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Sensitized effects of neuropeptide Y on multiple ingestive behaviors in P rats following ethanol abstinence

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Abstract

Neuropeptide Y (NPY) suppresses ethanol drinking in alcohol-preferring (P) rats, an effect which is augmented following a single ethanol abstinence period. The present experiment tests both ethanol drinking and feeding in P rats following multiple abstinence periods. Female P rats had continuous access to 15% (v/v) ethanol and water for 6 weeks followed by 3 ethanol access cycles of 2 weeks with no ethanol and 2 weeks with ethanol. Following intracerebroventricular cannula implantation during the third period of ethanol abstinence, groups ($n = 12–13$/dose) were infused with NPY (2.5, 5.0, 10.0 μg) or aCSF prior to ethanol reinstatement. Two additional groups ($n = 11–12$/dose) were treated similarly except that ethanol access was uninterrupted, and they were infused with a single NPY dose (10.0 μg) or aCSF. NPY increased food intake in all groups, and this effect was greater following ethanol abstinence. NPY suppressed ethanol intake, and this suppression lasted longer (24 h post-infusion) in rats with a history of ethanol abstinence periods than rats with a history of continuous ethanol access (4 h post-infusion). These results confirm past findings and indicate that global dysregulation of brain NPY systems during ethanol abstinence may render P rats more sensitive to the behavioral effects of NPY.

Keywords: Neuropeptide Y; Alcohol-preferring (P) rats; Ethanol drinking; Feeding; Ethanol abstinence

Alcohol abuse and alcoholism are typically expressed by a progression through stages of increased alcohol drinking, and alcoholics often endure multiple cycles of relapse drinking and abstinence (Finney and Moos, 1991; McMillen, 1997; Nezlek et al., 1994). The allostasis theory of drug addiction postulates that the transition from drug use to drug abuse is characterized by repeated cycles of drug consumption and abstinence (Koob and LeMoal, 1997, 2001; Koob, 2003). This cyclic pattern of chronic consumption, abstinence and relapse leads to alterations in brain reward systems (decreasing the positive reinforcing effects of the drug) and the dysregulation of brain stress systems (providing a basis for the negative reinforcing effects of the drug). During acute withdrawal and protracted abstinence from alcohol, increases in the activity of corticotropin-releasing factor (CRF) systems, coupled with decreases in the activity of neuropeptide Y (NPY) systems, may result in a state of enhanced anxiety and stress that promotes relapse to alcohol drinking (Koob, 2003).

Evidence for a genetic contribution to ethanol consumption comes from studies in which rats are selectively bred for extremes of high and low oral ethanol drinking (see Lumeng et al., 1995 for a review). At Indiana University, selective breeding has produced the alcohol-preferring (P) and -nonpreferring (NP) lines of rats (Lumeng et al., 1977). In satisfaction of the criteria proposed as essential for an animal model of alcoholism (Cicero, 1979; Lester and Freed, 1973), P rats voluntarily consume ethanol for its pharmacological effects and not solely for its taste, smell or caloric properties, work for ethanol through operant responding; and develop tolerance and dependence through free-choice drinking (see Murphy et al., 2002 for a review).

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Following a period of free-choice continuous access to ethanol and a subsequent period of imposed ethanol abstinence, P rats exhibit a temporary increase in ethanol consumption upon reinstatement of ethanol as compared to pre-abstinence baseline levels, a phenomenon termed the alcohol deprivation effect (ADE; McKinzie et al., 1998; Sinclair and Li, 1989). A role for physical dependence has not been established in the context of the ADE model. However, signs of ethanol withdrawal-related behavior have been noted in P rats following periods of chronic ethanol drinking that would be expected to produce an ADE, for example, increases in anxiety as measured by the elevated plus-maze (Kampov-Polevoy et al., 2000) and increases in behavioral excitability as measured by activity in an open field (Waller et al., 1982). The ADE has been extended to a model in which P rats are given multiple periods of ethanol drinking, punctuated by periods of imposed ethanol abstinence (Rodd-Hendricks et al., 2000). This approximates typical drinking patterns in human alcoholics (Sinclair, 1987). Prospective pharmacological treatments have been tested with variations of this model to determine their efficacy in preventing relapse to ethanol drinking (Heyser et al., 1998; Spanagel et al., 1996; Spanagel and Hölter, 2000). Due to recent findings concerning the relationship between NPY and ethanol drinking (Thiele and Badia-Elder, 2003), it is of interest to examine the effects of NPY on ethanol drinking within this model.

Neuropeptide Y (NPY) is widely distributed in the mammalian central nervous system (Gray and Morley, 1986; DeQuidt and Emson, 1986; Miyazaki and Funakoshi, 1988; Heilig and Widerlöv, 1995), and is involved in the regulation of anxiety-like (Heilig et al., 1993; Sajdyk et al., 2002) and feeding behaviors (Clark et al., 1984; Jolicoeur et al., 1991; Zarjevski et al., 1993). Studies involving genetically altered rodents have suggested a role for NPY in ethanol drinking behavior. NPY knock-out (KO) mice consume significantly more ethanol than wild-type mice, whereas transgenic mice which overexpress (OX) NPY consume less ethanol than wild-type mice (Thiele et al., 1998). Intracerebroventricular (ICV) administration of NPY decreases ethanol intake in P rats with limited (2 h/day) access to ethanol (Badia-Elder et al., 2001). A more recent study examined the effects of NPY on ethanol intake in a free-choice continuous access procedure with and without a period of imposed ethanol abstinence and ethanol reinstatement (Gilpin et al., 2003a). Following 6 weeks of continuous access to ethanol, and 2 weeks of imposed ethanol abstinence, P rats received a single ICV infusion of either aCSF or NPY (10.0 μg) immediately prior to ethanol reinstatement. A second group was treated in a parallel manner except that ethanol was never removed. NPY reduced ethanol intake in both groups but the size and duration of the effect were markedly enhanced in the group that underwent the period of imposed ethanol abstinence. This suggests that the imposition of an abstinence period alters NPY systems such that rats are sensitized to the behavioral effects of NPY.

However, that study had certain limitations that the present investigation seeks to resolve. First, in the Gilpin et al. (2003a) study, groups of rats which did and did not undergo periods of ethanol abstinence were not tested at the same time, thus direct statistical comparisons between the two groups could not be conducted. In addition, the previous study examined the effects of only a single dose of NPY on ethanol drinking. Finally, in the previous study, feeding following NPY infusion was only measured in rats that did not undergo periods of ethanol abstinence. In the current study, all experimental groups were tested concurrently so that the behavioral effects of NPY in rats with and without periods of ethanol abstinence could be compared statistically. Further, the present study examined the behavioral effects of multiple doses of NPY. Feeding behavior was also examined in all experimental groups to determine whether the altered behavioral effectiveness of NPY following periods of ethanol abstinence might extend to other ingestive behaviors known to be affected by the peptide. Thus, P rats with multiple cycles of chronic ethanol exposure and ethanol abstinence were compared to controls with continuous access to ethanol to test the hypothesis that the behavioral effects of NPY are enhanced by a history of ethanol drinking, imposed abstinence and relapse.

1. Methods

1.1. Subjects

Subjects were 95 experimentally naïve female P rats (bred at the Indiana University School of Medicine) of the 54th generation of selective breeding that weighed between 230–326 g at the end of the initial 6-week baseline drinking period. All rats were individually housed in plastic tub-style cages in a vivarium maintained on a 12:12 h light/dark cycle (lights off at 1400 h). Food (Lab Diet 5001, PMI Nutrition International Inc., Brentwood, MO) and water were available ad libitum at all times. The protocol for this study was approved by the IUPUI School of Science IACUC and was conducted in accordance with NIH guidelines (National Research Council, 1996).

1.2. Stereotaxic surgery

Surgical implantation of intracerebroventricular cannulae was conducted using aseptic procedures as previously described (Badia-Elder et al., 2001), with the exception that rats were anesthetized via inhalation of isoflourane (IsoFlo, Abbott Laboratories, North Chicago, IL) before and during surgery. The stereotaxic coordinates were adjusted to accommodate the smaller female rats used in the present study (AP-1.0, ML±1.5, DV-3.8). At the completion of all experimental manipulations, anatomic localization was

confirmed by infusion of angiotensin II (100 ng in 5 μl aCSF; Human; Sigma, St. Louis, MO). Placement was accurate if rats initiated immediate, sustained (at least 30 s), and uninterrupted water drinking following ICV infusion of angiotensin; those rats which did not meet these criteria were excluded from all data analysis.

1.3. Infusion parameters

A Harvard 33 microinfusion pump was used for all drug infusions at a rate of 2.5 μl/min, and the injection cannula was left in the guide cannula for one additional minute to allow for adequate diffusion of the solution. Infusions were delivered to the cannula via polyethylene tubing (PE 50) that was connected to a Hamilton 25 μl syringe. Rats were immediately placed in a clean plastic cage with 15% (v/v) ethanol, water, and food available ad libitum.

1.4. Procedure

Refer to Fig. 1 for a timeline showing periods of ethanol availability, periods of imposed ethanol abstinence, surgery days, and behavioral testing days. The study had three main components. First, prior to surgery, a baseline of chronic ethanol drinking was established in which rats either had periods of imposed abstinence or had continuous access to ethanol. The second component was a dose-response study to compare the effectiveness of different doses of NPY in reducing ethanol drinking, when administered immediately prior to reinstatement of ethanol availability following a period of imposed ethanol abstinence. The third component was the comparison of the effects of NPY in rats with and without histories of multiple periods of imposed ethanol abstinence.

1.4.1. Baseline period

Rats were given continuous (24 h/day) access to both 15% (v/v) ethanol and water. During this time, the side on which the ethanol bottle was placed was alternated daily. The rats were allowed 6 weeks of free-choice continuous access to ethanol and water, the final 6 days of which entailed both 4-h (1400 to 1800 h) and 24-h (1400 h) measurements that were used as the baseline intakes for each fluid. At this time, rats were assigned to two groups, an abstinence (ABST) group and continuous access (CONT) group, that were matched for ethanol intake and body weight. Twice as many rats were assigned to the ABST group ($n=64$) as were assigned to the CONT ($n=31$) group in anticipation of later subdivision into NPY treatment groups.

1.4.2. ABST group

Following the 6 weeks of ethanol access, rats underwent a 14-day period with no ethanol (abstinence period 1), 14 days with ethanol, 14 more days with no ethanol (abstinence period 2), 14 more days with ethanol, and 14 more days with no ethanol (abstinence period 3). The final 6 days of each period of ethanol availability entailed both 4-h and 24-h measurements that were used as baseline fluid intakes for comparison with fluid intakes upon subsequent reinstatement of ethanol. During the third abstinence period, rats were assigned to 4 NPY dose groups ($n=16$/group) matched for ethanol intake (g/kg/day) during the preceding baseline period. Stereotaxic surgeries were conducted during the first seven days of abstinence period 3. During the last seven days of abstinence period 3, sham infusions (rats treated as if receiving infusion but nothing infused) were implemented daily at 1400 h to acclimate the rats to the infusion procedure. On the fourteenth day, immediately preceding the return of ethanol, rats received ICV infusions of either aCSF [5.0 μl; Plasma-Lyte (Electrolyte) Solution, Baxter, Deerfield, IL] or one of four doses (2.5, 5.0, or 10.0 μg/5.0 μl aCSF) of NPY (Porcine; American Peptide Company, Sunnyvale, CA).

1.4.3. CONT group

Rats were treated in a similar manner as rats in the ABST group except that they did not undergo any periods of imposed ethanol abstinence (with the exception that ethanol was removed for the 24 h following stereotaxic surgery). They were assigned to two NPY groups (15–16/group) matched for ethanol intake (g/kg/day) during the same

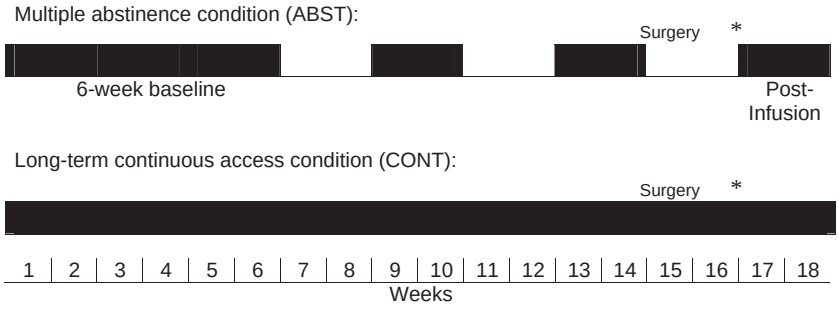

Fig. 1. Timeline of ethanol access schedules (by weeks) for rats which underwent periods of imposed ethanol abstinence (ABST) and rats with uninterrupted continuous access to ethanol (CONT). Segments of the lines which are thicker represent periods during which ethanol was available to the rats of the specified group. Segments of the line which are thinner represent imposed ethanol abstinence periods. Baseline and post-infusion periods are denoted appropriately. The time point at which rats underwent stereotaxic surgeries is also marked. The asterisk (*) denotes NPY infusion day.

baseline period as rats in the ABST group. Rats underwent stereotaxic surgeries and sham infusions in parallel with rats in the ABST group. Rats were then infused with either aCSF (5.0 μl) or NPY (10.0 μg/5.0 μl aCSF) on the same day as rats in the ABST group.

1.4.4. Fluid and food intake

Following each return of ethanol to the ABST group, ethanol and water intakes were recorded at 4 and 24 h. Ethanol, water, and food intakes were recorded at 4 and 24 h on infusion day, and ethanol and water intakes were recorded for thirteen days post-infusion.

1.5. Data analysis

All 95 P rats (ABST group: $n=64$; CONT group: $n=31$) were included in data analysis of pre-surgery drinking measures. Several rats from each NPY treatment group were excluded from infusion day data analysis due to loss of headcap during experimentation, or failure to confirm viability/placement of cannulae during angiotensin testing. A total of 73 P rats were included in the analysis of infusion day data ($n=11-13$/group).

The amount of fluid consumed was determined by weighing the drinking bottles at 1400 h every day, just before the start of the dark cycle, for the 24-h measurement, and at 1800 h for the 4-h measurement. Water intake (ml), ethanol intake (ml and g/kg) and ethanol preference on reinstatement days and infusion day, and food intake on infusion day were analyzed using one-way, two-way, and three-way split-plot repeated measure analyses of variance (RM ANOVA) where appropriate. In all cases, significance was determined at $p<0.05$. Post hoc analyses were conducted using Bonferroni simultaneous test method where appropriate.

2. Results

2.1. Pre-surgery ethanol drinking

2.1.1. Reinstatement of ethanol drinking: 4-h measures

Fig. 2 shows ethanol intake (g/kg) by rats in the ABST and CONT groups that was measured for the first 4 h that ethanol was available to the ABST group following abstinence periods 1 and 2. For each abstinence period, separate two-way RM ANOVA were carried out with drinking history (ABST vs. CONT groups) as a between subject factor and drinking day (baseline vs. reinstatement day) as a within-subjects factor. The analysis for abstinence period 1 indicated that, regardless of drinking history, rats exhibited higher 4 h g/kg ethanol intake on the reinstatement day relative to baseline, $F(1,93)=26.79$, $p<0.001$. The RM ANOVA on 4 h g/kg ethanol intake data from abstinence period 2 yielded a significant effect of drinking day and a significant drinking history × drinking day interaction,

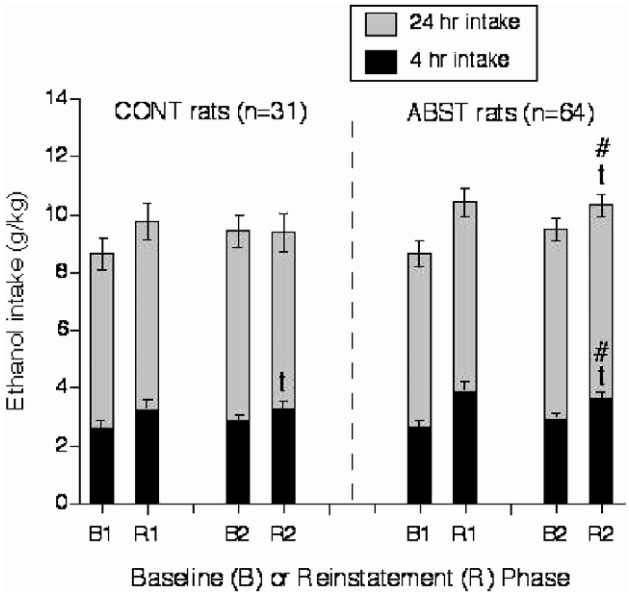

Fig. 2. Mean (±SEM) intake of 15% (v/v) ethanol solution (g ethanol/kg body weight) by P rats in the CONT group (left panel) and the ABST group (right panel). The bars labeled B1 and B2 represent the last six days before the respective period of imposed ethanol abstinence which directly followed. The bars labeled R1 and R2 represent ethanol consumption following the return of ethanol to the ABST group. The black lower portions of the stacked bars represent 4 h ethanol intakes (1400–1800 h). The open upper portions of the stacked bars represent ethanol intakes during the remainder of the 24 h period (i.e. the total height of the stacked bars represent 24 h ethanol intakes). Baseline intakes were calculated as the grand mean of the average consumptions for individual rats during this period. #$p<0.05$ significant difference from CONT rats. $^{t}p<0.05$ significant difference from baseline.

$F(1,93)=39.76$, $p<0.001$ and $F(1,93)=4.21$, $p=0.04$, respectively. Bonferroni post hoc analyses revealed that ABST rats exhibited higher 4 h g/kg ethanol intake on reinstatement day relative to baseline ($p<0.001$), and relative to CONT rats ($p<0.05$). CONT rats also consumed significantly more ethanol (g/kg) during this time period relative to their own baseline ($p<0.05$). A separate series of two-way RM ANOVA yielded similar results with respect to 4 h ethanol ($E:T$) preference (not shown).

2.1.2. Reinstatement of ethanol drinking: 24-h measures

Fig. 2 also shows ethanol intake (g/kg) by rats in the ABST and CONT groups that was measured for the first 24 h that ethanol was available to the ABST group following abstinence periods 1 and 2. A two-way RM ANOVA (drinking history × drinking day) of data from the abstinence period 1 revealed that, regardless of drinking history, rats exhibited higher 24 h g/kg ethanol intake on the reinstatement day relative to baseline, $F(1,93)=20.73$, $p<0.001$. The RM ANOVA of 24 h g/kg ethanol intake data from abstinence period 2 yielded a significant effect of drinking day and a significant drinking history × drink-drinking day interaction, $F(1,93)=5.68$, $p=0.019$ and $F(1,93)=6.37$, $p=0.013$, respectively. Bonferroni post hoc analyses revealed that ABST rats exhibited higher

24 h g/kg ethanol intake on the reinstatement day relative to baseline ($p<0.001$), and relative to CONT rats ($p<0.05$). A separate series of two-way RM ANOVAs yielded similar results with respect to 24 h ethanol ($E:T$) preference (not shown), with the exception that, during abstinence period 2, CONT rats also exhibited significantly higher ethanol preference on the reinstatement day relative to baseline ($p<0.05$).

2.2. Post-NPY infusion fluid intake

Two sets of analyses were performed on infusion day fluid intake data. First, data for rats from only the ABST group were analyzed with two-way RM ANOVA with NPY dose (0.0, 2.5, 5.0, 10.0 μg) as a between subjects factor and drinking day (baseline vs. infusion day) as a within-subjects factor. Second, data from rats in the ABST and CONT group infused with either 10.0 μg or aCSF were analyzed with three-way RM ANOVA with NPY dose (0.0, 10.0 μg) and drinking history (CONT vs. ABST) as between subjects factors and drinking day (baseline vs. infusion day) as a within-subjects factor. For analyses that examined fluid intake further into the post-infusion drinking period, analyses were similar except that the levels of the drinking day factor included post-infusion days 0, 1, 2 and 3, but not baseline data.

2.2.1. 4-h infusion day g/kg ethanol intake

2.2.1.1. ABST dose-response. Fig. 3 shows ethanol intake (g/kg) measured during the 4 h baseline period and 4 h following infusions (first 4 h of ethanol availability following infusion). A two-way RM ANOVA yielded significant effects of NPY dose, $F(3,46)=5.61$, $p=0.002$, drinking day, $F(1,46)=38.84$, $p<0.001$, and a significant dose × day interaction, $F(3,46)=6.49$, $p<0.001$. Bonferroni post hoc analyses revealed that ABST rats which were infused with either 10.0 or 5.0 μg NPY exhibited significantly lower 4 h g/kg ethanol intake on infusion day relative to ABST rats infused with either aCSF or 2.5 μg NPY ($p<0.05$ in all cases), and also relative to their own respective baseline measures ($p<0.001$ in both cases).

2.2.1.2. ABST vs. CONT rats. A three-way (NPY dose × drinking history × drinking day) RM ANOVA of data obtained from rats infused with either aCSF or 10.0 μg NPY revealed a significant effect of NPY dose, $F(1,43)=18.87$, $p<0.001$, and drinking day, $F(1,43)=92.42$, $p<0.001$, and a significant dose × drinking day interaction effect, $F(1,43)=44.30$, $p<0.001$. Bonferroni post hoc analyses revealed that, regardless of drinking history, rats which were infused with 10.0 μg NPY exhibited significantly lower g/kg ethanol intake relative to aCSF controls ($p<0.01$), and relative to baseline measures ($p<0.01$).

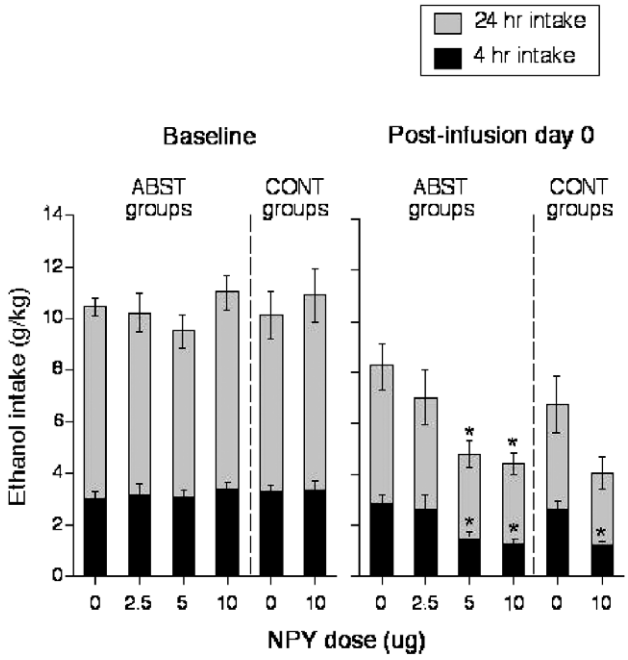

Fig. 3. Mean (±SEM) intake of 15% (v/v) ethanol solution (g ethanol/kg body weight) by P rats in ABST groups and CONT groups during baseline, and on post-infusion day 0 (infusion day). The baseline period represents the last six days before the third period of imposed ethanol abstinence for the ABST groups. Groups are divided according to the NPY dose with which they were infused on post-infusion day 0: ABST groups were infused with 0.0 (aCSF), 2.5, 5.0, or 10.0 μg NPY, and CONT groups were infused with either 0.0 or 10.0 μg NPY. The black lower portions of the stacked bars represent 4 h ethanol intakes (1400–1800 h), while the open upper portions of the stacked bars represent ethanol intakes during the remainder of the 24 h period (i.e. the total height of the stacked bars represent 24 h ethanol intakes). *$p<0.05$ significant difference from aCSF controls.

2.2.2. 4-h infusion day g/kg ethanol intake

2.2.2.1. ABST dose-response. Fig. 3 also shows ethanol intake (g/kg) measured during the 24 h baseline period and 24 h following infusions (first 24 h of ethanol availability following infusion). A two-way RM ANOVA yielded significant effects of NPY dose, $F(3,46)=3.71$, $p=0.018$, and drinking day, $F(1,46)=115.55$, $p<0.001$, and a significant dose × day interaction effect, $F(3,46)=5.87$, $p=0.002$. Bonferroni post hoc analyses revealed that all infused ABST rats (aCSF, 2.5, 5.0, 10.0 μg NPY) consumed significantly less ethanol (g/kg) on infusion day relative to their own respective baseline measures ($p<0.01$ in all cases). Further, rats which were infused with 10.0 μg NPY consumed significantly less ethanol (g/kg) on infusion day relative to rats infused with either aCSF or 2.5 μg NPY ($p<0.05$ in both cases), and rats which were infused with 5.0 μg NPY consumed significantly less ethanol (g/kg) on infusion day relative to aCSF controls ($p<0.01$).

2.2.2.2. ABST vs. CONT rats. A three-way (NPY dose × drinking history × drinking day) RM ANOVA of data obtained from rats infused with either aCSF or 10.0 μg NPY revealed a significant effect of NPY dose,

$F(1,43)=4.49$, $p=0.04$, and drinking day (baseline vs. day 0), $F(1,43)=128.98$, $p<0.001$, and a significant dose × drinking day interaction effect, $F(1,43)=20.02$, $p<0.001$.

2.2.3. 4-day post-infusion g/kg ethanol intake

2.2.3.1. ABST dose-response. Analyses of 4-day post-infusion g/kg ethanol intake data are represented in Fig. 4a by the points labeled post-infusion days 0 through 3. A two-way RM ANOVA (NPY dose × drinking day) yielded a significant effect of drinking day, $F(3,138)=53.47$, $p<0.001$, and a significant NPY dose × day interaction effect, $F(9,138)=2.73$, $p=0.006$. Bonferroni post hoc analyses once again confirmed that rats infused with either 5.0 or 10.0 μg NPY consumed less ethanol (g/kg) on infusion day than rats infused with aCSF ($p<0.05$ in both cases).

2.2.3.2. ABST vs. CONT rats. A three-way (NPY dose × drinking history × drinking day) RM ANOVA revealed a significant effect of drinking day, $F(3,132)=69.75$, $p<0.001$, NPY dose, $F(1,44)=4.49$, $p=0.04$, drinking history, $F(1,44)=4.95$, $p=0.031$, and a significant dose × day interaction effect, $F(3,132)=7.74$, $p<0.001$.

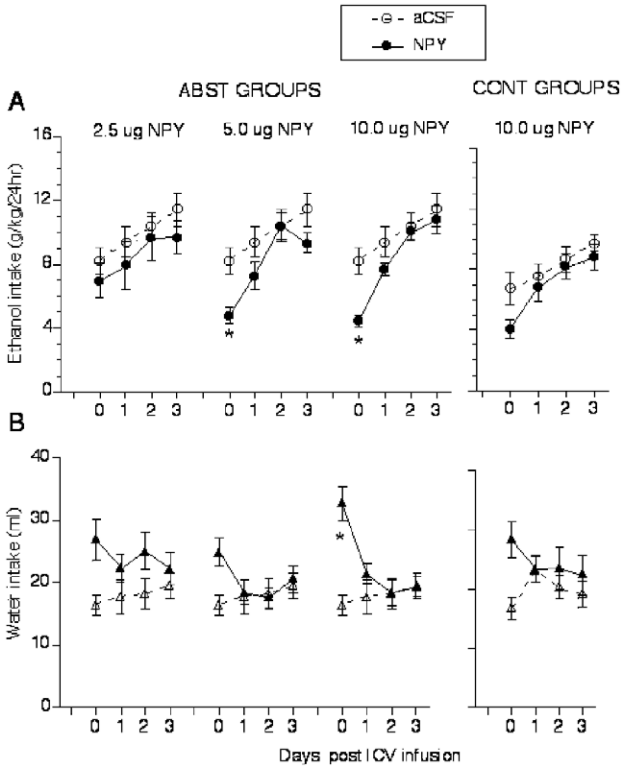

Fig. 4. (a) Mean (±SEM) intake of 15% (v/v) ethanol solution (g ethanol/kg body weight) during infusion day (day 0) and three post-infusion days by P rats in the ABST groups (left panel) following infusion of one of three doses of NPY (closed circles) and P rats in the CONT groups (right panel) infused with 10.0 μg NPY (closed circles) relative to appropriate aCSF controls (open circles). (b) Mean (±SEM) water intake during infusion day (day 0) and three post-infusion days by P rats in the ABST groups (left panel) following infusion of one of three doses of NPY (closed triangles) and P rats in the CONT groups (right panel) infused with 10.0 μg NPY (closed triangles) relative to appropriate aCSF controls (open triangles). *$p<0.05$ significant difference from aCSF.

2.2.3.3. Duration of NPY effect within drinking history groups. An a priori hypothesis of this experiment was that the same dose of NPY would reduce ethanol drinking for a longer duration in rats that had undergone periods of imposed ethanol abstinence relative to those that did not. Since the three-way ANOVA yielded a significant effect of drinking history (see above), separate two-way (NPY dose × drinking day) RM ANOVAs were conducted within each drinking history group in order to determine the duration of the suppressive effects of 10.0 μg NPY on ethanol drinking relative to each group's respective aCSF controls. The analysis of the ABST group's ethanol intake (g/kg) revealed a significant effect of day, $F(3,69)=43.97$, $p<0.001$, and a significant dose × day interaction effect, $F(3,69)=6.07$, $p<0.001$. Bonferroni post hoc analyses indicated that NPY reduced ethanol drinking on infusion day ($p<0.001$) relative to aCSF controls, but not on subsequent days. The analysis of the CONT group's ethanol intake (g/kg) revealed a significant effect of day, $F(3,63)=27.13$, $p<0.001$, but no effect of NPY dose or dose × day interaction effect.

2.2.4. 4-day post-infusion water intake

2.2.4.1. ABST dose-response. Analyses of 4 day post-infusion water intake data are represented in Fig. 4b by the points labeled post-infusion days 0 through 3. A two-way RM ANOVA (NPY dose × drinking day) yielded a significant effect of drinking day, $F(3,138)=8.55$, $p<0.001$, and a significant NPY dose × day interaction effect, $F(9,138)=4.83$, $p<0.001$. Bonferroni post hoc analyses revealed that rats infused with 10.0 μg drank significantly more water than rats infused with aCSF on infusion day only ($p<0.001$).

2.2.4.2. ABST vs. CONT rats. A three-way (NPY dose × drinking history × drinking day) RM ANOVA revealed a significant effect of drinking day, $F(3,132)=4.80$, $p=0.003$, NPY dose, $F(1,44)=6.64$, $p=0.013$, a significant NPY dose × drinking day interaction effect, $F(3,132)=16.79$, $p<0.001$, and a significant drinking history × drinking day interaction effect, $F(3,132)=3.35$, $p=0.021$.

2.3. Post-NPY infusion food intake

2.3.1. 4 h post-infusion food intake

2.3.1.1. ABST dose-response. Fig. 5 illustrates 4 h and 24 h post-NPY infusion food intake in all rats. A one-way (NPY dose) ANOVA of data obtained from ABST rats yielded an NPY dose-dependent increase, $F(3,46)=25.17$, $p<0.001$, in food intake. Bonferroni post hoc analyses

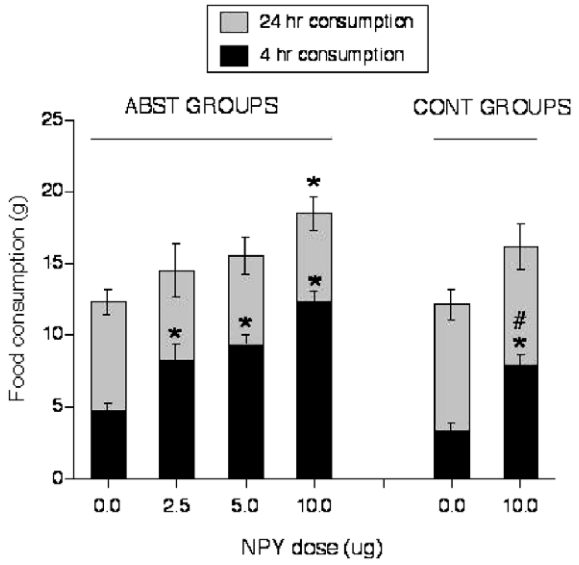

Fig. 5. Mean (±SEM) food consumption by rats in the ABST (left panel) and CONT (right panel) groups on infusion day. Groups are divided according to the NPY dose with which they were infused: ABST groups were infused with 0.0 (aCSF), 2.5, 5.0, or 10.0 μg NPY, and CONT groups were infused with either 0.0 or 10.0 μg NPY. The black lower portions of the stacked bars represent 4 h food intakes (1400–1800 h). The open upper portions of the stacked bars represent food intakes during the remainder of the 24 h period (i.e. the total height of the stacked bars represent 24 h food intakes). *$p<0.01$ significant difference from appropriate aCSF control group. #$p<0.01$ significant difference between ABST and CONT groups infused with 10.0 μg NPY.

revealed that ABST rats infused with any dose of NPY (2.5, 5.0, 10.0 μg) consumed significantly more food 4 h post-infusion than aCSF controls ($p<0.01$ in all cases). Further, ABST rats infused with 10.0 μg NPY consumed significantly more food than rats infused with either 5.0 or 2.5 μg NPY ($p<0.01$ in both cases).

2.3.1.2. ABST vs. CONT rats. A two-way (NPY dose × drinking history) ANOVA of data obtained from rats infused with either aCSF or 10.0 μg NPY yielded significant effects of NPY dose, $F(1,44)=144.97$, $p<0.001$, and drinking history, $F(1,44)=32.53$, $p<0.001$, and a significant dose × history interaction effect, $F(1,44)=9.62$, $p=0.003$. Bonferroni post hoc analyses revealed that ABST rats infused with 10.0 μg NPY consumed significantly more food than CONT rats infused with 10.0 μg NPY ($p<0.01$). Also, CONT rats infused with 10.0 μg NPY consumed significantly more food than aCSF controls ($p<0.001$).

2.3.2. 24 h post-infusion food intake

2.3.2.1. ABST dose-response. A one-way ANOVA of data obtained from ABST rats yielded a significant effect of NPY, $F(3,46)=4.18$, $p=0.011$, on food intake. Bonferroni post hoc analyses revealed that ABST rats infused with 10.0 μg NPY consumed significantly more food than aCSF controls ($p<0.01$).

2.3.2.2. ABST vs. CONT rats. A two-way (NPY dose × drinking history) ANOVA of data obtained from rats infused with either aCSF or 10.0 μg NPY yielded a significant effect of NPY dose, $F(1,44)=22.42$, $p<0.001$.

3. Discussion

In the present investigation, P rats were given either uninterrupted long-term continuous access to ethanol (CONT) or long-term continuous ethanol access interrupted by intermittent two-week periods of imposed ethanol abstinence (ABST). Ethanol intake and ethanol preference consistently increased during the pre-surgery drinking phase of the experiment. Rats with repeated cycles of ethanol access and imposed ethanol abstinence exhibited transient increases in ethanol drinking over baseline upon reinstatement of ethanol access, indicative of an alcohol deprivation effect, and this effect was more pronounced following the second abstinence cycle compared to the first.

Directly prior to reinstatement of ethanol, following the third period of imposed ethanol abstinence for the ABST group, rats were infused ICV with NPY or aCSF. Rats in the ABST group infused with aCSF exhibited higher ethanol intake than aCSF-infused rats in the CONT group, consistent with the alcohol deprivation effects seen following abstinence periods 1 and 2. In ABST rats, the middle (5.0 μg) and highest (10.0 μg) NPY doses suppressed ethanol drinking and preference for 24 h relative to baseline and aCSF controls. In CONT rats, NPY (10.0 μg) suppressed ethanol drinking for 4 h and ethanol preference for 24 h relative to aCSF controls. It should be noted that absolute levels of ethanol intake following infusion of the highest NPY dose were similar in the ABST and CONT groups, but the magnitude of the suppression was greater in the ABST group relative to the CONT group due to different levels of ethanol intake by respective aCSF controls. These results replicate past findings that the suppressive effects of NPY on ethanol drinking are of increased magnitude and duration in P rats with a history of ethanol abstinence relative to rats that have experienced uninterrupted access to ethanol (Gilpin et al., 2003a). In that study, the suppressive effects of NPY on ethanol intake lasted 72 h in P rats that had undergone a single abstinence period.

Perhaps the most interesting findings from the present investigation concern the observed changes in food intake following infusion of NPY. Four hours following infusion, NPY dose-dependently increased food intake in ABST rats, and also increased food intake in CONT rats relative to their respective controls. Further, ABST rats infused with the highest dose of NPY consumed significantly more food than CONT rats infused with the same dose. At the 24 h measurement only ABST rats infused with the highest dose still showed significantly higher food intake than controls. These findings indicate that, following multiple periods of

imposed ethanol abstinence, systems which mediate not only ethanol drinking, but also feeding, become sensitized to the behavioral effects of NPY.

The increased food intake seen in all NPY-infused rats argues against any malaise- or activity-related cause for the observed reductions in ethanol intake. The enhanced effect of NPY on food intake in ABST rats is interesting for two reasons. First, the differential magnitude of the behavioral effects of NPY, which appears to be dependent on whether rats underwent periods of ethanol abstinence, concurrently affects two distinct ingestive behaviors in opposite directions. Second, the sensitized feeding response in ABST rats relative to CONT rats illustrates that the dysregulation of NPY during withdrawal is not limited to systems which mediate ethanol-seeking behavior and may be manifested in a variety of NPY-related behaviors.

Since NPY is a potent orexigenic, it is important to note several pieces of evidence that indicate the NPY-induced suppressive effects on ethanol drinking are not secondary to NPY-induced increases in food intake. For example, the lowest (2.5 μg) NPY dose produced increases in food intake, but did not affect ethanol intake. In some cases, suppression of ethanol intake and ethanol preference by NPY outlasted the effects of NPY on food intake. Also, short-term decreases in ethanol intake are not necessarily correlated with increases in food intake. For example, four hours following infusion the highest dose of NPY caused a more pronounced increase in food intake in ABST rats than in CONT rats, while concurrently producing similar decreases in ethanol intake in both groups. These temporal distinctions between the various behavioral effects of NPY imply that its effects on ethanol drinking behavior are direct effects that are separate from its effects on feeding.

It has been previously shown that, in the absence of food, NPY administered ICV produces a decrease in ethanol intake in P and high alcohol drinking (HAD1) rats, but not in NP, low alcohol drinking (LAD1) or unselected Wistar rats, in a limited access procedure (Badia-Elder et al., 2001, 2003). The same NPY treatment significantly increased sucrose intake in Wistar rats and in HAD1 and LAD1 rats (P and NP rats were not tested), suggesting that different neural mechanisms mediate the appetite-stimulating and ethanol-suppressing effects of NPY. In support of this notion, NPY infused directly into the paraventricular nucleus of the hypothalamus increases ethanol intake in HAD1 and LAD1 rats (Gilpin et al., 2004) and in unselected rats (Kelley et al., 2001). This indicates that NPY, when infused directly into the brain structure known to mediate the orexigenic effects of the peptide, does not cause a secondary decrease in ethanol drinking, and further suggests that the suppressive effects of ICV NPY on ethanol drinking are not mediated by the PVN. Thus far, attempts at localizing the effects of NPY on ethanol consumption to a discrete neuroanatomical structure have presented conflicting and/or negative results (Gilpin et al., 2003b, 2004; Katner et al., 2002a,b; Kelley et al., 2001; Thorsell et al., 2003).

A potential mechanism for augmented behavioral responses to exogenously administered NPY following ethanol abstinence is Y receptor upregulation due to NPY hypoactivity during abstinence. Decreased protein levels of NPY are observed in several brain regions (e.g., amygdaloid nuclei) during acute ethanol withdrawal (Roy and Pandey, 2002; Bison and Crews, 2003). However, in rats which undergo ethanol withdrawal seizures, sharp increases in hippocampal NPY immunoreactivity are observed 72 h following withdrawal, likely as a neuroprotective response to seizure activity (Bison and Crews, 2003). Indeed, ICV administered NPY reduces the severity of ethanol withdrawal symptoms (Woldbye et al., 2002). It is not yet known what specific effects, if any, protracted ethanol abstinence has on endogenous NPY activity or Y receptor regulation.

Koob and LeMoal (1997) have proposed a model of addiction in which hedonic reward systems may be dysregulated by repeated cycles of drug taking and abstinence. This dysregulation, defined as allostasis in brain reward systems, is characterized by increased drug reward thresholds during dependence and increased likelihood to relapse during periods of abstinence from the drug. These abstinence periods may be further defined by increases in CRF (anxiogenic peptide) activity and reciprocal decreases in NPY (anxiolytic peptide) activity, which might contribute to the motivational basis (i.e. negative affect) for relapse to alcohol drinking during periods of alcohol abstinence (for recent review, see Koob, 2003). This model is consistent with evidence that imposed ethanol abstinence has anxiogenic effects in rats (Hölter et al., 1998), and that microinjections of CRF antagonists administered directly into the CeA reverse these effects (Rassnick et al., 1993). Within this model of allostasis, it is not surprising that exogenous NPY-induced suppression of ethanol intake is amplified following a period without ethanol since NPY might be expected to alleviate the anxiogenic effects of a period of ethanol abstinence.

In conclusion, the present study confirms previous findings that ICV administered NPY suppresses ethanol intake in P rats in a continuous access procedure, and that this suppression is augmented by the inclusion of periods of imposed ethanol abstinence (Gilpin et al., 2003a). This study further shows that the orexigenic effects of NPY are also amplified following periods of imposed ethanol abstinence, indicating global dysregulation of brain NPY systems during ethanol abstinence. The results of this study, taken together with past findings (Badia-Elder et al., 2001, 2003; Gilpin et al., 2004), suggest that NPY exerts direct suppressive effects on ethanol drinking that are not secondary to the appetite-stimulating effects of the peptide. It is possible that NPY hypoactivity, coupled with dysregulation of brain CRF systems, during abstinence might contribute to the motivational basis for relapse to alcohol

drinking (Koob, 2003), and that NPY administration might block relapse by alleviating the anxiogenic effects of ethanol abstinence.

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