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Neuropeptide Y response to alcohol is altered in nucleus accumbens of mice selectively bred for drinking to intoxication

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Abstract

The High Drinking in the Dark (HDID) mice have been selectively bred for drinking to intoxicating blood alcohol levels and represent a genetic model of risk for binge-like drinking. Presently, little is known about the specific genetic factors that promote excessive intake in these mice. Previous studies have identified neuropeptide Y (NPY) as a potential target for modulating alcohol intake. NPY expression differs in some rodent lines that have been selected for high and low alcohol drinking phenotypes, as well as inbred mouse strains that differ in alcohol preference. Alcohol drinking and alcohol withdrawal also produce differential effects on NPY expression in the brain. Here, we assessed brain NPY protein levels in HDID mice of two replicates of selection and control heterogeneous stock (HS) mice at baseline (water drinking) and after binge-like alcohol drinking to determine whether selection is associated with differences in NPY expression and its sensitivity to alcohol. NPY levels did not differ between HDID and HS mice in any brain region in the water-drinking animals. HS mice showed a reduction in NPY levels in the nucleus accumbens (NAc) – especially in the shell – in ethanol-drinking animals vs. water-drinking controls. However, HDID mice showed a blunted NPY response to alcohol in the NAc core and shell compared to HS mice. These findings suggest that the NPY response to alcohol has been altered by selection for drinking to intoxication in a region-specific manner. Thus, the NPY system may represent a potential target for altering binge-like alcohol drinking in these mice.

Keywords

neuropeptide Y; alcohol; binge drinking; drinking in the dark; selective breeding; behavioral genetics; immunohistochemistry; nucleus accumbens

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1. Introduction

Binge drinking as defined by the NIAAA is a pattern of alcohol (ethanol) intake that results in blood ethanol concentrations (BECs) at or above the legal limit of 0.08 g% [1]. The High Drinking in the Dark (HDID) mice have been selectively bred for reaching high BECs after a limited access drinking in the dark (DID) procedure. These mice readily drink to intoxicating BECs and are a novel genetic model of risk for binge drinking [2][3]. Because the genetic and neurobiological factors underlying binge drinking are not yet known, identifying specific genetic contributions to the HDID phenotype may have translational value for understanding excessive drinking in humans.

Studies in humans and animal models have suggested a role for NPY in alcohol consumption. Multiple polymorphisms in the *NPY* gene have been associated with ethanol drinking and dependence in humans [4][5][6], although this association is not always replicated [7]. In rodent studies, *Npy* gene expression and peptide levels in several brain regions have been found to differ between lines of mice and rats selected for high ethanol preference and also between inbred strains with disparate ethanol intakes [8][9][10]. Similarly, genetic manipulation of *Npy* can alter ethanol drinking as well. Knocking out *Npy* has been shown to increase ethanol consumption, and overexpression of *Npy* can decrease ethanol consumption [11]. There is some evidence, though, that these effects may be at least partially dependent on genetic background [12].

There is also evidence that ethanol influences NPY peptide levels in the brain. Ethanol exposure can result in decreased NPY expression in some regions including the central nucleus of the amygdala (CeA), the hippocampus, and the motor cortex [13][14][15][16]. Ethanol withdrawal can produce an opposite effect, with elevated NPY levels reported in the amygdala, frontal cortex, dorsomedial striatum, and hippocampus during withdrawal in mice and rats [13][16][17]. The ethanol metabolite acetaldehyde has also been shown to produce decreases in NPY levels in the hippocampus and nucleus accumbens (NAc) following acute treatment, and increases in these same regions during withdrawal [18].

A majority of the work on NPY and ethanol has been done using animals selectively bred for, or tested on, ethanol preference in a two-bottle choice continuous access test. However, continuous access preference drinking may only result in limited experience of intoxicating BECs and might not always reflect consumption that is driven by the pharmacological effects of ethanol [19]. Continuous access preference drinking and limited access DID are also believed to be genetically distinct traits [20][21]. Therefore, investigating the neurobiological factors relevant to DID binge-like drinking is useful for fully understanding drinking to intoxication. There is some evidence to suggest that NPY is relevant to binge-like drinking. Central administration of NPY or a selective NPY Y1 receptor agonist reduces intake and BEC in the DID test in B6 mice [15], and a similar effect has been seen with NPY Y1 receptor activation in the bed nucleus of the stria terminalis (BNST) [22]. In the HDID mice, gene network connectivity analysis of striatal tissue from naïve mice of both selection replicates and unselected heterogeneous stock (HS) mice found selection-dependent alteration of connectivity of the gene encoding the NPY Y2 receptor [20]. A microarray study measuring NPY mRNA in naïve HDID-1 and HS mice showed greater

Npy gene expression in the NAc, CeA, and BNST of HDID-1 mice than HS mice [23]. Taken together, these data suggest that selection may have produced alterations in the NPY system of the HDID mice as compared to controls, and that NPY may therefore be related to drinking to intoxication in these animals.

The present studies were designed to explore further the potential relationship between NPY and binge-like ethanol drinking in the HDID mice. In two separate experiments, we compared NPY protein expression in HDID-1 and HDID-2 mice to HS mice after either water or ethanol DID. Immunohistochemistry was used to assess NPY levels in six brain regions: the NAc (core and shell), the amygdala (CeA and basolateral amygdala [BLA]), the BNST, and the paraventricular nucleus of the hypothalamus (PVN). We hypothesized that HDID mice would differ from HS mice in baseline (water-drinking) NPY expression in the extended amygdala areas where NPY mRNA levels were previously found to be higher. We were also interested in whether ethanol drinking during the DID test would affect brain NPY expression differently in the HDID replicate lines vs. the HS mice.

2. Materials and methods

2.1 Animals and husbandry

Male mice from the HDID-1 and HS lines were bred and housed in the Veterinary Medical Unit of the Veterans Affairs Portland Health Care System (Portland, OR). Mice were between the ages of 55 and 90 days at the time of brain dissection. Experiment 1 used male HDID-1 mice from generations S27-S28 and HS mice from filial generation G75. Experiment 2 used male HDID-2 mice from selection generation S25 and HS mice from filial generation G80. HS mice are the starting population of the HDID selection and are the result of an 8-way inbred strain cross (see [2] for details). These mice are maintained without selective breeding and are used as the comparator control line for the HDID animals. During all experiments, mice were singly housed in standard polycarbonate shoebox cages on Bed-o-cob bedding and were provided with *ad libitum* access to water and food (Purina 5001 chow, LabDiet, St. Louis, MO). Mice were habituated to single housing conditions for at least 1 week prior to the start of testing. Throughout the habituation and experimental periods, mice were maintained on a 12 h/12 h reverse light-dark cycle with lights off at 09:30. All procedures were approved by the local Institutional Animal Care and Use Committee and were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

2.2 Drinking in the Dark

A standard 4-day drinking in the dark (DID) procedure was used for these experiments [24]. Mice were weighed each day approximately 1 h before testing. At 3 h into the dark cycle, the water bottles were removed from each cage and replaced with a 10 ml drinking tube containing 20% ethanol (200 proof [molecular-sieve dehydrated], Decon Labs, King of Prussia, PA, dissolved in tap water v/v) or water depending on group assignment. Fluid levels were recorded and tubes were left in place for 2 h on Days 1-3. On Day 4, tubes were left in place for 4 h and fluid levels were recorded at the 2 h time point and again at the end of the drinking session. Immediately following drinking on Day 4, a 20 μ l blood sample was

taken from the peri-orbital sinus of all ethanol-drinking animals. Blood samples were processed according to standard lab protocols for gas chromatography determination of BEC [25]. Mice were then moved in squads of two-four to a separate procedure room and were deeply anesthetized via intraperitoneal injection with rat cocktail (ketamine (100 mg/ml), xylazine (20 mg/ml), and acepromazine (10 mg/ml) diluted 1:6 in saline) and transcardially perfused with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) according to previously published methods for NPY immunohistochemistry [26]. After perfusion, brains were dissected and stored at -8°C in 15 ml conical centrifuge tubes containing 4% PFA. All ethanol group animals were perfused first, followed by all water group animals. This was done to minimize the time between the end of the drinking session and brain dissection to limit any potential impact of ethanol withdrawal on NPY expression.

2.3 Experiment 1: HDID-1 and HS NPY immunohistochemistry after water or binge ethanol drinking

Fifty-three male HDID-1 and HS mice were singly housed and allowed to acclimate for a week. Mice were then tested on the standard 4-day DID procedure, with half the animals drinking 20% ethanol and half drinking water ($n=13-14/\text{group/line}$). Groups were matched for age on Day 4 (mean $\pm$ SEM: HDID-1 water, 58.31 ± 1.05 ; HDID-1 ethanol, 58.85 ± 0.95 , HS water, 58.0 ± 0.61 ; HS ethanol, 59.15 ± 0.78). After drinking on Day 4, blood samples were collected from the ethanol group and animals were perfused for brain dissection.

2.4 Experiment 2: HDID-2 and HS NPY immunohistochemistry after water or binge ethanol exposure

Fifty-two HDID-2 and HS mice were singly housed and allowed to acclimate for a week. Mice were then tested on the standard 4-day DID procedure, with half the animals drinking 20% ethanol and half drinking water ($n=12-14/\text{group/line}$). Groups were matched for age on Day 4 (mean $\pm$ SEM: HDID-2 water, 77.58 ± 2.40 ; HDID-2 ethanol, 77.08 ± 2.40 , HS water, 76.86 ± 0.78 ; HS ethanol, 76.46 ± 0.70). After drinking on Day 4, blood samples were collected from the ethanol group and animals were perfused for brain dissection.

2.5 NPY immunohistochemistry

Brains were cut into 40 micron sections on a Leica cryostat and were stored in 0.1% sodium azide in PBS at -8°C until the time of assay. NPY immunohistochemistry (IHC) was conducted according to previously published methods [26]. Briefly, floating sections were rinsed in PBS and then incubated in 0.3% hydrogen peroxide for 15 minutes. Slices were then rinsed again in PBS and incubated in a blocking solution (2% bovine serum albumin [BSA] and 5mg/ml heparin in 0.003% Triton X100 and PBS) for 5 hours. After blocking, slices were incubated overnight in rabbit polyclonal antibody targeted against NPY (anti-NPY N9528, Sigma-Aldrich, St. Louis, MO; 1:10,000 dilution) in a Triton and BSA PBS solution. This primary antibody dilution was chosen based on a previous titration experiment and was found to yield robust NPY staining with minimal background staining. The next day, slices were washed in PBS and incubated for 1 hour in biotinylated-goat anti-rabbit secondary antibody in PBS and Triton. Slices were washed again in PBS and then incubated in avidin/biotin solution (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) in PBS and Triton for 1 hour. Slices were washed once more in PBS and then were stained

using a 3,3'-diaminobenzidine (DAB) reaction. Slices were then slide-mounted on the same day as the assay.

For each region, we aimed to analyze both hemispheres from two to four slices per animal. Optical density values and cell counts from all slices for each animal were averaged so that there was only a single data point per measure for each brain region. Analysis was done using ImageJ (National Institutes of Health) by an experimenter blinded to treatment and genotype. Each region of interest was selected manually using anatomical reference points from a mouse brain atlas [27]. NAc core and shell were quantified as separate regions, as were the dorsal and ventral BNST. Photomicrographs were analyzed using a standardized background subtraction and the threshold function in ImageJ (threshold was kept constant across slices within a given region for each experiment). Optical density of staining (NPY-positive fibers and cell bodies) was then automatically quantified by the software and reported as a percent of total area (i.e., selected brain region). Cell bodies were counted only in the NAc as this was the only area with a significant number of NPY-positive cell bodies and also low enough density of fiber staining that they could be reliably counted. All NPY-positive cell bodies within each NAc subdivision (core vs. shell) were manually counted.

2.6 Statistical analyses

Optical density of NPY staining (% area) and NPY-positive cell counts were analyzed for each region separately using a two-way analysis of variance (ANOVA) for treatment group and genotype. Significant group $\times$ genotype interactions were followed up with one-way ANOVAs for each genotype separately. Because of an *a priori* interest in potential differences in NPY expression between water drinking and ethanol drinking animals within each genotype, we made the decision to follow up genotype $\times$ group interactions that showed statistical trends towards significance ($p < 0.1$) with one-way ANOVAs for each genotype. Conducting pairwise comparisons in the absence of a significant interaction in the omnibus F test can be justified, but there is a potential for elevated risk of Type I errors [28]. To minimize this possibility, a Bonferroni correction was used to adjust the significance level (α) for follow-up ANOVAs when the initial genotype $\times$ group interaction was not statistically significant. Ethanol intake was converted to a g/kg body weight dose and water intake was converted to ml/kg. Day 4 intake was analyzed with one-way ANOVA for an effect of genotype separately for each fluid group, and BECs were analyzed for an effect of genotype as well. The significance level was set at $\alpha = 0.05$ unless otherwise specified.

3. Results

In some instances, brain slices could not be quantified for NPY immunoreactivity due to either damage to the tissue in the region of interest or incomplete staining. These slices were excluded from the analysis, and final group sizes for each brain region in both experiments ranged from $n=9$ -14/drinking group/genotype.

3.1 Experiment 1

Figure 1 shows ethanol intake (a) and BECs (b) during the DID test. The HDID-1 and HS mice showed the expected genotypic difference in ethanol consumption ($F_{1,24}=22.937$,

$p < 0.001$) and BEC ($F_{1,24} = 37.851$, $p < 0.001$). Water intake on Day 4 did not differ significantly between the genotypes ($F_{1,25} = 1.529$, $p > 0.1$; data not shown). Figure 2 shows representative photomicrographs of NPY staining in the NAc of water-drinking (a) and ethanol-drinking (b) HDID-1 mice and water-drinking (c) and ethanol-drinking (d) HS mice. Figure 3 shows NPY immunoreactivity (a and b) and NPY-positive cell counts (c and d) in the NAc core and shell. NPY immunoreactivity in the NAc core showed a significant main effect of drinking group ($F_{1,43} = 4.681$, $p = 0.036$), a statistical trend toward a main effect of genotype ($F_{1,43} = 3.934$, $p = 0.054$), and a significant group $\times$ genotype interaction ($F_{1,43} = 5.112$, $p = 0.029$). Follow-up ANOVAs showed that HS mice had a robust decrease in NAc core NPY levels in the ethanol group as compared to the water drinking animals ($F_{1,22} = 13.396$, $p = 0.001$), whereas HDID-1 showed no difference between the treatment groups ($F_{1,21} = 0.004$, $p = 0.952$). Analysis of NPY immunoreactivity in the NAc shell showed a significant main effect of genotype ($F_{1,43} = 4.904$, $p = 0.032$), no significant effect of group ($F_{1,43} = 2.829$, $p = 0.1$), and a trend toward a significant group $\times$ genotype interaction ($F_{1,43} = 3.358$, $p = 0.074$). Because of our interest in group differences within each genotype, this statistical trend was followed up with one-way ANOVAs for each genotype (Bonferroni-corrected $\alpha = 0.0125$). Again, HS mice showed significantly lower NPY levels after ethanol drinking than after water drinking ($F_{1,22} = 9.337$, $p = 0.006$). HDID-1 mice showed no difference between the groups ($F_{1,21} = 0.008$, $p = 0.928$). The number of NPY-positive cells in the NAc core showed no significant main effects and no significant interaction ($F_{1,42} = 1$, $p > 0.1$ for all). The number of NPY-positive cells in the NAc shell showed only a significant group $\times$ genotype interaction ($F_{1,42} = 4.462$, $p = 0.041$). Follow-up ANOVAs showed that HS mice did not differ in the number of cells between groups, and HDID-1 mice had a trend toward a reduction in the number of cells in the ethanol group as compared to water ($F_{1,20} = 3.533$, $p = 0.075$).

Striatal levels of NPY immunoreactivity and mRNA have been shown to vary with age in rats [29]. To determine whether age was potentially affecting our findings, we used linear regression to assess the relationship between age at time of brain dissection and NPY immunoreactivity in the NAc core and shell of ethanol drinking and water drinking animals. For both drinking groups, no significant correlations were found between age and NPY immunoreactivity in either NAc subregion ($r = -0.188$ – -0.225 , $n = 22$ – 25 , $p = 0.279$ for all). This suggests that NPY levels in these mice do not systematically vary over the age range of animals used under baseline or ethanol-drinking conditions.

Figure 4 shows NPY immunoreactivity in the CeA (a), BLA (b), BNST (dorsal and ventral) (c), and PVN (d) of HDID-1 and HS mice of each group. Only NPY levels in the CeA showed a significant main effect of genotype ($F_{1,43} = 6.974$, $p = 0.011$), with HDID-1 mice having higher NPY immunoreactivity than HS mice. No regions showed a significant main effect of drinking group and no significant group $\times$ genotype interactions were found ($F_{1,40-46} = 1$, $p > 0.1$ for all).

3.2 Experiment 2

Figure 5 shows ethanol intake (a) and BECs (b) during the DID test. HDID-2 and HS mice showed the expected genotypic difference in both ethanol consumption ($F_{1,24} = 10.808$,

$p=0.003$) and BEC ($F_{1,24}=71.091$, $p<0.001$). Water intake did not differ between the genotypes ($F_{1,24}=0.072$, $p=0.791$; data not shown). Figure 6 shows representative photomicrographs of NPY staining in the NAc of water-drinking (a) and ethanol-drinking (b) HDID-2 mice and water-drinking (c) and ethanol-drinking (d) HS mice. Figure 7 shows NPY immunoreactivity (a and b) and NPY-positive cell counts (c and d) in the NAc core and shell. Analysis of NPY immunoreactivity in the NAc core showed no significant main effects of either group or genotype ($p=0.372$ for both), but there was a trend toward a significant group $\times$ genotype interaction ($F_{1,48}=3.565$, $p=0.065$). As with Experiment 1, statistical trends toward significant interactions were followed up to assess potential group differences within each genotype (Bonferroni-corrected $\alpha=0.0125$). Follow-up ANOVAs showed a trend toward an effect of group in the HS mice ($F_{1,25}=3.33$, $p=0.08$), with water drinking animals tending to have higher NPY expression than ethanol drinking animals, but this difference did not reach statistical levels of significance. HDID-2 mice showed no significant difference between drinking groups ($F_{1,23}=0.612$, $p=0.442$). Analysis of NPY immunoreactivity in the NAc shell found no significant main effect of group or genotype ($F_{1,46}=1.583$, $p=0.215$ for both), but there was a significant group $\times$ genotype interaction ($F_{1,46}=5.05$, $p=0.029$). Follow-up ANOVAs found no effect of group in the HDID-2 mice ($F_{1,22}=0.573$, $p=0.457$). There was a significant main effect of group for the HS mice, with water drinking animals having significantly higher NPY levels in the NAc shell than ethanol drinking animals ($F_{1,24}=5.499$, $p=0.028$). The number of NPY-positive cells in the NAc core and NAc shell showed no significant main effects and no significant interaction ($F_{1,45-47}=1.592$, $p>0.2$ for all). As with Experiment 1, linear regression analysis showed no significant correlations between animal age and NPY immunoreactivity in the NAc core or shell for either drinking group ($r=-0.05-0.06$, $n=25-26$, $p=0.777$ for all).

Figure 8 shows NPY immunoreactivity in the CeA (a), BLA (b), BNST (dorsal and ventral) (c), and PVN (d) of HDID-2 and HS mice of each drinking group. There were no significant main effects or interactions for NPY expression in either the CeA or BLA ($F_{1,47-48}=2.481$, $p=0.122$ for all). Analysis of NPY expression in the BNST found a significant main effect of genotype in both the dorsal and ventral BNST, with HDID-2 mice having lower NPY levels overall than the HS mice ($F_{1,48}=4.448$, $p=0.04$ for both). There was no main effect of drinking group and no significant group $\times$ genotype interaction ($F_{1,48}=2.559$, $p=0.116$ for all). Analysis of the PVN found only a statistical trend toward an effect of drinking group, with ethanol-drinking animals trending towards lower NPY levels ($F_{1,43}=3.199$, $p=0.081$). There was no main effect of genotype and no significant group $\times$ genotype interaction ($F_{1,43}=1.380$, $p=0.247$ for all).

To determine whether the reduction from baseline levels of NPY in the NAc of HS mice was related to ethanol consumption, we pooled the HS mice from Experiments 1 and 2 to increase the number of data points and allow for analysis with linear regression. Figure 9 shows correlations between NAc NPY levels in HS mice and Day 4 ethanol intake (left) and water intake (right). Because absolute staining levels cannot be readily compared across assays performed at different times, NPY levels for ethanol-drinking HS mice were normalized within each experiment to a percent of maximum staining observed for that given group. This approach was used to normalize scores for water-drinking HS mice in

both experiments as well. Linear regression showed a significant negative correlation between Day 4 ethanol intake and NPY expression in both the NAc core ($r=-0.447$, $n=24$, $p=0.029$) and shell ($r=-0.509$, $n=23$, $p=0.013$) for HS mice. In contrast, water intake was not significantly correlated with NPY levels in either the NAc core or shell in these animals ($r=0.191$, $n=27$, $p>0.3$).

4. Discussion

In these experiments, we showed that selection for high BECs in the HDID mice has resulted in blunted NPY response to ethanol in the NAc in both replicate lines. In contrast, HS mice show a reliable reduction in NAc shell NPY levels after ethanol DID and a less consistent reduction in NAc core NPY levels. This reduction in NPY expression after ethanol drinking is consistent with previous findings of ethanol effects on NPY expression in other brain areas [13][14][15][16]. Additionally, NAc NPY immunoreactivity in the HS mice in both the core and shell was negatively correlated with ethanol intake, suggesting that the decreases in NPY expression are related to their ethanol consumption. This change in NPY response to ethanol is one of the few reported examples of a specific neurobiological difference between the HDID and HS mice at the protein level and provides possible evidence for a relationship between NPY and binge-like drinking in these genotypes.

The role of NPY in the NAc has been relatively unexplored compared to other brain areas, but there is evidence that NPY in this region is relevant to reward and addiction. NPY infusion into the NAc has been shown to condition a place preference in rats, indicating that NPY signaling in this area is perceived as rewarding [30]. Additionally, NPY administered centrally or into the NAc shell stimulates dopamine release, which is generally associated with reward [31][32]. NPY administration into the NAc shell can also enhance morphine reward, whereas administration of the NPY Y1 receptor antagonist BIBP 3226 decreases morphine reward [33]. In a recent study, NPY infusion into the NAc shell was found to increase self-administration of ethanol into the posterior ventral tegmental area in rats [34]. In the present studies the most consistent difference in NPY immunoreactivity between both HDID lines and the HS mice was seen in the NAc shell. Thus, it is possible that the sustained high levels of NPY expression in NAc shell of HDID mice during ethanol drinking could similarly enhance the perceived rewarding value of ethanol and could promote high DID intake. HDID and HS mice have been shown not to differ in ethanol place preference conditioned with a 2 g/kg injection [35], though there could still be differences in ethanol reward sensitivity at different doses or on a more direct measurement of reward (e.g. progressive ratio self-administration). Furthermore, HDID and HS male mice do show robust differences in an ethanol-conditioned taste aversion [35], and there is evidence to suggest that dopamine release in the NAc may also be involved in encoding responses to aversive stimuli e.g. [36]. Altered NPY signaling in the NAc of HDID mice (particularly the shell) might therefore be involved in the reduced ethanol aversion sensitivity of these animals via downstream effects on dopamine, though this will need to be tested directly in the future.

Previous studies of NPY region-specific effects on ethanol intake have been predominantly focused on the CeA and other extended amygdala structures [15][22][37]. Based on this

literature, the putative role of NPY in altering ethanol drinking has been as an anxiolytic neuropeptide: high drinking, high anxiety animals show low levels of NPY in the CeA and treatments that increase these levels (NPY administration, Y2 receptor antagonism, etc.) reduce both anxiety and ethanol intake [38][39][40]. However, this does not appear to be the mechanism by which NPY may modulate drinking in the HDID-1 mice as HDID-1 males actually show *lower* basal levels of anxiety-like behavior than HS mice [41]. This is consistent with the genotypic difference in NPY immunoreactivity in the CeA seen in Experiment 1 (HDID-1 > HS). The HDID-2 mice did not show this difference from HS in the CeA, but they did have overall lower NPY levels in both the dorsal and ventral BNST regardless of drinking group. Activation of NPY receptors in the BNST has been shown to suppress binge drinking [22], so lower levels of endogenous NPY in this region could conceivably be related to greater ethanol intake in the DID test. However, the lack of concordance in NPY findings between the HDID-1 and HDID-2 mice in the CeA and BNST suggests that changes in these regions may be less relevant to the specific binge-like drinking phenotype of HDID mice than the blunted NAc shell response, which does appear to be a correlated response to selection. It should be noted that a correlated response to selection indicates a *genetic* correlation and cannot show that the correlated trait directly influences the selection phenotype. While the present findings are highly suggestive of a link between NPY and binge-like drinking in the HDID mice, future experiments will be needed to determine whether direct manipulation of the NPY system can differentially alter ethanol drinking in the HDID and HS lines, as well as whether these effects are specific to ethanol.

Interestingly, no significant differences were seen between the genotypes for the number of NPY positive cell bodies in either the NAc core or shell. This suggests that sustained higher levels of NPY in these brain regions of HDID mice are not due to an increase in local production/release of NPY, but rather may reflect upstream alterations in NPY transmission. The arcuate nucleus and the CeA are both potential sources of NPY to the NAc shell [34]. The arcuate nucleus has not yet been examined for either NPY immunoreactivity or gene expression in the HDID and HS lines, and this region may be an additional target to consider in the future. Alternatively, these findings could reflect changes in NPY processing in HDID mice compared to the HS. NPY can be cleaved at various points, and the resulting peptides show altered affinity for the NPY receptor subtypes [42] [43]. C-terminal fragments are a common cleavage product and they can produce feedback inhibition of NPY release due to their high affinity for the Y2 autoreceptor [43]. Many of these cleaved peptides cannot readily be differentiated from intact NPY through immunohistochemistry. Therefore, apparent higher levels of NPY in the HDID mice may also reflect alterations in NPY processing and/or degradation. This could explain in part the fact that the NPY IHC findings from the water-drinking animals largely did not parallel the genetic differences in *Npy* mRNA levels previously seen in ethanol-naïve HDID-1 and HS mice. In the previous gene expression study, HDID-1 mice had greater *Npy* gene expression in NAc, CeA, and BNST than HS mice [23]. There are many post-transcriptional processing steps in the translation of mRNA to peptides (for review, see [44]), and it therefore should not necessarily be expected that mRNA and protein levels show a one-to-one relationship. However, the difference between measures of mRNA and protein levels could also indicate altered regulatory

processes or compensatory feedback mechanisms in the NPY system of HDID-1 mice that allow for only limited basal differences in NPY peptide levels between the lines despite different gene expression. Future experiments will be needed to determine what these changes might be and how they could relate to the high drinking phenotype of the HDID mice.

5. Conclusions

Selective breeding for binge-like ethanol drinking has resulted in an attenuated NPY response to ethanol in the NAc of HDID mice, particularly in the NAc shell. This altered NPY response is seen in both the HDID-1 and HDID-2 lines and appears to be a correlated response to selection. Although the exact relationship between sustained high levels of NPY in the NAc and binge-like drinking are not known, NPY in the NAc shell could be affecting the perceived motivational effects of ethanol and therefore overall levels of consumption. The NPY system in general – and NPY in the NAc specifically – therefore represents a potential target for reducing drinking to intoxication in the HDID lines.

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References

- [1]. NIAAA Newsletter. NIAAA Council Approves Definition of Binge Drinking. Vol. 3. DHHS-NIH; Bethesda, MD: 2004. 2004. p. 3Winter
- [2]. Crabbe JC, Metten P, Rhodes JS, Yu C-H, Brown LL, Phillips TJ, Finn DA. A line of mice selected for high blood ethanol concentrations shows drinking in the dark to intoxication. *Biological Psychiatry*. 2009; 65(8):662–670. doi:10.1016/j.biopsych.2008.11.002. [PubMed: 19095222]
- [3]. Crabbe JC, Metten P, Belknap JK, Spence SE, Cameron AJ, Schlumbohm JP, Phillips TJ. Progress in a replicated selection for elevated blood ethanol concentrations in HDID mice. *Genes, Brain, and Behavior*. 2014; 13(2):236–246. doi:10.1111/gbb.12105.
- [4]. Bhaskar LVKS, Thangaraj K, Kumar KP, Pardhasaradhi G, Singh L, Rao VR. Association between neuropeptide Y gene polymorphisms and alcohol dependence: a case-control study in two independent populations. *European Addiction Research*. 2013; 19(6):307–313. doi: 10.1159/000346679. [PubMed: 23652361]
- [5]. Francès F, Guillen M, Verdú F, Portolés O, Castelló A, Sorlí JV, Corella D. The 1258 G>A polymorphism in the neuropeptide Y gene is associated with greater alcohol consumption in a Mediterranean population. *Alcohol (Fayetteville, N.Y.)*. 2011; 45(2):131–136. doi:10.1016/j.alcohol.2010.08.009.
- [6]. Mottagui-Tabar S, Prince JA, Wahlestedt C, Zhu G, Goldman D, Heilig M. A novel single nucleotide polymorphism of the neuropeptide Y (NPY) gene associated with alcohol dependence. *Alcoholism, Clinical and Experimental Research*. 2005; 29(5):702–707.
- [7]. Zill P, Preuss UW, Koller G, Bondy B, Soyka M. Analysis of single nucleotide polymorphisms and haplotypes in the neuropeptide Y gene: no evidence for association with alcoholism in a German population sample. *Alcoholism, Clinical and Experimental Research*. 2008; 32(3):430–434. doi:10.1111/j.1530-0277.2007.00586.x.

- [8]. Caberlotto L, Thorsell A, Rimondini R, Sommer W, Hyytiä P, Heilig M. Differential expression of NPY and its receptors in alcohol-preferring AA and alcohol-avoiding ANA rats. *Alcoholism, Clinical and Experimental Research*. 2001; 25(11):1564–1569.
- [9]. Hayes DM, Knapp DJ, Breese GR, Thiele TE. Comparison of basal neuropeptide Y and corticotropin releasing factor levels between the high ethanol drinking C57BL/6J and low ethanol drinking DBA/2J inbred mouse strains. *Alcoholism, Clinical and Experimental Research*. 2005; 29(5):721–729.
- [10]. Hwang BH, Zhang JK, Ehlers CL, Lumeng L, Li TK. Innate differences of neuropeptide Y (NPY) in hypothalamic nuclei and central nucleus of the amygdala between selectively bred rats with high and low alcohol preference. *Alcoholism, Clinical and Experimental Research*. 1999; 23(6):1023–1030.
- [11]. Thiele TE, Marsh DJ, Ste Marie L, Bernstein IL, Palmiter RD. Ethanol consumption and resistance are inversely related to neuropeptide Y levels. *Nature*. 1998; 396(6709):366–369. doi: 10.1038/24614. [PubMed: 9845072]
- [12]. Thiele TE, Miura GI, Marsh DJ, Bernstein IL, Palmiter RD. Neurobiological responses to ethanol in mutant mice lacking neuropeptide Y or the Y5 receptor. *Pharmacology, Biochemistry, and Behavior*. 2000; 67(4):683–691.
- [13]. Criado JR, Liu T, Ehlers CL, Mathé AA. Prolonged Chronic Ethanol Exposure Alters Neuropeptide Y and Corticotropin-Releasing Factor Levels in the Brain of Adult Wistar Rats. *Pharmacology, Biochemistry, and Behavior*. 2011; 99(1):104–111. doi:10.1016/j.pbb.2011.04.005.
- [14]. Slawecki CJ, Jiménez-Vasquez P, Mathé AA, Ehlers CL. Effect of ethanol on brain neuropeptides in adolescent and adult rats. *Journal of Studies on Alcohol*. 2005; 66(1):46–52. [PubMed: 15830902]
- [15]. Sparrow AM, Lowery-Gionta EG, Pleil KE, Li C, Sprow GM, Cox BR, Thiele TE. Central Neuropeptide Y Modulates Binge-Like Ethanol Drinking in C57BL/6J Mice via Y1 and Y2 Receptors. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*. 2012 doi:10.1038/npp.2011.327.
- [16]. Bison S, Crews F. Alcohol withdrawal increases neuropeptide Y immunoreactivity in rat brain. *Alcoholism, Clinical and Experimental Research*. 2003; 27(7):1173–1183. doi:10.1097/01.ALC.0000075827.74538.FE.
- [17]. De Pauli RF, Coelho CC, Tesone-Coelho C, Linardi A, Mello LE, Silveira DX, Santos JG. Differential patterns of expression of neuropeptide Y throughout abstinence in outbred Swiss mice classified as susceptible or resistant to ethanol-induced locomotor sensitization. *Alcohol (Fayetteville, N.Y.)*. 2014; 48(1):63–72. doi:10.1016/j.alcohol.2013.11.004.
- [18]. Plescia F, Brancato A, Marino RAM, Vita C, Navarra M, Cannizzaro C. Effect of Acetaldehyde Intoxication and Withdrawal on NPY Expression: Focus on Endocannabinoidergic System Involvement. *Frontiers in Psychiatry*. 2014; 5:138. doi:10.3389/fpsy.2014.00138. [PubMed: 25324788]
- [19]. Dole VP, Gentry RT. Toward an analogue of alcoholism in mice: scale factors in the model. *Proceedings of the National Academy of Sciences of the United States of America*. 1984; 81(11):3543–3546. [PubMed: 6587369]
- [20]. Iancu OD, Darakjian P, Walter NA, Malmanger B, Oberbeck D, Belknap J, Hitzemann R. Genetic diversity and striatal gene networks: focus on the heterogeneous stock-collaborative cross (HS-CC) mouse. *BMC Genomics*. 2010; 11(1):585. doi:10.1186/1471-2164-11-585. [PubMed: 20959017]
- [21]. Phillips TJ, Reed C, Burkhart-Kasch S, Li N, Hitzemann R, Yu C-H, Belknap JK. A method for mapping intralocus interactions influencing excessive alcohol drinking. *Mammalian Genome: Official Journal of the International Mammalian Genome Society*. 2010; 21(1-2):39–51. doi: 10.1007/s00335-009-9239-9. [PubMed: 20033183]
- [22]. Pleil KE, Rinker JA, Lowery-Gionta EG, Mazzone CM, McCall NM, Kendra AM, Kash TL. NPY signaling inhibits extended amygdala CRF neurons to suppress binge alcohol drinking. *Nature Neuroscience*. 2015; 18(4):545–552. doi.org/10.1038/nn.3972. [PubMed: 25751534]

- [23]. Zhang L, Wang S, Crabbe JC, Harris RA, Mayfield RD, Ponomarev I. ACER. 2011; 35249(Issue supplement s1) Gene expression profiles in High Drinking in the Dark mice and their control. Abstract 0994.
- [24]. Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. *Physiology & Behavior*. 2005; 84(1):53–63. doi:10.1016/j.physbeh.2004.10.007. [PubMed: 15642607]
- [25]. Rustay NR, Crabbe JC. Genetic Analysis of Rapid Tolerance to Ethanol's Incoordinating Effects in Mice: Inbred Strains and Artificial Selection. *Behavior Genetics*. 2004; 34(4):441–451. doi: 10.1023/B:BEGE.0000023649.60539.dd. [PubMed: 15082941]
- [26]. Hostetler CM, Hitchcock LN, Anacker AMJ, Young LJ, Ryabinin AE. Comparative distribution of central neuropeptide Y (NPY) in the prairie (*Microtus ochrogaster*) and meadow (*M. pennsylvanicus*) vole. *Peptides*. 2013; 40:22–29. doi:10.1016/j.peptides.2012.12.008. [PubMed: 23262357]
- [27]. Franklin, KBJ.; Paxinos, G. *The Mouse Brain in Stereotaxic Coordinates*. Academic Press; San Diego: 2001.
- [28]. Hancock GR, Klockars AJ. The Quest for α : Developments in Multiple Comparison Procedures in the Quarter Century Since Games. *Review of Educational Research*. 1996; 1971; 66(3):269–306.<http://doi.org/10.3102/00346543066003269>
- [29]. Higuchi H, Yokokawa K, Iwasa A, Yoshida H, Miki N. Age-dependent increase in neuropeptide Y gene expression in rat adrenal gland and specific brain areas. *Journal of Neurochemistry*. 1991; 57(6):1840–1847. [PubMed: 1940903]
- [30]. Brown CM, Coscina DV, Fletcher PJ. The rewarding properties of neuropeptide Y in perifornical hypothalamus vs. nucleus accumbens. *Peptides*. 2000; 21(8):1279–1287. [PubMed: 11035216]
- [31]. Quarta D, Leslie CP, Carletti R, Valerio E, Caberlotto L. Central administration of NPY or an NPY-Y5 selective agonist increase in vivo extracellular monoamine levels in mesocorticolimbic projecting areas. *Neuropharmacology*. 2011; 60(2-3):328–335.<http://doi.org/10.1016/j.neuropharm.2010.09.016> [PubMed: 20868698]
- [32]. Sørensen G, Wegener G, Hasselstrøm J, Hansen TVO, Wörtwein G, Fink-Jensen A, Woldbye DPD. Neuropeptide Y infusion into the shell region of the rat nucleus accumbens increases extracellular levels of dopamine. *Neuroreport*. 2009; 20(11):1023–1026. [PubMed: 19579268]
- [33]. Desai SJ, Upadhyaya MA, Subhedar NK, Kokare DM. NPY mediates reward activity of morphine, via NPY Y1 receptors, in the nucleus accumbens shell. *Behavioural Brain Research*. 2013; 247:79–91. doi:10.1016/j.bbr.2013.03.018. [PubMed: 23511250]
- [34]. Borkar CD, Upadhyaya MA, Shelkar GP, Subhedar NK, Kokare DM. Neuropeptide Y system in accumbens shell mediates ethanol self-administration in posterior ventral tegmental area. *Addiction Biology*. 2015<http://doi.org/10.1111/adb.12254>
- [35]. Barkley-Levenson AM, Cunningham CL, Smitasin PJ, Crabbe JC. Rewarding and aversive effects of ethanol in High Drinking in the Dark selectively bred mice. *Addiction Biology*. 2015; 20(1):80–90.<http://doi.org/10.1111/adb.12079> [PubMed: 23910826]
- [36]. McCutcheon JE, Ebner SR, Loriaux AL, Roitman MF. Encoding of Aversion by Dopamine and the Nucleus Accumbens. *Frontiers in Neuroscience*. 2012; 6 doi:10.3389/fnins.2012.00137.
- [37]. Gilpin NW. Neuropeptide Y (NPY) in the extended amygdala is recruited during the transition to alcohol dependence. *Neuropeptides*. 2012; 46(6):253–259. doi:10.1016/j.npep.2012.08.001. [PubMed: 22938859]
- [38]. Primeaux SD, Wilson SP, Bray GA, York DA, Wilson MA. Overexpression of neuropeptide Y in the central nucleus of the amygdala decreases ethanol self-administration in “anxious” rats. *Alcoholism, Clinical and Experimental Research*. 2006; 30(5):791–801. doi:10.1111/j.1530-0277.2006.00092.x.
- [39]. Schroeder JP, Olive F, Koenig H, Hodge CW. Intra-amygdala infusion of the NPY Y1 receptor antagonist BIBP 3226 attenuates operant ethanol self-administration. *Alcoholism, Clinical and Experimental Research*. 2003; 27(12):1884–1891. doi:10.1097/01.ALC.0000098875.95923.69.
- [40]. Sparta DR, Fee JR, Hayes DM, Knapp DJ, MacNeil DJ, Thiele TE. Peripheral and central administration of a selective neuropeptide Y Y1 receptor antagonist suppresses ethanol intake by C57BL/6J mice. *Alcoholism, Clinical and Experimental Research*. 2004; 28(9):1324–1330.

- [41]. Barkley-Levenson AM, Crabbe JC. Genotypic and sex differences in anxiety-like behavior and alcohol-induced anxiolysis in High Drinking in the Dark selected mice. *Alcohol* (Fayetteville, N.Y.). 2015; 49(1):29–36. doi:10.1016/j.alcohol.2014.07.022.
- [42]. Grandt D, Schimiczek M, Rascher W, Feth F, Shively J, Lee TD, Michel MC. Neuropeptide Y 3-36 is an endogenous ligand selective for Y2 receptors. *Regulatory Peptides*. 1996; 67(1):33–37. [PubMed: 8952003]
- [43]. Michel MC, Beck-Sickinger A, Cox H, Doods HN, Herzog H, Larhammar D, Westfall T. XVI. International Union of Pharmacology Recommendations for the Nomenclature of Neuropeptide Y, Peptide YY, and Pancreatic Polypeptide Receptors. *Pharmacological Reviews*. 1998; 50(1): 143–150. [PubMed: 9549761]
- [44]. Vogel C, Marcotte EM. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet*. 2012; 13(4):227–232. doi:10.1038/nrg3185. [PubMed: 22411467]

Research Highlights

- Neuropeptide Y (NPY) is potentially involved in alcohol intake.
- HDID mice were selectively bred for binge-like drinking to intoxication.
- NPY protein levels were measured after water or binge-alcohol drinking.
- Unselected mice have lower NPY in NAc after alcohol drinking than water drinking.
- HDID mice have sustained high NAc NPY levels and blunted NPY response to alcohol .

Figure 1.

Ethanol intake (a) across each day of the 4-day DID test and BECs (b) after drinking on Day 4 for the ethanol group HDID-1 and HS mice in Experiment 1. Intake data (g/kg) are shown for each 2 h session on Days 1-3, and for the total 4 h session on Day 4. Means $\pm$ SEM shown. N=13/line. *** indicates statistically significant difference from HS mice ($p < 0.001$).

Figure 2.

Representative photomicrographs of NPY immunohistochemistry in the nucleus accumbens (NAc) (core and shell) of water-drinking HDID-1 mice (a), ethanol-drinking HDID-1 mice (b), water-drinking HS mice (c) and ethanol-drinking HS mice (d) from Experiment 1. Scale bar represents 500 μm .

Figure 3.

NPY immunoreactivity in the nucleus accumbens core (a) and shell (b) for HDID-1 and HS mice in each drinking group in Experiment 1. Panels c and d show the average number of NPY-positive cell bodies in the core and shell, respectively. Means $\pm$ SEM shown. N=11-13/line/group. ** indicates statistically significant difference ($p < 0.01$).

Figure 4.

NPY immunoreactivity in the central nucleus of the amygdala (a), basolateral amygdala (b), bed nucleus of the stria terminalis (c), and paraventricular nucleus of the hypothalamus (d) of HDID-1 and HS mice of both drinking groups in Experiment 1. Means $\pm$ SEM shown. N=9-13/line/group. * indicates statistically significant difference from HS mice ($p < 0.05$).

Figure 5.

Ethanol intake (a) across each day of the 4-day DID test and BECs (b) after drinking on Day 4 for the ethanol group HDID-2 and HS mice in Experiment 2. Intake data (g/kg) are shown for each 2 h session on Days 1-3, and for the total 4 h session on Day 4. Means $\pm$ SEM shown. N=12-14/line. *** indicates statistically significant difference from HS mice ($p < 0.001$).

Figure 6.

Representative photomicrographs of NPY immunohistochemistry in the nucleus accumbens (NAc) (core and shell) of water-drinking HDID-2 mice (a), ethanol-drinking HDID-2 mice (b), water-drinking HS mice (c) and ethanol-drinking HS mice (d) from Experiment 2. Scale bar represents 500 μm .

Figure 7.

NPY immunoreactivity in the nucleus accumbens core (a) and shell (b) for HDID-2 and HS mice in each drinking group in Experiment 2. Panels c and d show the average number of NPY-positive cell bodies in the core and shell, respectively. Means $\pm$ SEM shown. N=13/line/group. * indicates statistically significant difference ($p<0.05$).

Figure 8.

NPY immunoreactivity in the central nucleus of the amygdala (a), basolateral amygdala (b), bed nucleus of the stria terminalis (c), and paraventricular nucleus of the hypothalamus (d) of HDID-2 and HS mice of both drinking groups in Experiment 2. There was a significant main effect of genotype for BNST (dorsal and ventral), with lower NPY levels in HDID-2 than HS mice. Means $\pm$ SEM shown. N=11-14/line/group.

Figure 9.

Correlations between ethanol (left) and water (right) intake and nucleus accumbens NPY immunoreactivity in HS mice of both experiments. Regression lines are shown for each nucleus accumbens sub-region (core and shell). N=23-27/region/group.