thus unable to provide data, only the first two infusion cycles were included in the data analysis. Data in tables and graphs are presented as mean ± standard error of the mean.

3.1. Effects of stress on consummatory behaviors

Four-way mixed factorial ANOVAs with stress, ethanol access pattern, and NPY dose as between-subjects factors and infusion cycle as the within-subjects factor revealed no significant main effects of nor interactions involving stress for any measure at either 2- or 24-hour post-infusion (Fig. 2). More specifically, stress did not alter ethanol, food, or water consumption during continuous ethanol access or following ethanol deprivation. In addition, stress did not alter the effects of NPY on consumption. Further, analysis of ethanol drinking during the two days between stress administration and infusion in rats given continuous access to alcohol failed to detect acute effects of restraint stress. Since there were no effects or interactions involving stress, data were collapsed across the levels of stress and subsequent analyses were performed with three-way (ethanol access pattern: continuous vs. intermittent; NPY dose: aCSF vs. 5 μg NPY; and infusion cycle: baseline, cycle 1, cycle 2) mixed factorial ANOVAs (n = 16–19 per group).

3.2. Effects of NPY, ethanol access pattern, and infusion cycle on ethanol intake and preference

3.2.1. 2 h post-infusion

NPY significantly reduced ethanol intake (g/kg) [$F(1,64) = 9.274$, $p = 0.003$)] (Fig. 3a). No significant interactions between NPY and

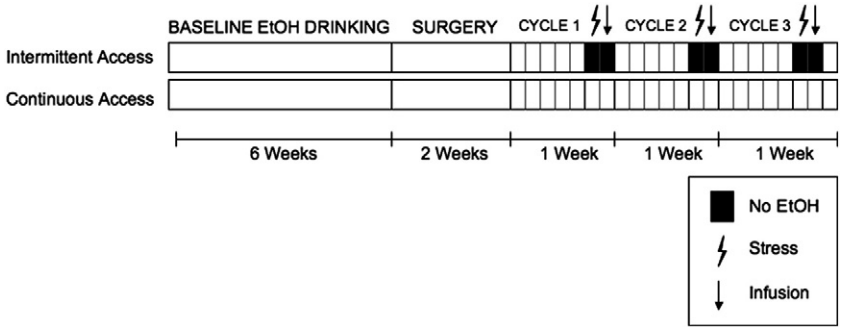

Fig. 1. Timeline for experimental procedures.

Table 1
Mean (SEM) water intake (ml), ethanol preference (E/T), and food intake (g) in rats undergoing intermittent (INT) or continuous (CONT) ethanol access. Rats were infused with either aCSF or NPY during 2 cycles. Intakes were measured at 2- and 24-hours post-infusion.

	Baseline	Cycle 1	Cycle 2
Water intake (ml)			
2 h post-infusion			
CONT/aCSF		4.59(0.58)	4.17(0.59)
CONT/NPY		10.73(0.68)*	8.58(0.82)*
INT/aCSF		2.07(0.29)	1.82(0.31)
INT/NPY		9.11(1.5)*	9.95(2.19)*
24 h post-infusion			
CONT/aCSF	21.37(2.04)	18.91(1.53)	15.23(1.65)#
CONT/NPY	23.96(1.51)	23.71(1.86)*	19.58(2.42)*
INT/aCSF	19.95(0.97)	15.75(1.51)	13.96(1.06)#
INT/NPY	22.80(1.58)	25.35(4.08)*	24.75(3.11)*
Ethanol preference (E/T)			
2 h post-infusion			
CONT/aCSF		0.40(0.04)	0.42(0.05)
CONT/NPY		0.17(0.02)*	0.19(0.02)*
INT/aCSF		0.67(0.04)$	0.71(0.05)$
INT/NPY		0.22(0.03)*, $	0.26(0.07)*, $
24 h post-infusion			
CONT/aCSF	0.32(0.05)	0.32(0.04)	0.37(0.03)
CONT/NPY	0.28(0.04)	0.21(0.04)*	0.21(0.02)*
INT/aCSF	0.35(0.03)	0.51(0.04)$	0.55(0.03)$
INT/NPY	0.30(0.03)	0.28(0.04)*, $	0.36(0.07)*, $
Food intake (g)			
2 h post-infusion			
CONT/aCSF		2.65(0.24)	2.05(0.21)
CONT/NPY		7.53(0.75)*	7.24(0.52)*
INT/aCSF		1.69(0.32)	2.03(0.33)
INT/NPY		6.54(0.79)*	6.80(1.07)*
24 h post-infusion			
CONT/aCSF		13.33(0.67)	11.74(1.33)
CONT/NPY		14.43(1.35)	12.36(1.60)
INT/aCSF		13.36(0.52)	13.05(0.61)
INT/NPY		16.28(1.02)*, $	15.57(0.63)*, $

* = $p<0.05$ vs. aCSF.
\# = $p<0.05$ vs. baseline.
$ = $p<0.05$ vs. CONT.

ethanol access pattern or infusion cycle on ethanol intake were found, nor was an ADE present at this timepoint. Significant main effects of NPY dose [$F(1,64)=124.283$, $p<0.001$] and ethanol access pattern [$F(1,64)=30.03$, $p<0.001$] on ethanol preference were found (Fig. 3a; Table 1). Pairwise comparisons indicated that NPY reduced preference compared to aCSF and that deprived rats had increased preference compared to those given continuous ethanol access. A significant NPY dose by ethanol access pattern interaction [$F(1,64)=13.76$, $p<0.001$] demonstrated that the suppressive effects of NPY on ethanol preference were more pronounced in rats given intermittent access to ethanol.

3.2.2. 24 h post-infusion

NPY continued to reduce ethanol intake [$F(1,64)=21.275$, $p<0.001$, Fig. 3b] and preference [$F(1,64)=19.368$, $p<0.001$, Table 1] at 24 h post-infusion. Post hoc oneway ANOVAs revealed that this effect persisted until the third post-infusion day [$F(1,67)>4$, $p<0.033$ for each day] (Fig. 4). A main effect of ethanol access pattern was also found for both measures [$F(1,64)=10.555$, $p=0.002$, ethanol intake; $F(1,64)=12.584$, $p<0.001$, ethanol preference], with post hoc analyses showing increased ethanol intake and preference in deprived rats (Fig. 3b; Table 1). Subsequent oneway ANOVAs showed that this effect was present for each of the five ensuing post-infusion days during the second cycle [$F(1,67)>9$, $p<0.013$ for each day] (Fig. 4). Interactions between NPY and cycle for ethanol intake [$F(2,128)=8.541$, $p<0.001$] and preference [$F(2,128)=7.746$, $p=0.001$] indicated that NPY, but not aCSF, decreased ethanol intake during cycles 1 and 2 compared to

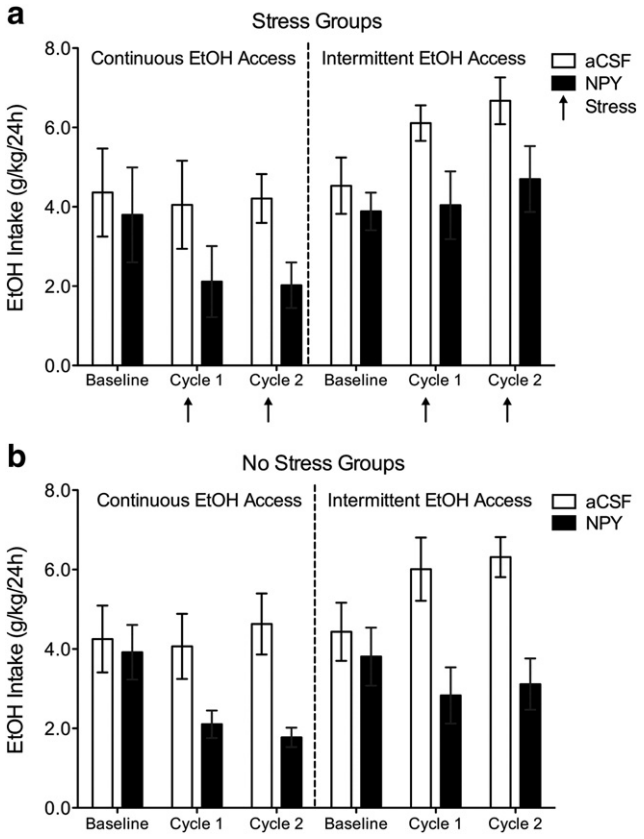

Fig. 2. Ethanol intake in stressed (a) and unstressed (b) rats 24-hours post-infusion. Stress failed to alter ethanol intake regardless of ethanol access pattern or NPY treatment.

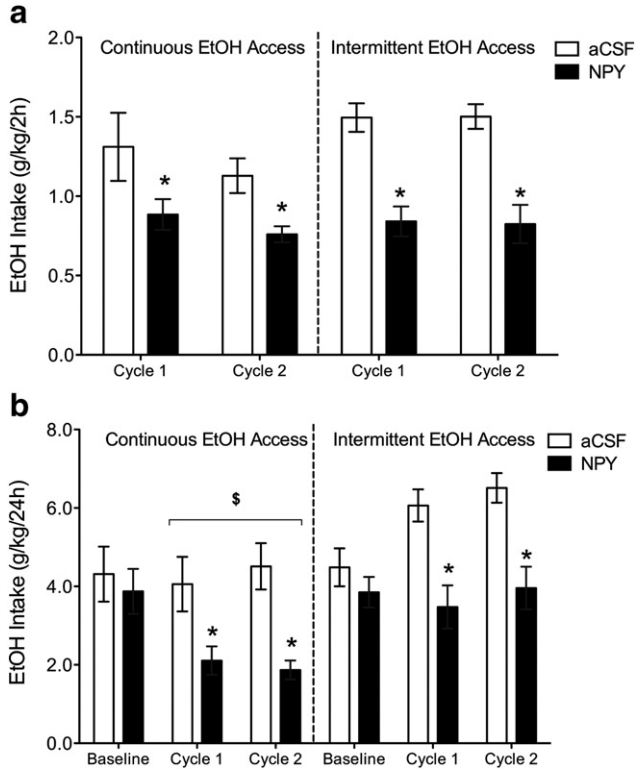

Fig. 3. Ethanol intake at 2 (a) and 24 (b) hours post-infusion. Data were combined across stress and no stress conditions. Significant main effects of NPY were seen for ethanol intake at both timepoints. Intermittent ethanol access led to elevated ethanol intake at 24 h post-infusion. * = $p<0.05$ vs. aCSF; $ = $p<0.05$ vs. intermittent access.

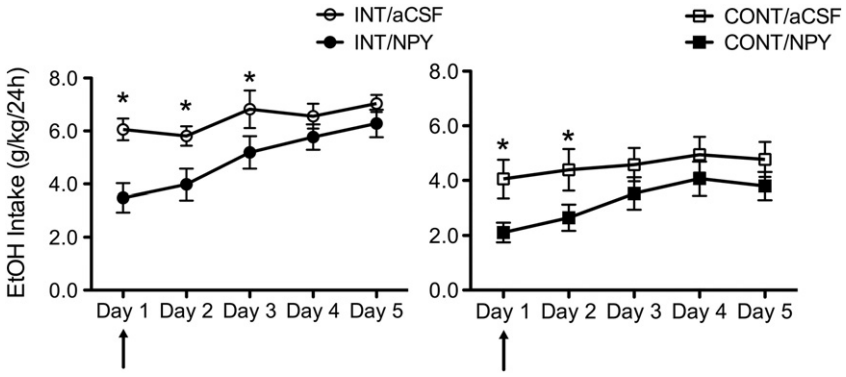

Fig. 4. Daily ethanol intake during cycle 1 of the experiment. Day 1 represents the 24-hour post-infusion/reinstatement measure. Data were combined across stress and no stress conditions. NPY suppressed ethanol intake for 3 days following infusion when ethanol access groups were combined. Ethanol deprivation increased ethanol intake for all 5 post-infusion days when NPY and aCSF groups were combined. * = $p<0.05$ vs. NPY.

baseline. Interactions between ethanol access pattern and cycle for ethanol intake [$F(2,128)=7.687, p<0.001$] and preference [$F(2,128)=7.561$, $p=0.001$] indicated that intermittent, but not continuous ethanol access, increased ethanol drinking during cycles 1 and 2 compared to baseline.

3.3. Effects of NPY, ethanol access pattern, and infusion cycle on food intake

3.3.1. 2 h post-infusion

A significant main effect of NPY dose was found for food intake [$F(1,64)=113.272$, $p<0.001$] with rats infused with NPY consuming significantly more food than rats infused with aCSF (Table 1).

3.3.2. 24 h post-infusion

The orexigenic effects of NPY persisted at 24 h post-infusion [$F(1,64)=6.238$, $p=0.015$, Table 1). In addition, a significant main effect of ethanol access pattern was also found for food intake [$F(1,64)=4.962$, $p=0.029$], with rats having intermittent ethanol access consuming significantly more food than rats with continuous ethanol access.

3.4. Effects of NPY, ethanol access pattern, and infusion cycle on water intake

3.4.1. 2 h post-infusion

A significant main effect of NPY dose was found for water intake [$F(1,64)=57.753$, $p<0.001$], with rats infused with NPY consuming significantly more water than rats infused with aCSF (Table 1). A significant interaction between ethanol access pattern and cycle on water intake was also found [$F(1,64)=4.028$, $p=0.049$], indicating that water intake by deprived rats increased with each cycle, while rats given continuous ethanol access tended to decrease their water intake as a function of cycle.

3.4.2. 24 h post-infusion

NPY continued to increase water intake at 24 h post-infusion [$F(1,64)=14.797$, $p<0.001$, Table 1]. Though no main effects of deprivation were found, a significant main effect of cycle [$F(1,128)=6.251$, $p=0.002$] was seen, with post hoc comparisons showing a general decrease in water intake across cycles. Specifically, water drinking during cycle 2 was significantly lower than drinking during baseline. A cycle by NPY dose interaction [$F(1,128)=3.287$, $p=0.041$] illustrates that NPY blocked cycle-related decreases in water intake evidenced in the aCSF group.

4. Discussion

As predicted, intermittent ethanol access produced an ADE evident at 24 h post-infusion, although an acute (2-hour) deprivation effect was not seen. NPY decreased ethanol intake and preference while increasing water and food intake at both 2 and 24 h following infusion. Food intake was also elevated at 24 h post-infusion in rats given intermittent exposure to ethanol. The effects of NPY and ethanol deprivation on ethanol drinking and preference were augmented with repeated cycles. However, contrary to our hypothesis, there was no effect of stress on ethanol consumption or preference.

Emergence of an ADE in the present study confirms the efficacy of the shortened repeated deprivation protocol of Breese et al. (2004). In P rats, continuous access to ethanol for 5 days followed by a 2-day deprivation period effectively increased ethanol intake and preference, and this effect lasted throughout the subsequent 5-day ethanol access period. Further, the effects of ethanol deprivation on increased ethanol drinking were enhanced with repeated cycles, which is consistent with the hypothesis that multiple abstinence periods lead to neuroadaptation, or "kindling", of systems associated with excessive ethanol drinking (Breese et al., 2005). Increases in ethanol intake as a function of repeated deprivation cycles has been shown consistently in P rats (Breese et al., 2004; Gilpin et al., 2005; Overstreet et al., 2007; Rodd et al., 2003; Rodd-Henricks et al., 2000b). Significant elevations in the breakpoint value elicited by repeatedly deprived P rats (Rodd et al., 2003) indicates that this line exhibits greater motivation to respond for ethanol after multiple periods of abstinence, and that the P rat is a useful model of craving-like behavior. Given the impact cyclic exposure to ethanol has on subsequent drinking, it would be of interest to investigate the effects of a greater number of abstinence periods on ethanol intake, a task facilitated by the shortened deprivation protocol of Breese and colleagues.

Our findings on the role of NPY in reducing ethanol drinking replicates and extends previous studies in P rats (Badia-Elder et al., 2001; Gilpin et al., 2003, 2005, 2008a,b,c). This effect is apparent even when food is not available (Badia-Elder et al., 2001, 2003) and therefore does not appear to be a compensatory mechanism for increased food consumption. Rather, it has been suggested that NPY reduces drinking through pathways associated with anxiety (Pandey et al., 2005). Supportive of this idea is the finding that NPY levels are reduced following one hour of restraint stress in outbred rats (Thorsell et al., 1998) and basally in the P rat amygdala (Ehlers, 1998; Hwang et al., 1999; Suzuki et al., 2004), a brain structure that is highly implicated in the anxiolytic effects of NPY (Heilig et al., 1993; Heilig and Widerlöv, 1995; Primeaux et al., 2005, 2006; Sajdyk et al., 2002, 2006, 2008; Thorsell et al., 2007). In addition, abstinence from ethanol causes significant decreases in NPY protein and mRNA in the CeA (Roy

and Pandey, 2002; Zhang and Pandey, 2003) and may contribute to the stress effects of ethanol withdrawal. Not surprisingly, an interactive effect between ethanol deprivation and NPY administration on ethanol intake has been shown in several studies (Gilpin et al., 2003, 2005, 2008a,b,c). While no such interaction was found to be significant in the present study, the duration of the abstinence period in the Gilpin et al. (2003, 2005, 2008b,c) studies was two weeks, and this extended length of deprivation could have led to a more pronounced effect. Nonetheless, the ability of NPY to suppress drinking in the present experiment was enhanced with successive cycles, which indicates that either repeated NPY infusion or repeated ethanol deprivation could contribute to this effect. As such, future studies that explicitly examine changes in NPY peptide and receptor levels as a function of concurrent ethanol deprivation and stress exposure will help determine whether deprivation-induced sensitization of the effects of NPY on consummatory behaviors occurs.

Perhaps the most surprising finding in the present experiment was that exposure to restraint stress failed to alter ethanol consumption and preference alone or in interaction with alcohol deprivation or NPY. First, no effects of stress were found in rats given continuous ethanol access during the two intervening days between stress and infusion, indicating that there were no acute stress effects on ethanol drinking. Further, despite using similar procedures, we were unable to replicate the results of the Breese et al. (2004) and Overstreet et al. (2007) studies that showed the effects of ethanol deprivation and stress to be additive in P rats. Given these findings, the lack of an interaction between stress and ethanol deprivation in the present investigation was unexpected. The application of restraint stress at approximately 5 h into ethanol deprivation, a time when post-deprivation anxiety is evident (Breese et al., 2004; Kampov-Polevoy et al., 2000; Knapp et al., 1998; Moy et al., 1997, 2000; Overstreet et al., 2002, 2007), was intended to maximize the stress effect. Similarly, an interaction between stress and NPY has been demonstrated in a recent study that showed NPY to suppress yohimbine-induced reinstatement of alcohol seeking in Wistar rats (Cippitelli et al., 2010), which indicates that NPY might similarly block the effects of restraint stress on alcohol drinking and/or the alcohol deprivation effect. It is possible that administration of NPY at a time more proximal to the stress exposure, as is done in the reinstatement procedure, would be more effective in blocking anxiety-related increases in ethanol drinking. However, as no main effect of stress was found in our investigation, the absence of an interaction between stress and NPY is more likely due to an ineffective stress procedure rather than an incongruity with the findings of the Cippitelli et al. (2010) study.

Despite substantial evidence to support a relationship between stress and alcohol drinking, null effects of stress have been previously reported (Fidler & Lolordo, 1996; Myers & Holman, 1967). Several factors may have contributed to the lack of an effect in the present study. First, it is possible that the extensive handling and mild restraint involved in the mock infusion procedure designed to habituate the rats to the infusion procedure inadvertently habituated the animals to the effects of restraint. However, the restraint associated with the infusion procedure was mild and lasted only 5 min, while the restraint associated with the stress procedure was more severe and lasted 1 h, demonstrating both qualitative and quantitative differences in the restraint experience. Alternatively, the rats could have habituated to the restraint procedure itself following repeated exposures. The likelihood of this is low, however, since previous research suggests that the effects of stress are more pronounced with subsequent cycles (Overstreet et al., 2007). Further, if habituation was problematic in the present study, a stress effect on drinking may have appeared during the first, but not subsequent, cycles; this was not what was found. Rather, when considering that stress effects on drinking were most prominent during the third stress cycle in the Overstreet et al. (2007) study, it is possible that the two cycles included in the present experiment were not sufficient to reveal a main effect of stress. In addition, the use of smaller female rats may have lessened the stressful impact of the restraint tubes. If this is the case, future studies that involve microinjections that necessitate such intense handling might benefit from the use of other stressors, such as footshock or injection of the pharmacological stressor, yohimbine. Finally, since a substrain of inbred P rats was used in the Breese et al. (2004) study, it is possible that these rats were more sensitive to stress than outbred P rats due to genetic factors.

In addition to a smaller body size, hormonal differences between the female rats used in the present study and the males used in the Breese et al. (2004) study are likely to have contributed to the lack of replication. While we used females to maintain consistency with previous research on the effects of NPY on deprivation-induced alcohol consumption (Gilpin et al., 2003, 2005), sex differences in the responsiveness to stress have been well documented. However, the directionality of this effect varies, with some studies indicating blunted (Duncko et al., 2001; Laviola et al., 2002; Mashoodh et al., 2008; Weiss et al., 2004), enhanced (Dalla et al., 2005; Iwasaki-Sekino et al., 2009; Weinstock et al., 1998; Wilson and Biscardi, 1994), or equivocal (Conrad et al., 2004) glucocorticoid responses to stress in female rats. Specifically, restraint stress typically leads to increased corticosterone levels in female rats compared to males (Aloisi et al., 1994; Chadda and Devaud, 2005; Doremus-Fitzwater et al., 2009; Kant et al., 1983). Females also show sensitization to the behavioral and hormonal response to repeated restraint stress, whereas males tend to show habituation (Khurana and Devaud, 2007; Dallman, 2007; Kennett et al., 1986; Haleem et al., 1988). When one takes these findings into consideration, it is even more surprising that repeated restraint stress failed to alter consummatory behaviors. However, the rats in the present study were exposed to ethanol for six weeks prior to experimental manipulations. While acute ethanol exposure leads to HPA activation to a greater extent in females than in males (Ogilvie et al., 1997; Rivier, 1993), following 6 months of chronic alcohol exposure, neither male nor female rats showed elevated corticosterone levels compared to ethanol-naïve rats, a result which indicates habituation of the HPA axis in response to alcohol (Silva et al., 2009). Further, in ovarectomized female rats, exposure to ethanol in a liquid diet led to a blunted ACTH response to mild footshock (Lee and Rivier, 1993). While the females in this study were not intact, in contrast to the females in the present experiment, the evidence still suggests that habituation to the stress effects of alcohol led to a blunted stress effect to subsequent restraint stress. The period of chronic ethanol exposure in the present experiment represents a key procedural difference from the Breese et al. (2004) study and could account for the discrepant findings on the effects of restraint on alcohol drinking. Given these caveats, it is critical to assess the efficacy of the stress procedure by a secondary means, such as by evaluating anxiety-like behavior (e.g., the social interaction test performed in the Breese et al., 2004 study) or analyzing glucocorticoid levels.

Although blood alcohol concentrations (BACs) were not determined, the rats in the present experiment were drinking amounts of ethanol at both the 2- and the 24-hour time point that are consistent with previous research. For example, peak BACs are reflective of large (>1 g/kg) bouts of drinking exhibited by P rats at the beginning and end of the dark cycle, resulting in BACs between 50–200 mg% (Bell et al., 2006; Murphy et al., 1986). Ethanol intake in female P rats is highly correlated with BACs (Bell et al., 2006), so it is reasonable to assume that rats in the present experiment were achieving the pharmacological effects of ethanol. Given that ethanol-experienced P rats metabolize ethanol at a rate of 9 mmol/kg/hr (~0.415 g/kg/h; Lumeng & Li, 1986), it is likely that BACs in rats deprived of ethanol were near zero at the time of stress application, reducing the possibility that residual anxiolytic effects of ethanol blunted the efficacy of the stress procedure. Of course, this potential does exist in rats given continuous access to ethanol, and could explain the lack of an effect of stress on subsequent ethanol intake. However, since stress

was ineffective in both the deprived and non-deprived groups, it is not likely that the presence of ethanol was responsible for the absent stress effect.

The present study confirms the efficacy of ethanol deprivation to augment, and NPY administration to diminish, ethanol drinking in P rats. However, enhanced effects of NPY in rats deprived of alcohol were not seen with the ethanol access pattern used. While NPY had opposite effects on ethanol and food intake, exposure to ethanol deprivation augmented consumption of both. This is further evidence that the mechanism by which NPY and ethanol deprivation alter consummatory behaviors may not be common. Nonetheless, while the ADE has been characterized as a model of relapse, dependence on alcohol is not necessary for the effect to occur. Using models that specifically target dependence-level ethanol exposure, alterations in the responsiveness to NPY as a function of alcohol deprivation are more pronounced (Gilpin et al., 2008c). In addition, while restraint stress, by itself or in interaction with other factors, failed to alter consummatory behaviors, this was likely due to idiosyncratic alterations in endogenous stress systems as a function of gender, prior exposure to ethanol, or habituation to the restraint as a result of handling. It is also unknown whether or not implicit stress effects of ethanol deprivation were present. Subsequent research that specifically assesses stress reactivity, uses longer periods of ethanol exposure and deprivation, and targets site- and receptor-specific aspects of the NPY system could reveal that NPY plays a modulatory role in the anxiety-related effects of alcohol exposure and withdrawal. Nonetheless, as NPY was able to block the ADE and to reduce ethanol drinking in non-abstinent P rats, the present findings support the potential for NPY receptor ligands in the treatment of alcohol relapse.

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