Published in final edited form as:

Addict Biol. 2012 March ; 17(2): 338–350. doi:10.1111/j.1369-1600.2011.00336.x.

Neuropeptide Y Signaling Modulates the Expression of Ethanol-Induced Behavioral Sensitization in Mice

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Abstract

Neuropeptide Y (NPY) and Protein Kinase A (PKA) have been implicated in neurobiological responses to ethanol. We have previously reported that mutant mice lacking normal production of the RII β subunit of PKA (RII β ^{−/−} mice) show enhanced sensitivity to the locomotor stimulant effects of ethanol and increased behavioral sensitization relative to littermate wild-type RII β ^{+/+} mice. We now report that RII β ^{−/−} mice also show increased NPY immunoreactivity in the nucleus accumbens (NAc) core and the ventral striatum relative to RII β ^{+/+} mice. These observations suggest that elevated NPY signaling in the NAc and/or striatum may contribute to the increased sensitivity to ethanol-induced behavioral sensitization that is characteristic of RII β ^{−/−} mice. Consistently, NPY^{−/−} mice failed to display ethanol-induced behavioral sensitization that was evident in littermate NPY^{+/+} mice. To more directly examine the role of NPY in the locomotor stimulant effects of ethanol, we infused a recombinant adeno-associated virus (rAAV) into the region of the NAc core of DBA/2J mice. The rAAV-FIB-NPY₁₃₋₃₆ vector expresses and constitutively secretes the NPY fragment NPY₁₃₋₃₆ (a selective Y₂ receptor agonist) from infected cells *in vivo*. Mice treated with the rAAV-FIB-NPY₁₃₋₃₆ vector exhibited reduced expression of ethanol-induced behavioral sensitization compared to mice treated with a control vector. Taken together, the current data provide the first evidence that NPY signaling in the NAc core and the Y₂ receptor modulate ethanol-induced behavioral sensitization.

Keywords

Ethanol; Neuropeptide Y; Nucleus Accumbens; PKA; Sensitization; Y₂ Receptors

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Authors' Contribution DMH, JRF, FC, JML-C, and MN each contributed to the acquisition of animal data. DMH and TET were responsible for data analyses and were the primary writers of the manuscript. TJM, DJK, GRB, and IC provided critical input to the interpretation of the data. All authors critically reviewed content and approved the final version for publication.

INTRODUCTION

Behavioral sensitization is defined as the long-lasting and progressive enhancement of the locomotor and motivational responses to psychostimulant drugs following repeated administration (Kalivas and Stewart, 1991). Repeated administration of psychostimulants is thought to trigger progressive and persistent neuroadaptations in neural circuitry underlying the reinforcing and motivational properties of drugs, and it is these neuroadaptations that modulate behavioral sensitization (Robinson and Berridge, 2000). As such, the development and expression of behavioral sensitization may reflect an underlying mechanism that increases the risk for developing drug dependence (Robinson and Berridge, 1993).

Some of the neurochemical systems that have been implicated in drug-induced locomotor stimulation and behavioral sensitization involve G-protein-coupled receptors that recruit cAMP-dependent protein kinase A (PKA) signaling, including dopamine (Broadbent et al., 1995; Broadbent et al., 2005; Hamamura et al., 1991; Itzhak and Martin, 1999; Lessov and Phillips, 2003; Mattingly et al., 1994; Palmer et al., 2003), adenosine (Chen et al., 2003), serotonin (Auclair et al., 2004), opioids (Camarini et al., 2000), and more recently corticotropin releasing factor (Fee et al., 2007; Pastor et al., 2008). Thus, PKA signaling may be critical for the neuroplastic changes that are associated with drug-induced behavioral sensitization. Consistent with this view, we recently found that mutant mice lacking normal production of the regulatory RII β subunit of PKA (RII β ^{-/-} mice) showed increased sensitivity to the locomotor stimulant effects of ethanol and behavioral sensitization resulting from repeated ethanol administration (Fee et al., 2006). RII β ^{-/-} mice display increased basal PKA signaling resulting from unregulated activity of the catalytic α subunit in brain regions where RII β is usually expressed, including the nucleus accumbens (NAc) and striatum (Cadd and McKnight, 1989; Czyzyk et al., 2008). As such, increased sensitivity to ethanol-induced behavioral sensitization in RII β ^{-/-} mice may result from enhanced PKA signaling in critical brain regions.

Accumulating evidence suggests that PKA signaling drives the production of neuropeptide Y (NPY). NPY is a 36 amino acid neuromodulator with activity in a number of brain regions (Berglund et al., 2003; Colmer and Wahlestedt, 1993; Gray and Morley, 1986) and which has been implicated in a wide range of biological functions including neurobiological responses to ethanol (Clark et al., 1984; Hansel et al., 2001; Heilig et al., 1993; Pandey et al., 2003a; Thiele and Badia-Elder, 2003; Woldbye et al., 1996). Interestingly, NPY arises from a cAMP-inducible gene which contains a CRE-binding domain and is regulated by the cAMP-responsive element binding protein (CREB) gene transcription factor (Pandey et al., 2004). Consistently, amygdalar infusion of the PKA inhibitor, Rp-cAMPS, decreased the local expression of NPY immunoreactivity in Sprague-Dawley rats (Pandey et al., 2003b; Zhang and Pandey, 2003), and partial deletion of the gene responsible for CREB production in mice decreased NPY levels throughout the brain (Pandey et al., 2004).

Because PKA signaling stimulates NPY synthesis, and because RII β ^{-/-} mice show enhanced ethanol-induced behavioral sensitization (Fee et al., 2006), the first aim of the present investigation was to determine if, relative to RII β ^{+/+} mice, RII β ^{-/-} mice have altered NPY immunoreactivity in brain regions implicated in behavioral sensitization, at baseline and/or in response to ethanol administration. To further investigate the relationship between NPY and behavioral sensitization, we studied the expression of ethanol-induced locomotor sensitization in NPY deficient (NPY^{-/-}) and in normal littermate wildtype control (NPY^{+/+}) mice. Finally, we utilized a recombinant adeno-associated viral (rAAV) vector containing the secretion signal sequence for the laminar protein fibronectin (FIB) and the coding sequence for the selective Y₂ receptor agonist NPY₁₃₋₃₆ (rAAV-FIB-NPY₁₃₋₃₆) in order to manipulate NPY Y₂ receptor signaling in the region of the NAc core. NPY₁₃₋₃₆ is

a Y₂ receptor selective agonist with 136-fold selectivity over the Y₁ receptor (Gerald et al., 1996). Cells infected with this virus exhibit subsequent gene expression leading to constitutive secretion of functionally active NPY₁₃₋₃₆ (Foti et al., 2007; Haberman et al., 2003). Here we found that ethanol-induced behavioral sensitization-prone RIIβ^{-/-} mice exhibited higher levels of NPY immunoreactivity in the NAc core and ventral striatum relative to RIIβ^{+/+} mice, while NPY^{-/-} mice showed blunted expression of ethanol-induced behavioral sensitization relative to NPY^{+/+} mice. Mice that received infusion of the rAAV-FIB-NPY₁₃₋₃₆ vector into the NAc core showed blunted ethanol-induced behavioral sensitization relative to mice treated with a control vector. Together, these results provide novel evidence that NPY signaling in the region of the NAc core and the Y₂ receptor modulate the expression of ethanol-induced behavioral sensitization in mice.

MATERIALS AND METHODS

Animals

RIIβ^{-/-} mice were created through the disruption of the RIIβ gene by homologous recombination in embryonic stem cells from 129/SvJ mice (Brandon et al., 1998). Male RIIβ^{-/-} and RIIβ^{+/+} littermate mice were backcrossed for over eight generations onto a C57BL/6J background and were 3-5 months of age at the initiation of procedures. NPY^{-/-} mice were created through the disruption of the NPY gene by homologous recombination in embryonic stem cells from 129/SvJ mice (Erickson et al., 1996). Male and female littermate NPY^{-/-} and NPY^{+/+} mice were backcrossed to a C57BL/6J background for over eight generations and were 3-8 months of age at the onset of study. While older mice have been shown to develop more robust ethanol-induced behavioral sensitization (Stevenson et al., 2008), age was balanced between groups to avoid an age-effect confound. Male 6-8 week old DBA/2J mice were purchased from Jackson Laboratory (Bar Harbor, ME). DBA/2J mice were housed individually immediately upon arrival to the laboratory and mice from the RIIβ and NPY breeding colonies were individually housed approximately 2 weeks before experimental procedures. Mice lived in polypropylene cages with corn cob bedding (Teklad, Madison, WI) and had *ad libitum* access to standard rodent chow (LabDiet, Brentwood, MO) and water. The colony room was maintained at 22°C with a 12 h:12 h light:dark cycle. All procedures were conducted in compliance with the National Institute of Health guidelines and each protocol was approved by the University of North Carolina Institutional Animal Care and Use Committee (IACUC).

Experiment 1: Assessment of NPY Immunoreactivity in RIIβ^{-/-} and RIIβ^{+/+} mice

Because we have previously shown that RIIβ^{-/-} mice show more robust expression of ethanol-induced behavioral sensitization relative to RIIβ^{+/+} mice and this genotype difference emerges within 5 ethanol injections (Fee et al., 2006), all mice received an intraperitoneal (i.p.) injection of either 2.0 g/kg ethanol (20% w/v in 0.9% NaCl) or equivolume isotonic saline on each of 5 consecutive days. All injections were administered in a volume of 10 ml/kg. Briefly, the control group received 5 separate saline injections (RIIβ^{-/-}, N = 8; RIIβ^{+/+}, N = 8), the acute ethanol group received 4 saline injections followed by a final ethanol injection on Day 5 (RIIβ^{-/-}, N = 8; RIIβ^{+/+}, N = 9), and the repeated ethanol group received 5 ethanol injections (RIIβ^{-/-}, N = 9; RIIβ^{+/+}, N = 8). Animals received injections and were immediately returned to their homecages.

Two hours after the final injection (Ogilvie et al., 1998), animals were anesthetized and perfused transcardially with 0.1 mM phosphate-buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde in buffered saline. Brains were sliced into 40 μm sections using a vibratome. Sections containing the NAc and striatum were collected because these regions have been implicated in ethanol-induced behavioral sensitization (Quadros et al., 2002;

Souza-Formigoni et al., 1999). Slices containing the central nucleus of the amygdala (CeA) and the paraventricular nucleus of the hypothalamus (PVN) were also collected to assess site-specificity of genotype differences in NPY immunoreactivity (IR). NPY IR was accomplished with a rabbit anti-NPY antibody (Peninsula Laboratories, San Carlos, CA; 1:1000), VectaStain rabbit ABC secondary antibody kit (Vector Laboratories, Burlingame, CA) and staining using 3,3'-Diaminobenzidine (DAB) kits (Vector Laboratories, Burlingame, CA) as detailed previously (Hayes et al., 2005). No evidence of staining was seen in slices from control assays lacking either primary or secondary antibody. Slices were then mounted onto microslides and images were visualized using a Nikon Digital Sight DS-U1 camera attached to a Nikon Eclipse E400 microscope (Melville, NY). Image J (NIH, Bethesda, MD) software was used to process background (non-cellular regions or corpus colossum) and signal (cell body or processes) intensities and data were presented as background-corrected standardized image densities for each brain region.

Experiment 2: Assessment of Ethanol-Induced Locomotor Activity and Ethanol-Induced Behavioral Sensitization in NPY^{-/-} and NPY^{+/+} Mice

NPY^{-/-} (male, N = 10; female, N = 10) and NPY^{+/+} (male, N = 10; female, N = 10) mice were transported to the locomotor testing room in their home cages during the light phase of their light:dark cycle and allowed to habituate for 30 minutes prior to testing. A fan provided masking noise in the testing room. For each 10 min locomotor activity trial, mice were removed from their homecages, given an i.p. injection of ethanol or equivolume saline (all injections were given in a volume of 10 ml/kg) and placed into the center of an open-field arena that automatically recorded activity via photobeam breaks (Harvard Apparatus, Inc., Holliston, MA). Injections were given once every other day with one rest day provided between injections.

In order to allow for habituation to testing procedures and to establish a baseline locomotor activity, mice were run through procedures on 3 days immediately after i.p. injection of saline. Over the next 5 trials, mice received i.p. injection of a 1.5 g/kg dose of ethanol just prior to being placed in the locomotor activity chamber (15% w/v in 0.9% NaCl) to measure initial sensitivity to the locomotor stimulant effects of a low dose of ethanol. Two weeks later, mice were exposed to a behavioral sensitization protocol similar to what has been described previously (Fee et al., 2006; Lessov et al., 2001; Phillips et al., 1994). First, mice received 5 trials in which injection of a 2.0 g/kg dose of ethanol was administered prior to placement in the activity chambers (20% w/v in 0.9% NaCl) to establish a baseline of pre-sensitization ethanol-induced locomotor activity in response to the 2.0 g/kg dose of ethanol. Next, mice were given 10 i.p. injections of a 2.5 g/kg dose of ethanol (25% w/v in 0.9% NaCl), one injection per day (mice were given injections and returned to their homecages). Finally, mice were given an i.p. injection of ethanol (2.0 g/kg) or saline just prior to placement into the locomotor activity apparatus on the post-sensitization test trial. For all mice, ethanol or saline were given in a counterbalanced order on separate days with 3 days in between each session.

In a separate set of animals, NPY^{-/-} (male, N = 9; female, N = 8) and NPY^{+/+} (male, N = 9; female, N = 10) mice were given the same regimen of ethanol injections as described above (with respect to the doses and number of injections), except that on the final day of the experiment all mice were given an i.p. injection of a 2.0 g/kg dose of ethanol. Ten minutes later, 10 µl of blood was collected from a small incision made to the tail vein of each mouse. Gentle pressure was applied to the incision with sterile gauze until bleeding stopped. Samples were centrifuged, and 5 µl of serum from each sample was analyzed for blood ethanol concentration (BEC) measured in mg/dL (Analox Instruments, Lunenburg, MA).

Experiment 3: Assessment of ethanol-induced locomotor activity and ethanol-induced behavioral sensitization in DBA/2J mice with synthesis and constitutive secretion of NPY₁₃₋₃₆ in the NAc core

The AAV2 vectors were constructed as previously described (Haberman et al., 2003; McCown, 2006). Specific details on production of the rAAV-FIB-NPY₁₃₋₃₆ construct have recently been published (Foti et al., 2007). The rAAV-FIB-NPY₁₃₋₃₆ construct (3.3×10^{12} viral particles/ml) contains the coding sequence for the Y₂ fragment of NPY and when neurons are infected *in vivo* these cells constitutively secrete the gene product (Foti et al., 2007). Infusion of a viral vector containing the coding sequence for Green Fluorescent Protein (AAV-FIB-GFP; 4.2×10^{12} viral particles/ml) served as the control, which has been shown to not have any effect on behavior (Foti et al., 2007; Haberman et al., 2003). Further, GFP is not endogenous to mammals and has no known neurobiological effects.

Mice were anesthetized with a cocktail of ketamine (117 mg/kg) and xylazine (7.92 mg/kg) and infused bilaterally with either the control virus rAAV-FIB-GFP (N = 19) or the rAAV-FIB-NPY₁₃₋₃₆ virus (N = 20) via a 33 gauge cannula (Plastics One, Roanoke, VA). The injector was aimed at the region of the core of the NAc (1.4 mm anterior to bregma, ± 0.9 mm lateral to midline, and 4.7 mm ventral to skull surface) and delivered 0.5 μ l per side at a rate of 0.1 μ l/min via microsyringe attached to a Harvard Apparatus PHD 2000 microinfusion pump. Animals recovered for 2 weeks which ensured ample time for the transduction and gene expression by the viral vector. The infusion volume and rate were chosen from pilot experiments with a rAAV-GFP vector (see Figure 5). At the end of the experiment, brains from 4 mice treated with the rAAV-FIB-GFP vector and 6 mice treated with the rAAV-FIB-NPY₁₃₋₃₆ vector were removed and the region of the NAc core was dissected out from a slice that was approximately 440 μ m thick (cut between 1.18 and 0.74 mm relative to bregma). The dissected samples were approximately 300 to 350 μ m in diameter and were cut manually. RT-PCR was used to verify the transduction of vector into brain tissue using primers designed to span the FIB-NPY₁₃₋₃₆ sequence which can only be derived from the AAV vector (Foti et al., 2007).

Locomotor sensitization procedures were similar to those described above. Briefly, after collecting baseline measures, mice were given 3 i.p. injections of 2.0 g/kg dose of ethanol (20% w/v in 0.9% NaCl; 10 ml/kg), one per day, just before placement into the locomotor activity chambers to establish pre-sensitization locomotor activity. Next, mice received 10 i.p. injections (one per day) of a 2.5 g/kg dose of ethanol (25% w/v ethanol in 0.9% NaCl; 10 ml/kg) and returned to their homecage. On the post-sensitization test day mice were again given an i.p. injection of a 2.0 g/kg dose of ethanol (20% w/v in 0.9% NaCl; 10 ml/kg) and placed in the locomotor arena for a 10 minute test. On the final day, mice were given an i.p. injection of saline (10 ml/kg) before the 10 minute locomotor activity test.

Data Analysis

All data are represented as mean $\pm$ standard error of the mean (SEM). Data were analyzed using analysis of variance (ANOVA) and Fischer's Least Significant Difference (LSD) tests were performed for post hoc analyses. Paired sample t-tests were used to compare ethanol-induced locomotor activity at pre- and post-sensitization time points to verify behavioral sensitization in raw data sets. Significance was accepted at $p < 0.05$ (two-tailed).

RESULTS

Experiment 1: Assessment of NPY Immunoreactivity in RII β $-/-$ and RII β $+/+$ mice

The RII β $-/-$ mice weighed significantly less than RII β $+/+$ mice at the onset of experimentation (23.72 ± 0.44 g vs. 25.33 ± 0.32 g [$F_{(1,48)} = 8.720$; $p = 0.005$]), which has

been reported previously (Cummings et al., 1996). Representative photomicrographs from slices collected through the NAc of RII β ^{+/+} (Fig. 1A) and RII β ^{-/-} (Fig. 1B) mice, and quantification of NPY IR in the NAc core (Fig. 1C) and shell (Fig. 1D) are depicted in Fig. 1. Relative to RII β ^{+/+} mice, RII β ^{-/-} mice displayed higher NPY IR in the core of the NAc (Fig. 1C). A two-way 2 $\times$ 3 (genotype $\times$ ethanol treatment) ANOVA performed on NPY IR data collected from the NAc core revealed a significant main effect of genotype [$F_{(1,35)} = 6.85$; $p = 0.014$], but the ethanol treatment and interaction effects did not attain statistical significance. Genotype differences in the NAc were specific to the core region as RII β ^{+/+} and RII β ^{-/-} mice did not exhibit significant differences in NPY IR in the shell of the NAc (Fig. 1D), though the trend was in the same direction. A two-way 2 $\times$ 3 (genotype $\times$ ethanol treatment) ANOVA performed on NPY IR data collected from the NAc shell failed to show any significant effects.

Representative photomicrographs of NPY IR in the striatum of RII β ^{+/+} (Fig. 2A) and RII β ^{-/-} (Fig. 2B) mice are shown in Fig. 2, and average NPY IR data are presented in Fig. 3. Separate two-way 2 $\times$ 3 (genotype $\times$ ethanol treatment) ANOVAs were performed on each of the 4 subregions defined within the striatum (see Fig. 2). RII β ^{-/-} mice displayed higher NPY IR in the ventromedial [$F_{(1,35)} = 15.67$; $p < 0.001$] and ventrolateral [$F_{(1,35)} = 8.35$; $p = 0.007$] subregions of the striatum relative to RII β ^{+/+} mice (Figs. 3C and 3D, respectively), indicated by significant main effects of genotype. Differences between genotypes were sub-region specific as there were no significant main effects of genotype in the dorsomedial (Fig. 3A) or dorsolateral striatum (Fig. 3B). Interestingly, there were significant main effects of ethanol treatment in the ventromedial [$F_{(2,35)} = 3.90$; $p = 0.032$] and ventrolateral [$F_{(2,35)} = 4.91$; $p = 0.015$] subregions of the striatum (Figs. 3C and D). Post hoc LSD tests revealed that mice treated with a single injection of a 2.0 g/kg dose of ethanol (acute ethanol) displayed significantly lower NPY IR than saline treated mice in both the ventromedial and ventrolateral subregions of the striatum, regardless of genotype. No other effects were statistically significant from ANOVAs performed on striatum NPY IR data. A summary of NPY immunoreactivity (IR) data from brain regions in which there were no statistically significant effects are depicted in Table 1.

Experiment 2: Assessment of Ethanol-Induced Locomotor Activity and Ethanol-Induced Behavioral Sensitization in NPY^{-/-} and NPY^{+/+} Mice

Mice weighed an average of 24.36 g at the onset of study and there were no significant genotype differences in body weight [$F_{(1,36)} = 2.74$; $p = 0.107$]. A comparison of locomotor responses between NPY^{-/-} and NPY^{+/+} mice (Fig. 4A) and male and female mice (Fig. 4B) are presented in Fig. 4. Because locomotor activity did not differ significantly between NPY^{-/-} and NPY^{+/+} over the days of baseline saline (S) injections [genotype $\times$ days interaction effect results were $F_{(2,66)} = 1.03$; $p = 0.36$] or over days of pre-sensitization exposure to the 1.5 (E1.5) or 2.0 (preE2) g/kg doses of ethanol [genotype $\times$ days interaction effects results were $F_{(4,144)} = 0.80$; $p = 0.53$ and $F_{(4,144)} = 1.81$; $p = 0.23$, respectively], data from each of these phases were averaged to yield a single value per animal at each phase. A three-way 2 $\times$ 2 $\times$ 5 (genotype $\times$ sex $\times$ phase) repeated-measures ANOVA revealed a main effect of phase [$F_{(4,136)} = 14.76$; $p < 0.001$] and sex [$F_{(1,34)} = 4.71$; $p = 0.037$], and significant interaction effects of genotype $\times$ phase [$F_{(4,136)} = 5.68$; $p < 0.001$] and sex $\times$ phase [$F_{(4,136)} = 2.90$; $p = 0.024$]. No other effects achieved statistical significance. Post hoc analysis of the genotype $\times$ phase interaction revealed that NPY^{-/-} mice were less active than NPY^{+/+} mice during post-sensitization exposure to the 2.0 g/kg dose of ethanol (postE2 in Fig. 4A), while a similar analysis of the sex $\times$ phase interaction revealed that female mice exhibited significantly greater locomotor activity during the initial exposure to the 2.0 g/kg dose of ethanol (preE2 in Fig. 4B) and to the post-sensitization exposure to the 2.0 g/kg dose of ethanol (postE2 in Fig. 4B). Paired sample t-tests performed as planned

comparisons indicated that both NPY^{-/-} and NPY^{+/+} mice (Fig. 4A) and male and female mice (Fig. 4B) exhibited ethanol-induced sensitization indicated by a significant increase in locomotor activity from preE2 to postE2.

Fig. 4C depicts locomotor activity in NPY^{-/-} and NPY^{+/+} mice where locomotor activity on the post-sensitization ethanol test day (postE2) and on the day of the final saline injection were defined as the change in activity relative to baseline activity during the initial saline injections (postE2 or Final Saline – initial saline). A three-way $2 \times 2 \times 2$ (drug $\times$ sex $\times$ genotype) repeated-measures ANOVA revealed a significant main effect of drug [$F_{(1,34)} = 30.87$; $p < 0.001$] reflecting the greater activity following post-sensitization injection of the 2.0 g/kg dose of ethanol (postE2) relative to the final saline injection. An examination of the data shows that increased locomotor activity was specific to ethanol injection. Additionally, there was a significant drug $\times$ genotype interaction [$F_{(1,34)} = 14.02$; $p = 0.001$], and post hoc analysis of this interaction revealed that NPY^{-/-} mice displayed reduced activity relative to NPY^{+/+} mice at postE2 but there were no genotype differences during the final saline injection. No other effects achieved statistical significance. Taken together, these data reinforce the observation that NPY^{-/-} mice are less sensitive to the locomotor stimulant effects of ethanol, and that stimulation of locomotor behavior is specific to ethanol injection as increased activity did not generalize to a saline injection.

Data in Fig. 4D depicts locomotor activity in NPY^{-/-} and NPY^{+/+} mice where locomotor activity on the post-sensitization ethanol test day (postE2) and on the day of the final saline injection were defined as the change in activity relative to activity during pre-sensitization exposure to the 2.0 g/kg dose of ethanol (postE2 or Final Saline – preE2). A three-way $2 \times 2 \times 2$ (drug $\times$ sex $\times$ genotype) ANOVA revealed a significant main effect of drug [$F_{(1,34)} = 30.87$; $p < 0.001$] and of a drug $\times$ genotype interaction effect [$F_{(1,34)} = 14.02$; $p = 0.001$]. No other effects achieved statistical significance. Post hoc analysis confirmed that NPY^{-/-} mice displayed a significant attenuation of ethanol-induced locomotor activity relative to NPY^{+/+} mice on the postE2 test day. There were also significant differences between genotypes on the final saline injection with NPY^{+/+} mice showing significantly lower levels of activity relative to NPY^{-/-} mice, though change in activity data for both genotypes did not noticeably increase above zero. These observations show that NPY^{-/-} mice are less sensitive to ethanol-induced sensitization and that sensitized behavior is specific to ethanol injections. Finally, analysis of BEC data showed that NPY^{-/-} (male, 206.43 ± 9.58 mg/dL; female, 220.60 ± 10.16 mg/dL) and NPY^{+/+} (male, 214.91 ± 9.58 mg/dL; female, 216.36 ± 9.09 mg/dL) mice had similar BECs, as a two-way 2×2 (genotype $\times$ sex) ANOVA performed on BEC data failed to show any significant effects.

Experiment 3: Assessment of ethanol-induced locomotor activity and ethanol-induced behavioral sensitization in DBA/2J mice with synthesis and constitutive secretion of NPY₁₃₋₃₆ in the NAc core

Mice weighed an average of 23 g at onset of experimentation. Fig. 5 depicts a slice through the NAc core from a mouse that was injected with a rAAV-GFP vector using the coordinates and parameters described in the methods section. GFP expression was restricted to the core region of the NAc. Importantly, the expression of GFP also confirmed the transduction of the rAAV vector in mouse brain tissue 2 weeks after injection. Fig. 6A shows the locomotor responses of DBA/2J mice infused with either the rAAV-FIB-GFP or rAAV-FIB-NPY₁₃₋₃₆ vectors into the NAc core. A two-way 2×2 (phase $\times$ viral treatment) repeated-measures ANOVA revealed a significant main effect of phase [$F_{(3,99)} = 37.12$; $p < 0.001$] and a significant phase $\times$ viral treatment interaction effect [$F_{(3,99)} = 2.90$; $p = 0.039$]. The viral treatment main effect was not significant. Furthermore, both the rAAV-FIB-NPY₁₃₋₃₆ and rAAV-FIB-GFP treated mice displayed increased ethanol-induced locomotor activity on the ethanol test day (postE2) relative to initial exposure to the 2.0 g/kg dose of ethanol (preE2)

indicated by significant paired sample t-tests performed as planned comparisons (both $p < 0.01$).

Fig. 6B depicts ethanol-induced locomotor activity in DBA/2J mice treated with either the rAAV-FIB-GFP or rAAV-FIB-NPY₁₃₋₃₆ vector into the region of the NAc core, where locomotor activity on the post-sensitization ethanol test day (postE2) and on the day of the final saline injection were defined as the change in activity relative to baseline activity during the initial saline injections (postE2 or Final Saline – initial saline). A two-way 2×2 (phase $\times$ viral treatment) repeated-measures ANOVA revealed a significant effect of phase [$F_{(1,33)} = 72.32$; $p < 0.001$], reflecting that the increased activity was specific to ethanol administration. There was also a significant phase $\times$ viral treatment interaction effect [$F_{(1,33)} = 4.21$; $p = 0.048$], and post hoc analysis revealed that mice treated with the rAAV-FIB-NPY₁₃₋₃₆ vector displayed reduced ethanol-induced locomotor activity when compared to the rAAV-FIB-GFP-treated mice at postE2. The viral treatment main effect did not achieve statistical significance.

Fig. 6C depicts locomotor activity in DBA/2J mice treated with either the rAAV-FIB-GFP or rAAV-FIB-NPY₁₃₋₃₆ vector into the region of the NAc core, where locomotor activity on the post-sensitization ethanol test day (postE2) and on the day of the final saline injection were defined as the change in activity relative to activity during pre-sensitization exposure to the 2.0 g/kg dose of ethanol (postE2 or Final Saline – preE2). A two-way 2×2 (phase $\times$ viral treatment) repeated-measures ANOVA revealed a significant main effect of phase [$F_{(1,33)} = 72.32$; $p < 0.001$] and a phase $\times$ treatment interaction [$F_{(1,33)} = 4.21$; $p = 0.048$] but the viral treatment main effect did not achieve statistical significance. However, post hoc analysis showed that mice treated with the rAAV-FIB-NPY₁₃₋₃₆ vector displayed blunted ethanol-induced locomotor sensitization on the postE2 test day relative to mice treated with the rAAV-FIB-GFP vector. Taken together, data from Experiment 3 show that injection of the rAAV-FIB-NPY₁₃₋₃₆ vector into core region of the NAc blunts ethanol-induced locomotor activity and behavioral sensitization in DBA/2J mice. The expression of sensitized behavior and the effects of the rAAV-FIB-NPY₁₃₋₃₆ vector on behavioral sensitization were specific to ethanol injection and not evident after post-sensitization injection of saline.

RT-PCR Validation of Vector Activity In Vivo

As previously described (Foti et al., 2007; Haberman et al., 2003), the vector-derived NPY₁₃₋₃₆ and GFP cannot be visualized *in vivo* by immunohistochemistry, as secreted protein is rapidly degraded and washed out during the perfusion procedure. Thus, the duration of rAAV-FIB-NPY₁₃₋₃₆ vector activity *in vivo* was validated by demonstrating the presence of appropriate vector-derived mRNA in microdissected NAc tissue of DBA/2J mice (see Foti et al. 2007). Microdissection procedures are described above. All of the 6 samples collected from mice treated with the rAAV-FIB-NPY₁₃₋₃₆ vector exhibited the 143 base pair band while none of the 4 samples collected from mice treated with the rAAV-FIB-GFP showed the 143 base pair band (data not shown). Since brain tissue was collected after the experiment, these results verify that vector-derived mRNA was present throughout the course of the study.

DISCUSSION

In the present report, we combined immunohistological, genetic, and viral vector approaches to provide novel evidence that central NPY signaling modulates ethanol-induced behavioral sensitization in mice. In Experiment 1, RII β ^{-/-} mice on a C57BL/6J background exhibited significantly greater NPY IR in the NAc core and ventral striatum relative to littermate RII β ^{+/+} mice. Since the NAc core and striatum have previously been implicated in ethanol-

induced behavioral sensitization (Quadros et al., 2002; Souza-Formigoni et al., 1999), increased NPY IR in these regions may modulate the enhanced sensitivity to ethanol-induced locomotor activity and behavioral sensitization that are characteristic of $R11\beta^{-/-}$ mice (Fee et al., 2006). Importantly, increased NPY IR in $R11\beta^{-/-}$ mice was region-specific as genotype differences were not evident in the dorsal striatum, the shell of the NAc, the CeA, or the PVN. In Experiment 2, we directly tested the role of NPY signaling by assessing ethanol-induced locomotor activity and behavioral sensitization in littermate $NPY^{-/-}$ and $NPY^{+/+}$ mice on a C57BL/6J background. $NPY^{-/-}$ mice exhibited blunted expression of ethanol-induced locomotor activity and behavioral sensitization relative to $NPY^{+/+}$ mice, suggesting that NPY plays a facilitatory role in the expression of these behaviors. Importantly, altered locomotor activity was specific to ethanol injection (and not seen following saline injections), and genotype differences were not secondary to alterations of BECs. Based on the results from Experiment 1, in Experiment 3 we tested the role of NPY signaling within the region of the NAc core in behavioral sensitization. We focused on the NAc core because the large size of the striatum would require multiple injection sites, and thus the striatum will be the focus of future investigations. Here, DBA/2J mice that were given NAc core injection of the rAAV-FIB-NPY₁₃₋₃₆ vector exhibited blunted ethanol-induced locomotor activity on the ethanol test day relative to DBA/2J mice treated with a control vector. Blunted activity was specific to ethanol injection (and was not seen following saline injections). Since the NPY₁₃₋₃₆ fragment is a selective agonist for the Y_2 receptor, the present results suggest that Y_2 receptor signaling in the region of the NAc core modulates the expression of ethanol-induced behavioral sensitization. Previous evidence showed that the Y_2 receptor can function as a presynaptic autoreceptor that inhibits endogenous NPY release (Chen et al., 1997; King et al., 1999). Thus, it is tempting to speculate that observations from the viral vector experiment are consistent with the idea that endogenous NPY signaling in the region of the NAc core facilitates ethanol-induced locomotor activity and behavioral sensitization, and this facilitatory effect is blunted by the rAAV-FIB-NPY₁₃₋₃₆ vector, though we have no direct evidence for a pre- or post-synaptic role of the Y_2 receptor in the present report.

In Experiment 1, we used male mice only because we have previously found that the enhanced sensitivity to the locomotor stimulant effects of ethanol in $R11\beta^{-/-}$ mice, relative to $R11\beta^{+/+}$ mice, did not depend on sex (Fee et al., 2006). However, since sex could play a role in altered sensitivity to the locomotor stimulant effects of ethanol between $NPY^{-/-}$ and $NPY^{+/+}$ mice, we studied both male and female mice in Experiment 2. We found that there was a sex effect that interacted with the phase of the experiment, reflecting the observation that female mice showed greater locomotor activity in response to the 2.0 g/kg dose of ethanol before and after sensitization training. Importantly, while female mice tended to be more active, both sexes exhibited the expression of behavioral sensitization, and the sex effect did not significantly interact with genotype indicating that blunted sensitivity to ethanol-induced behavioral sensitization by $NPY^{-/-}$ mice did not depend on sex. Finally, we used male DBA/2J mice in Experiment 3 because these mice have previously been shown to exhibit robust ethanol-induced behavioral sensitization (Lessov et al., 2001; Phillips et al., 1994) and our interest was in manipulating this phenotype with the rAAV-FIB-NPY₁₃₋₃₆ vector. Interestingly, while C57BL/6J mice typically exhibit weaker ethanol-induced behavioral sensitization relative to DBA/2J mice (Lessov et al., 2001), wild-type mice on the C57BL/6J background in the current work showed robust behavioral sensitization, which may be the result of “hitchhiking” genes from the 129/SvJ stem cells used to create $R11\beta^{-/-}$ (Brandon et al., 1998) and $NPY^{-/-}$ (Erickson et al., 1996) mice.

In addition to observing genotype differences in NPY IR between $R11\beta^{-/-}$ and $R11\beta^{+/+}$ mice in Experiment 1, we found that a single injection of a 2.0 g/kg dose of ethanol significantly attenuated NPY IR in the ventromedial and ventrolateral striatum relative to

saline injected mice, an effect that did not depend on genotype. While acute injection of ethanol has been shown to increase NPY IR in the CeA and medial amygdala of rats (Pandey et al., 2005), the current finding is consistent with the observation that a single injection of ethanol decreased NPY mRNA in the arcuate nucleus of the hypothalamus in rats (Kinoshita et al., 2000). Nonetheless, the present observations suggest that acute ethanol exposure modulates NPY IR in brain regions that have been implicated in ethanol-induced behavioral sensitization, and lend additional support to the idea that NPY signaling in the striatum plays a modulatory role in ethanol-induced locomotor activation. The finding that NPY IR was altered after a single, but not after 5 repeated ethanol injections suggests the possibility that NPY plays a role in the initial acquisition of ethanol-induced locomotor sensitization but is not necessary for the ultimate expression of behavioral sensitization (which was evident after 5 ethanol injections in the Fee et al. 2006 study). However, two potential caveats must be considered. With immunohistochemistry procedures, decreased IR in response to ethanol exposure could indicate that ethanol blunts normal signaling via reduced production of NPY. Alternatively, ethanol-induced decreases of NPY IR could reflect increased NPY signaling via a facilitation of release. Thus, decreased NPY IR may reflect either increased or decreased signaling. Future studies that assess mRNA may be useful for determining if acute ethanol exposure attenuates NPY expression in the striatum. The second caveat that must be considered is that stress associated with i.p. injection of ethanol could produce non-specific effects on NPY IR. In fact, acute stress has been found to decrease NPY IR and mRNA in the brains of rats (Kim et al., 2003; Thorsell et al., 1998). Thus, IR data following ethanol injections must be interpreted with caution.

As previously discussed (Foti et al., 2007), the constitutive secretion of NPY₁₃₋₃₆ promoted by the rAAV-FIB-NPY₁₃₋₃₆ vector renders immunohistochemical identification of vector-derived NPY₁₃₋₃₆ untenable. However, our ability to find appropriate vector-derived NPY₁₃₋₃₆ mRNA in the area of vector infusion, in brains collected from subjects after the completion of Experiment 3, verifies that our vector treatment was functionally active throughout the course of the experiment. Another complication stemming from the constitutive secretion of NPY₁₃₋₃₆ (and of GFP in mice treated with the rAAV-FIB-GFP vector) is that it was not possible to verify the specific site of vector-derived gene product for each subject. However, we carefully performed pilot experiments with a rAAV-GFP vector, which induces the production of GFP in infected cells but does not induce the constitutive secretion of GFP given the absence of FIB (Haberman et al., 2003). Using the same coordinates and injection rate/volume used in the present experiment, we were able to confine GFP expression to the NAc core. However, since we cannot completely rule out the possibility that the viral vectors in the present experiment infected cells in regions adjacent to the NAc core, we cautiously interpret our results to indicate that NPY Y₂ receptor signaling in the “region” of the NAc core modulates ethanol-induced behavioral sensitization.

It is of interest to consider the possible mechanism by which NPY signaling modulates ethanol-induced behavioral sensitization. Dopamine (DA) signaling within the striatum and NAc have been implicated in behavioral sensitization associated with multiple drugs of abuse (Kalivas and Stewart, 1991; Pierce and Kalivas, 1997). In the striatum and NAc, NPY is synthesized by intrinsic aspiny neurons (Aoki and Pickel, 1988; Massari et al., 1988) and NPY signaling in these regions appears to stimulate local DA release. For example, in brain slices through rat striatum, NPY was found to augment KCl-induced [³H]dopamine overflow (Adewale et al., 2007). NPY may exert its effect on DA signaling in the striatum by interactions with NMDA receptor signaling, as NPY enhanced NMDA-stimulated [³H]dopamine release from rat striatum slices (Ault et al., 1998; Ault and Werling, 1997). Importantly, using *in vivo* microdialysis procedures, NPY infused into the NAc was found to dose-dependently increase extracellular DA levels (Sorensen et al., 2009). Together, these

observations suggest that NPY signaling in the striatum and NAc stimulate local DA activity, and this may be part of the mechanism by which NPY facilitates ethanol-induced locomotor sensitization.

In conclusion, the present set of experiments combined immunohistological, genetic, and viral vector approaches to provide the first evidence that central NPY signaling modulates ethanol-induced behavioral sensitization in mice. Furthermore, Y₂ receptor signaling in the region of the NAc core appears to be part of the mechanism by which NPY signaling modulates ethanol-induced behavioral sensitization. Future studies will take advantage of the rAAV-FIB-NPY₁₃₋₃₆ vector, as well as a rAAV-FIB-NPY vector which promotes the constitutive secretion of NPY, to further characterize the circuitry involved.

Acknowledgments

This work was supported by NIH grants AA013573, AA015148, AA016716, AA014949, AA014284, NS35633, and the Department of Defense grant W81XWH-06-1-0158.

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Fig. 1. NPY immunoreactivity in the nucleus accumbens (NAc). Representative photomicrographs are coronal brain slices (at approximately 1.0 mm anterior to bregma) taken from RIIP^{+/+} (A) and RIIP^{-/-} (B) mice which received saline injections. Images were photographed with 10x objectives and scale bar = 200 μm. Quantification of NPY immunoreactivity in the core (C) and shell (D) regions of the NAc were performed using Image J software and values are represented as mean density ± SEM. * indicates a genotype difference ($p < 0.05$).

Fig. 2. NPY immunoreactivity in the striatum. Representative photomicrographs are coronal brain slices (at approximately 1.0 mm anterior to bregma) taken from $RII\beta^{+/+}$ (A) and $RII\beta^{-/-}$ (B) mice which received saline injections. Rectangles represent approximate regions in which NPY immunoreactivity was quantified. Abbreviations: DM= dorsomedial; DL=dorsolateral; VM=ventromedial; VL=ventrolateral. Images were photographed with 10x objectives and scale bar = 200 μ m.

Fig. 3.

Quantification of NPY immunoreactivity in $RII\beta^{-/-}$ and $RII\beta^{+/+}$ mice in the dorsolateral (A), dorsomedial (B), ventrolateral (C), and ventromedial (D) striatum following saline, acute ethanol, or repeated ethanol injections. Quantification of NPY immunoreactivity was performed using Image J software and values are represented as mean density $\pm$ SEM. * indicates a significant main effect difference between genotypes ($p < 0.05$). # indicates a significant difference between saline and acute ethanol treated animals ($p < 0.05$). Abbreviations: DM=dorsomedial; DL=dorsolateral; VM=ventromedial; VL=ventrolateral.

Fig. 4.

Raw locomotor activity from NPY^{-/-} and NPY^{+/+} mice (A) and from males and females (B) following saline injection (S; average of 3 days), injection of a 1.5 g/kg dose of ethanol (E1.5; averaged over 5 days), initial injection of a 2.0 g/kg dose of ethanol (preE2; averaged over 5 days), test day injection of a 2.0 g/kg dose of ethanol (postE2), and the final saline injection (S). Mice received homecage injections of a 2.5 g/kg dose of ethanol (10, once per day) between preE2 and postE2. (C) Locomotor activity in NPY^{-/-} and NPY^{+/+} mice on the ethanol test day (postE2) or final saline injection corrected for activity during the initial saline baseline phase as a measure of ethanol-induced locomotor activity. (D) Ethanol-induced locomotor sensitization is presented as a change in activity on the ethanol test day (postE2) or final saline administration from activity following the initial ethanol injection (preE2). Values are represented as mean $\pm$ SEM. * indicates a significant genotype/sex difference ($p < 0.05$). # indicates a significant increase in locomotor activity between the initial preE2 and postE2 data ($p < 0.05$).

Fig. 5.

Representative photomicrograph of a 50 μm coronal slice cut through the core region of the nucleus accumbens (NAc) of a mouse that was given site-directed infusion of a rAAV-GFP vector aimed at the NAc core (0.5 μl). Ten days after infusion the mouse was perfused and its brain was removed. Slices display strong expression of GFP in cell bodies and terminals, a finding that verifies that the transduction of the rAAV vector into cells of the NAc core. The brain slice diagram indicates the location of vector infusions. Images were photographed with 20X objectives and the scale bar = 50 μm .

Fig. 6.

(A) Raw locomotor activity in DBA/2J mice following injection of saline (S; 3 day average), initial injection of a 2.0 g/kg dose of ethanol (preE2; 3 day average), test day injection of a 2.0 g/kg dose of ethanol (postE2), or a final saline injection (S). Mice received homecage injections of a 2.5 g/kg dose of ethanol (10, once per day) between preE2 and postE2. Subjects were treated with either rAAV-FIB-GFP or rAAV-FIB-NPY₁₃₋₃₆ infused into the NAc core. (B) Locomotor activity on the ethanol test day (postE2) or final saline injection corrected for basal activity during the initial saline injections as a measure of ethanol-induced locomotor activity. (C) Change in activity on the ethanol test day (postE2) or final saline injection from initial injection of ethanol (preE2) as a measure of ethanol-induced behavioral sensitization. Values are represented as mean $\pm$ SEM. * indicates a significant main effect of viral treatment ($p < 0.05$). # indicates a significant increase in locomotor activity between preE2 and postE2 ($p < 0.05$).

Table 1

Average densities of NPY immunoreactivity presented as percent of the area (Mean $\pm$ SEM) following saline, acute ethanol, or repeated ethanol treatment in select brain regions of male RIIP $-/-$ and RIIP $+/+$ mice

	RIIP $+/+$			RIIP $-/-$		
	Saline	Acute Ethanol	Repeated Ethanol	Saline	Acute Ethanol	Repeated Ethanol
Central nucleus of the amygdala	0.2 $\pm$ 0.02	0.3 $\pm$ 0.07	0.4 $\pm$ 0.08	0.3 $\pm$ 0.08	0.4 $\pm$ 0.08	0.4 $\pm$ 0.06
Paraventricular nucleus of the hypothalamus	51.2 $\pm$ 1.4	50.2 $\pm$ 1.1	50.1 $\pm$ 0.5	50.4 $\pm$ 1.3	51.8 $\pm$ 1.1	51.0 $\pm$ 1.3