HHS Public Access

Author manuscript

J Genet Genomics. Author manuscript; available in PMC 2016 October 07.

Published in final edited form as:

J Genet Genomics. 2016 July 20; 43(7): 421–430. doi:10.1016/j.jgg.2016.04.010.

Npy deletion in an alcohol non-preferring rat model elicits differential effects on alcohol consumption and body weight

Bin Qiu[a], **Richard L. Bell**[b], **Yong Cao**[a,e], **Lingling Zhang**[a], **Robert B. Stewart**[d], **Tamara Graves**[c], **Lawrence Lumeng**[c], **Weidong Yong**[a,*], and **Tiebing Liang**[c,*]

[a] Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100021, China

[b] Department of Psychiatry, Indiana University School of Medicine, Indianapolis, IN 46202, USA

[c] Department of Gastroenterology, Indiana University School of Medicine, Indianapolis, IN 46202, USA

[d] Department of Psychology, Purdue School of Science, Indiana University–Purdue University of Indianapolis, Indianapolis, IN 46202, USA

[e] Experimental Medicine Center, The First Affiliated Hospital of Sichuan Medical University, Luzhou 646000, China

Abstract

Neuropeptide Y (NPY) is widely expressed in the central nervous system and influences many physiological processes. It is located within the rat quantitative trait locus (QTL) for alcohol preference on chromosome 4. Alcohol-nonpreferring (NP) rats consume very little alcohol, but have significantly higher NPY expression in the brain than alcohol-preferring (P) rats. We capitalized on this phenotypic difference by creating an *Npy* knockout (KO) rat using the inbred NP background to evaluate NPY effects on alcohol consumption. Zinc finger nuclease (ZNF) technology was applied, resulting in a 26-bp deletion in the *Npy* gene. RT-PCR, Western blotting and immunohistochemistry confirmed the absence of *Npy* mRNA and protein in KO rats. Alcohol consumption was increased in $Npy^{+/-}$ but not $Npy^{-/-}$ rats, while $Npy^{-/-}$ rats displayed significantly lower body weight when compared to $Npy^{+/+}$ rats. In whole brain tissue, expression levels of Npy-related and other alcohol-associated genes, *Npy1r*, *Npy2r*, *Npy5r*, *Agrp*, *Mc3r*, *Mc4r*, *Crh* and *Crh1r*, were significantly greater in $Npy^{-/-}$ rats, whereas *Pomc* and *Crhr2* expressions were highest in $Npy^{+/-}$ rats. These findings suggest that the NPY-system works in close coordination with the melanocortin (MC) and corticotropin-releasing hormone (CRH) systems to modulate alcohol intake and body weight.

Keywords

Knockout rat; Alcohol drinking behavior; NPY and receptors; CRH and receptors; Melanocortin and receptors; BDNF

* Corresponding authors. wyong@cnilas.org (W. Yong), tliang@iu.edu (T. Liang).

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jgg.2016.04.010.

1. Introduction

The selectively bred alcohol-preferring (P) and alcohol-nonpreferring (NP) rat lines are one of the best characterized rodent models for studying divergent voluntary alcohol drinking behavior (Murphy et al., 2002; Bell et al., 2012; McBride et al., 2014). Using inbred alcohol-preferring (iP) and -nonpreferring (iNP) rats, genetic studies have successfully located quantitative trait loci (QTL) for alcohol preference, with the highest logarithm of odds score (LOD score 9.2) on chromosome 4 (Chr 4) in the iP × iNP F_2 rats (Bice et al., 1998; Carr et al., 1998). The development of Chr 4 congenic strains further demonstrated the importance of this genomic region for alcohol preference (Carr et al., 2006) as well as body weight (Spence et al., 2013). Additionally, a number of candidate genes within this genomic region, including neuropep-tide Y (*Npy*), corticotropin releasing hormone receptor 2 (*Crhr2*) (Yong et al., 2014), and others, are differentially expressed in various brain regions of the selectively bred and inbred rat lines of opposing alcohol preferences (Carr et al., 2007; Liang et al., 2010; Bell et al., 2012). Recent RNA-sequencing also revealed major genomic landscape differences between P and NP rats, finding greater genetic variability than previously identified (Zhou et al., 2013b). NPY is widely expressed in the central nervous system (CNS) and has been associated with various psychiatric disorders (Widerlov et al., 1988; Widdowson et al., 1992), including anxiety (Adrian et al., 1983; Heilig et al., 1989; Kask et al., 2002; Thorsell, 2008) and alcoholism (Thiele et al., 1998; Badia-Elder et al., 2001; Gilpin et al., 2005, 2008b; Zhang et al., 2010). NPY is also implicated in other physiological functions (Parker, 2013), including food intake (Clark et al., 1984; Jolicoeur et al., 1991; Zarjevski et al., 1993), bone density (Teixeira et al., 2009), and neuronal excitability (Woldbye et al., 1996).

Increasing evidence implicates NPY as a very important neuro-peptide in the biology of addiction. For instance, NPY levels in various brain regions are profoundly affected by chronic administration of cocaine (Wahlestedt et al., 1991) and alcohol (Olling et al., 2007). In P rats, intracerebroventricular (ICV) administration of NPY suppressed voluntary alcohol consumption (Badia-Elder et al., 2001; Thiele and Badia-Elder, 2003; Thorsell et al., 2005a, b; Gilpin et al., 2008c) and over-expression of *Npy* in the amygdala suppressed increased alcohol intake of animals after repeated alcohol deprivation (Thorsell et al., 2007). In agreement with studies in mice, our findings with P and NP rats indicate that NPY levels are inversely related to free-choice alcohol drinking (Thiele et al., 1998; Spence et al., 2005). These studies suggest that low NPY expression is a risk factor for high alcohol use; however, previous pharmacological experiments may have overlooked the effect of endogenous NPY. Thus, the development of an *Npy* knockout (KO) animal is an important advance for the research community.

NPY also functionally interacts with corticotropin releasing hormone (CRH) (Kash and Winder, 2006; Sajdyk et al., 2006) and decreases γ-aminobutyric acid (GABA)-ergic signaling in the central amygdala and bed nucleus of the stria terminalis (BNST) (Kash and Winder, 2006; Gilpin et al., 2011; Roberto et al., 2012). Additionally, NPY modulates catecholaminergic (Fuzesi et al., 2007), dopaminergic (Romano et al., 2014), glutamatergic (Patrylo et al., 1999; Smialowska et al., 2002), and serotonergic neuronal activities

(Yoshimura et al., 2014) within the mesocorticolimbic and extended amygdala reward circuits. Together, these studies indicate that *Npy* is a critical gene for various neurobiological functions including addiction.

Npy KO mice, surprisingly, did not show overt differences in feeding and body weight regulation, but they did show increased anxiety in elevated plus maze (EPM) (Palmiter et al., 1998), open field, and acoustic startle response tests (Bannon et al., 2000). They also showed increased susceptibility to seizures (Palmiter et al., 1998) and greater voluntary ethanol drinking, as well as resistance to ethanol-induced sedation (Thiele et al., 1998), though the magnitude of these differences depended, in part, on the genetic background of the KO mice (Thiele et al., 2000). In other words, some of the observed increases in alcohol consumption could be attributed to the predisposition of certain strains of mice to prefer alcohol. Our study addresses the confounding effect of high alcohol preference by establishing *Npy* KO rats on an iNP background, as they possess a higher level of NPY expression and voluntarily consume less ethanol. Thus, any increase in alcohol consumption resulting from *Npy* elimination cannot be attributed to a predisposition to high alcohol consumption.

In this study, we successfully created *Npy* KO rats using zinc finger nuclease (ZFN) technology (Geurts et al., 2009), confirmed by sequencing and expression quantification. KO rats were tested for alcohol consumption, body weight, and food intake. Anxiety level was assessed with EPM. In addition, specific gene expression levels in the whole brain were measured to broaden our understanding of how genes that interact with *Npy* to control alcohol consumption and body weight are affected by the absence of *Npy*. Based on previous research, the most relevant genes include *Npy* receptors, corticotropin-releasing hormone (*Crh*) and its receptors, agouti-related peptide (*Agrp*), pro-opiomelanocortin (*Pomc*) and melanocortin (*MC*) receptors, and brain-derived neurotrophic factor (*Bdnf*). To our knowledge, this is the first *Npy* KO rat model that has been created, and the KO model will be a very useful *in vivo* tool for research on Npy-related conditions, including anxiety-like behavior, alcohol and drug addiction, eating and feeding behaviors, and others.

2. Results

2.1. Creation of Npy KO rat

To test the efficiency of the 16 designed ZFN constructs targeting the rat *Npy* gene (Table S1), constructs were transfected into rat C6 cells. Surveyor nuclease analysis showed that design #6 had the highest ZFN activity and therefore was used for the single-cell embryo injection (Fig. 1A). After multiple attempts, a ZFN-induced KO rat (#65) was successfully created. *NPY* KO was confirmed by Surveyor assay (Fig. 1B). The sequenced result indicated a ZFN-induced 26-bp deletion at the targeted site, including 6 bp in intron 1 and 20 bp in exon 2 (Fig. 1C). The sequencing alignment depicts the difference in the *Npy* gene deletion region between the WT and KO (Fig. 1D). Genotyping was performed on each newborn animal (Fig. 1E). The successful creation of *Npy* KO rats was also confirmed by demonstrating only residual expression of *Npy* mRNA and no expression of neuropeptide protein in multiple tissues (Fig. 1F–H). A predicted reading frame shift happened due to the 20 bp deletion in exon 2, which resulted in earlier termination of NPY protein translation. In order to further confirm the null expression of NPY in the KO rats, immunohistochemical

staining (IHC) was performed using whole brain sections. Compared to WT ($Npy^{+/+}$), no expression of NPY in the hippo-campus of KO ($Npy^{-/-}$) was evident in IHC (Fig. 1I). Reduced NPY expression was observed in heterozygous ($Npy^{+/-}$) animals. Therefore, this ZFN targeted *Npy* KO allele was confirmed to be an *Npy* null allele.

2.2. Npy$^{-/-}$ rats show decreased body weight when compared to WT rats

Both *Npy*-deficient male and female rats were fertile. Body weight and food intake were measured each week for five weeks starting from seven weeks of age in naive animals. We observed lower body weight in male $Npy^{-/-}$ when compared to both $Npy^{+/-}$ and $Npy^{+/+}$ rats (Fig. 2A), with the mean body weights at five weeks of age for each genotype depicted in Fig. 2B. In females, $Npy^{-/-}$ rats also displayed lower body weight when compared to $Npy^{+/+}$ rats, but no difference was found between $Npy^{-/-}$ and $Npy^{+/-}$ rats (Fig. 2C), with mean body weight depicted in Fig. 2D. No significant differences in food intake between the genotypes of male rats were observed, while the only difference observed in females was at week 2, with significantly less food being consumed by $Npy^{-/-}$ when compared to the other two genotypes (Fig. S1).

2.3. Npy$^{+/-}$ rats have higher alcohol consumption than WT rats

Previous studies indicated that the iNP rats have higher *Npy* expression and lower alcohol consumption (Ehlers et al., 1998; Spence et al., 2005). Therefore, in order to identify whether *Npy* gene deletion would affect iNP rat alcohol consumption, voluntary alcohol consumption was measured using intermittent (12 h/day, 3 days/week) access to 5% and 10% EtOH (concurrently available) and water. The intermittent access protocol has been shown to increase ethanol consumption in different rat lines (Bell et al., 2014). Interestingly, significantly higher alcohol consumption was only observed in $Npy^{+/-}$ rats at 7–10 weeks compared to $Npy^{-/-}$ or WT rats. This test was initiated during early adolescence postnatal day (PND) 30–35 (i.e., 5 week old rats) (Fig. 3A). Alcohol preference significantly increased in 6-, 7-, and 10-week-old $Npy^{+/-}$ rats ($P < 0.05$), with trends of increases at 8 and 9 weeks as well (Fig. 3B). During alcohol testing session, body weights were significantly lower in 6-, 7-, and 8-week-old male and female $Npy^{-/-}$ rats when compared to $Npy^{+/-}$ and WT littermates (Fig. 3C and D). Voluntary alcohol consumption in adulthood also showed the same trend, with $Npy^{+/-}$ rats drinking more alcohol than the other two genotypes (Fig. S2).

2.4. Npy$^{-/-}$ rats show no alteration in anxiety-like behavior

ICV infusion of NPY can abolish the normal preference for the closed arm in the EPM test (Heilig et al., 1989), can reduce the level of punishment response in the Geller-Seifterpunished responding test (Heilig et al., 1993), and can inhibit or attenuate the fear-potentiated startle response (Broqua et al., 1995). These studies demonstrate Npy has a dose-dependent anxiolytic effect. We applied the EPM test, which involves exploration of a plus-shaped maze consisting of two open and two closed arms, where decreased time in the open arms is considered an indicator of elevated anxiety. When compared to $Npy^{-/-}$ and WT rats, $Npy^{+/-}$ rats spent a longer time in the open arms (Fig. S3A). However, no statistically significant difference was observed in either duration or percentage of time spent in the open arm when compared to total time of arm entries (Fig. S3A and C). Likewise, there was no

observed significant difference in open arm entries and percentage of open arm entries to total arm entries (Fig. S3B and D). Taken as a whole, the results indicate no difference in anxiety-like behavior between genotypes.

2.5. Npy elimination affects multiple neuropeptide systems

Genes known to interact with *Npy* (*Npy1r*, *Npy2r*, *Npy5r*, *Crh*, *Crhr1*, *Crhr2*, *Pomc*, *Mc3r*, *Mc4r*, *Agrp*, and *Bdnf*) were selected for gene expression measurement. Alcohol use disorder is a complex disease involving multiple neuropeptide systems (Bell et al., 2012; Roberto et al., 2012; Pleil et al., 2015). Evidence has shown that NPY and its associated Y1, Y2, and Y5 receptors (Heilig and Thorsell, 2002; Schroeder et al., 2003; Lin et al., 2004; Leising et al., 2009), as well as CRH and its receptors CRH-R1 and CRH-R2 (Ehlers et al., 1992; Gilpin et al., 2008a; Lowery et al., 2010), influence voluntary alcohol consumption and stress responses (Sajdyk et al., 2006). Gene expression analyses revealed that $Npy^{-/-}$ and $Npy^{+/-}$ rats have increased *Npy1r*, *Npy2r*, and *Npy5r* expressions in the whole brain relative to WT rats (Fig. 4A–C), indicating that the dose effect of knocking out *Npy* results in an increase in these three receptor transcripts, possibly as a means of compensation. Similarly, expression levels of *Crh*, *Crhr1*, and *Crhr2* in the whole brain (Fig. 4D–F) were increased in both $Npy^{-/-}$ and $Npy^{+/-}$ rats. Interestingly, *Crhr2* expression was the highest in $Npy^{+/-}$ rats (Fig. 4F). Studies on alcohol and drug addiction have discovered a clear role for MC neuropeptide system (Alvaro et al., 1997; Lindblom et al., 2002; Olney et al., 2014). Activation of neurons characterized by their expression of agouti-related peptide (AgRP), an antagonist of MC receptors (primarily MC3R and MC4R in the CNS), stimulates hypothalamic control of feeding behavior (Aponte et al., 2011). Expression levels of *Agrp* and *Mc3r* were significantly greater in both $Npy^{+/-}$ and $Npy^{-/-}$ rats while *Mc4r* was only increased in $Npy^{-/-}$ rats (Fig. 5A–C). Evidence obtained in humans and rodents indicates that β-endorphin (encoded by the *Pomc* gene) is critical in the regulation of alcohol drinking behavior (Topel, 1988; Racz et al., 2008). The expression of *Pomc* was higher in the $Npy^{+/-}$ rats, but lower in the $Npy^{-/-}$ rats relative to WT controls (Fig. 5D). BDNF modulates hippocampal plasticity and hippocampal-dependent memory (Egan et al., 2003), dopaminergic reward processing (Pecina et al., 2014), as well as cocaine and alcohol responses (Horger et al., 1999; Lu et al., 2004; Jeanblanc et al., 2009). Multiple studies have demonstrated that BDNF induces *Npy* expression (Scharfman and MacLusky, 2008; Yoshimura et al., 2009), and likewise, NPY administration up-regulates *Bdnf* expression (Corvino et al., 2012; Croce et al., 2013). In agreement with those findings, the expression of *Bdnf* was observed to be significantly lower in both $Npy^{-/-}$ and $Npy^{+/-}$ rats compared to WT controls (Fig. 5E).

3. Discussion

In this study, we successfully created *Npy* KO rats on an iNP background using ZFN technology. Body weight was decreased with no significant change in food intake in $Npy^{-/-}$ rats. Alcohol consumption was increased in the $Npy^{+/-}$ rats when tested during both adolescence and adulthood. The gene expressions of *Npy* receptors and *Crh* and its receptors were increased in $Npy^{-/-}$ and $Npy^{+/-}$ rats relative to WT controls. Significant increases in expression were also observed for *Agrp*, *Mc3r*, and *Mc4r* in the $Npy^{-/-}$ rats. *Pomc* and

Crhr2 were expressed highest in the $Npy^{+/-}$ rat compared to $Npy^{-/-}$ and WT controls. Additionally, *Bdnf* expression was decreased in the $Npy^{+/-}$ and $Npy^{-/-}$ rats, relative to WT controls.

Previous studies have shown that NP rats have lower alcohol intake as well as higher *Npy* gene expression when compared to P rats. Therefore, we hypothesized that higher ethanol consumption would be observed in $Npy^{-/-}$ KO rats. Surprisingly, we observed increased alcohol consumption only in the $Npy^{+/-}$ rats. Similar result was found in β-endorphin knockout study, in which only heterozygous mice displayed increased alcohol consumption because of β-endorphin dose effect (Grisel et al., 1999). Our results indicate that *Npy* indeed plays an important role in alcohol consumption, and its endogenous function seems to be dose-dependent. In order to understand this phenotype, several other alcohol drinking behavior related genes and addiction-relevant neuropeptides were analyzed in the $Npy^{+/+}$, $Npy^{+/-}$, and $Npy^{-/-}$ rats. In the NPY receptor family, *Npy1r*, *Npy2r*, and *Npy5r* have been posited to mediate the effect of central NPY on anxiety-like and alcohol-drinking behaviors (Sorensen et al., 2004; Wetherill et al., 2008; Morales-Medina et al., 2010; Tasan et al., 2010; Roseboom et al., 2014). We confirmed that *Npy* mRNA expression decreased in heterozygous rats and almost no expression was detectable in KO rats; however, NPY receptor mRNAs increased in heterozygous rats and to almost doubled in KO rats (Fig. 4). This indicates that the expressions of these NPY receptors appear to be negatively correlated with NPY levels. This supported our intuition that when NPY levels are reduced, there would be an up-regulation of receptor levels in an attempt to maintain equilibrium in receptor activity. Similarly, expressions of *Crh* and its receptors (*Crhr1* and *Crhr2*) were up-regulated following *Npy* reduction (+/−) and deletion (−/−). These results are consistent with previous studies indicating that the central actions of NPY and CRH have opposing functions in the regulation of emotional and reward-seeking behaviors (Sajdyk et al., 2004, 2006; Pleil et al., 2015). However, *Crhr2* levels were significantly higher in $Npy^{+/-}$ than both $Npy^{-/-}$ and WT control rats. The increases in ethanol consumption run counter to previous work, which indicated that activation of *Crhr2* decreases binge-like ethanol drinking (Lowery et al., 2010; Ryabinin et al., 2012) and that NP rats possess innately higher *Crhr2* expression in the brain (Yong et al., 2014). Studying the homeostatic balance between multiple neuropeptide systems will further reveal the systematic adaptations mediating alcohol-drinking behavior. On the other hand, higher expression of *Pomc* in the heterozygous animals appears to be consistent with a previous report that Sardinian alcohol-preferring (sP) rats have higher *Pomc* expression in specific brain regions (Zhou et al., 2013a). POMC is a polypeptide hormone precursor. Prior to alcohol consumption, sP rats had higher basal hypothalamic *Pomc* mRNA expression than Sardinian alcohol-nonpreferring (sNP) rats, and this expression increased following alcohol consumption. (Zhou et al., 2013a). Manipulation of the MC receptor using the melanocortin 3/4 receptor agonist MTII decreases alcohol intake in chronic ethanol-drinking Alko alcohol-accepting (AA) rats (Ploj et al., 2002; Polidori et al., 2006). High expression of *Mc3r* and *Mc4r* in $Npy^{-/-}$ rats may play a role in their lower alcohol consumption. This *Npy* KO rat model provides an opportunity to study *Npy* expression level-dependent effects on other neuropeptide systems and how these neuropeptide systems interact to control alcohol consumption and other behaviors.

Previous studies have indicated that NPY and BDNF interact to modulate alcohol addiction. For example, it was found that lower BDNF expression is associated with higher alcohol consumption (Pandey et al., 2005; Yan et al., 2005; Jeanblanc et al., 2009), and in the P rat, higher alcohol consumption is consistent with its lower BDNF level (Moonat et al., 2011). Other studies have demonstrated a positive association between BDNF and NPY CNS expression levels (Scharfman and MacLusky, 2008; Yoshimura et al., 2009; Corvino et al., 2012; Croce et al., 2013). In the present study, both $Npy^{+/-}$ and $Npy^{-/-}$ rats displayed dramatically reduced *Bdnf* expression levels (Fig. 5E). This suggests that deficits in NPY signaling and decreased BDNF appear to be important elements underlying molecular processes mediating alcoholism. However, unexpectedly only $Npy^{+/-}$ rats exhibited the higher alcohol drinking phenotype, which indicates that low *Bdnf* alone is insufficient to promote alcohol consumption. Importantly, this is the first evidence that the total or partial loss of NPY significantly reduces the expression of *Bdnf* in a KO model.

The relative expressions of *Npy*, *Agrp*, and *Pomc* were also investigated in relation to body weight differences in the $Npy^{-/-}$, $Npy^{+/-}$, and WT rats. *Npy* and *Agrp* are co-localized in the hypothalamus and function as orexigenic peptides (Krashes et al., 2011, 2013; Parker, 2013). However, some clinical studies have found that hypothalamic expression levels of *Agrp* and *Npy* are negatively correlated with body weight change, with higher *Agrp* expression level associated with lower body weights (Alkemade et al., 2012). NPY immunoreactivity is lower in obese subjects (Goldstone et al., 2002; Alkemade et al., 2012), while AgRP immunoreactivity shows either no change (Goldstone et al., 2002) or a U-shaped correlation with BMI (Alkemade et al., 2012). These mixed findings, along with the present results, indicate that multiple neuropeptide effects must be considered when investigating consumptive behaviors or their consequences. We observed that $Npy^{-/-}$ rats with higher *Agrp* and lower *Pomc* (Fig. 5B and D) expressions have lower body weights. Previous studies have shown that activation of AgRP neurons promotes feeding, while stimulation of POMC neurons reduces food intake and body weight (Aponte et al., 2011). In the present study, Agrp and *Pomc* expressions appear to counter each other in $Npy^{-/-}$ and $Npy^{+/-}$ animals. Maybe *Npy* dose is sufficient to maintain body weight. Additionally, this result implies that altered body weight in the KO rats is independent of food intake or alcohol preference. Thus, other possible mechanisms of energy utilization/balance should be considered (Parker et al., 2015).

In view of all the above described gene expression changes, we propose an inverted U-shaped curve model to understand the drinking behavior contributions of *Npy* and its related genes (Fig. 6). At both ends of the inverted U-shaped relationship, the WT and KO animals display low alcohol consumption compared to the *Npy* heterozygous animals. When *Npy* expression is completely abrogated, other gene expression levels begin to play a compensatory role to maintain the network balance that determines alcohol drinking behavior. In contrast, with the exception of the high expression levels of *Pomc* and *Crhr2*, $Npy^{+/-}$ animals expressed medium levels of *Npy* and other genes that were between the WT and KO levels. These heterozygous animals notably drank more alcohol than the other two genotypes, which indicates an interplay among *Npy*, *Pomc*, and *Crhr2*.

The NPY system mediates many physiological and behavioral processes and, as such, is a key pharmacological target for many psychiatric disorders (Wu et al., 2011). Creation of the *Npy* KO is a step forward in understanding NPY and its related neuropeptide systems. The KO model is a novel tool for the translational research field and will be useful as a genetic *in vivo* means for complex disease research.

4. Materials and methods

4.1. Animals

All animals were housed in facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The experimental procedures were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee and are in accordance with the guidelines of the Guide for the Care and Use of Laboratory Animals.

4.2. ZFN design and testing in cell lines

ZFN constructs were co-designed by Sigma-Aldrich (USA) and the authors. In brief, the constructs consisted of sequence-generated zinc-finger proteins that specifically bind to targeted sequences, and an endonuclease (*Fok* I) that forms a heterodimer to cleave the target gene. The constructs have been described in detail elsewhere (Cathomen and Joung, 2008; Maeder et al., 2008; Carbery et al., 2010). Sixteen oligos were designed to target the genomic region around exon 2 (Table S1), and all of them were tested for their efficiency in knocking out gene expression in rat cell lines. Rat C6 cells were transfected with these pre-designed ZFNs and were harvested one day after transfection. DNA and RNA were isolated and Surveyor nuclease cleavage analysis was performed according to the manufacturer's protocol (Transgenomic, USA). *CEL*-I digestions were performed to cut both strands of the DNA heteroduplex and samples were run on a 10% PAGE-TBE gel.

4.3. Creation of Npy KO rats

Background iNP rats were developed by selective breeding for low alcohol preference, followed by inbreeding for more than 15 generations (Carr et al., 2006). Embryo collection and RNA injection were performed as described previously (Geurts et al., 2009). Genomic DNA isolated from all F_1 rats was used to sequence areas around the ZFN targeted region using four primers (Table S2, Npy-ZFN primers). Primers were designed based on the *Npy* genomic sequence accessed from the Ensembl Genome Browser (accession No. ENSRNOG00000009768). These primers were utilized to amplify two overlapping DNA fragments that encompassed a 2-kb region including the promoter, exon 1, intron 1, and exon 2.

4.4. Food intake and body weight measurement

Food intake and body weight measurements were taken in naive adult animals for five weeks starting at about PND 50. Both male and female rats with three genotypes: $Npy^{+/+}$ (8 males and 3 females), $Npy^{+/-}$ (5 males and 3 females), and $Npy^{-/-}$ (8 males and 5 females) were measured twice per week for body weight and food intake. Differences were compared between genotypes independently for males and females.

4.5. Free-choice alcohol drinking protocol

Adolescent ethanol-naive male and female rats were tested for voluntary alcohol drinking following a previously described protocol (Spence et al., 2013). The three groups of rats, $Npy^{+/+}$ (16 males and 11 females), $Npy^{+/-}$ (6 males and 7 females), and $Npy^{-/-}$ (9 males and 2 females) rats were used. Alcohol consumption and preference were also tested with three groups of rats, $Npy^{+/+}$ (5 males and 6 females), $Npy^{+/-}$ (4 males and 6 females), and $Npy^{-/-}$ (2 females).

4.6. EPM test

Animals of adult age were used for the EPM test ($Npy^{+/+}$, 5 males and 5 females; $Npy^{+/-}$, 10 males and 6 females; $Npy^{-/-}$, 4 males and 5 females). An Accuscan (USA) EPM test apparatus was used. Briefly, the apparatus is made of white acrylic plastic with two open arms (50 × 10 cm) at right angles to two wall-enclosed and covered arms (50 × 10 × 50 cm). The test arena is elevated 90 cm off of the floor. Each rat was released in the center facing an open arm, with each test session lasting 5 min. The levels of brightness were approximately 45 lx and 10 lx, in the open and closed arms respectively.

4.7. Gene expression

Whole brains were extracted, snap frozen in dry ice-bathed isopentane, transferred on dry ice, and stored at −80°C until RNA isolation. RNA was isolated using TRIzol (Life Technologies, USA) and purified using the RNeasy Mini Kit (Qiagen, USA). Using the ABI PRISM 7300 Sequence Detection System (Life Technologies), the relative mRNA expression levels were normalized to *Gapdh*. The used primers are listed in Table S2. Mixed model ANOVA were used and statistical significance was set at $P < 0.05$.

4.8. ELISA and immunohistochemical staining

Npy levels were measured using the EZRMNPY-27K ELISA kit following the manufacturer's instructions (Millipore, USA). For immunohistochemical staining, rats were anesthetized by 0.6% sodium pentobarbital (0.08 mL/kg), and perfused transcardially with PBS (pH 7.4, 4°C) followed by 4% paraformaldehyde (pH 7.4) for 40 min. Coronal sections were prepared at 4 μm thickness using a microtome (Leica manual rotary microtome RM2235, Germany). Tissue sections were mounted on 3-aminopropyltriethoxysilane (APES) coated slices and dried by incubating the slices at 55°C. After the slices were deparaffinized and rehydrated, antigen retrieval was performed using a microwave. Nonspecific antibody binding was blocked with goat serum working solution (Zhongshan Goldenbridge Biotechnology, China). The slices were then incubated overnight at 4°C with the mouse anti-NPY primary antibody (2 mg/mL, SAB1404138, Sigma-Aldrich), and incubated with Polink-2 plus Polymer HRP Detection System following the manufacturer's instructions (Zhongshan Goldenbridge Biotechnology). Finally, slices were visualized with DAB chromagen (Zhongshan Golden-bridge Biotechnology). Photomicrographs were acquired using a CTR6000 microscope with a DFC450 C camera (Leica, Germany). For negative controls, the primary antibody was replaced with PBS.

4.9. Statistical analysis

ANOVA was used to compare alcohol consumption followed by post-hoc test. Student's *t*-test analysis was used for EPM and gene expression change differences between genotypes. The *P*-value indicated the significance of difference between two genotypes. Statistical significance was set at $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by grants from the National Basic Research Program (No. 2013CB945001), the National Science Foundation of China (No. 81272273), the State High-Tech Program (No. 2012AA022403), PUMC Youth Fund, and the National Institutes of Health (Nos. NIAAA P60AA007611, U01AA013522 and R24AA015512). We would also like to express our appreciation to Michelle Johnson, Shaoliang Jing and Dr. Loren Field, Transgenic and Knock-Out Mouse Core, for providing equipment and technical support, and Drs. David Crabb and Janice Froehlich, Indiana Alcohol Research Center, for their support in initiating this pilot project and providing useful discussion of this research.

References

Adrian TE, Allen JM, Bloom SR, Ghatei MA, Rossor MN, Roberts GW, Crow TJ, Tatemoto K, Polak JM. Neuropeptide Y distribution in human brain. Nature. 1983; 306:584–586. [PubMed: 6358901]

Alkemade A, Yi CX, Pei L, Harakalova M, Swaab DF, la Fleur SE, Fliers E, Kalsbeek A. AgRP and NPY expression in the human hypothalamic infundibular nucleus correlate with body mass index, whereas changes in alphaMSH are related to type 2 diabetes. J. Clin. Endocrinol. Metab. 2012; 97:E925–E933. [PubMed: 22492775]

Alvaro JD, Tatro JB, Duman RS. Melanocortins and opiate addiction. Life Sci. 1997; 61:1–9. [PubMed: 9200663]

Aponte Y, Atasoy D, Sternson SM. AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. Nat. Neurosci. 2011; 14:351–355. [PubMed: 21209617]

Badia-Elder NE, Stewart RB, Powrozek TA, Roy KF, Murphy JM, Li TK. Effect of neuropeptide Y (NPY) on oral ethanol intake in Wistar, alcohol-preferring (P), and -nonpreferring (NP) rats. Alcohol Clin. Exp. Res. 2001; 25:386–390. [PubMed: 11290849]

Bannon AW, Seda J, Carmouche M, Francis JM, Norman MH, Karbon B, McCaleb ML. Behavioral characterization of neuropeptide Y knockout mice. Brain Res. 2000; 868:79–87. [PubMed: 10841890]

Bell RL, Sable HJ, Colombo G, Hyytia P, Rodd ZA, Lumeng L. Animal models for medications development targeting alcohol abuse using selectively bred rat lines: neurobiological and pharmacological validity. Pharmacol. Biochem. Behav. 2012; 103:119–155. [PubMed: 22841890]

Bice P, Foroud T, Bo R, Castelluccio P, Lumeng L, Li TK, Carr LG. Genomic screen for QTLs underlying alcohol consumption in the P and NP rat lines. Mamm. Genome. 1998; 9:949–955. [PubMed: 9880658]

Broqua P, Wettstein JG, Rocher MN, Gauthier-Martin B, Junien JL. Behavioral effects of neuropeptide Y receptor agonists in the elevated plus-maze and fear-potentiated startle procedures. Behav. Pharmacol. 1995; 6:215–222. [PubMed: 11224329]

Carbery ID, Ji D, Harrington A, Brown V, Weinstein EJ, Liaw L, Cui X. Targeted genome modification in mice using zinc-finger nucleases. Genetics. 2010; 186:451–459. [PubMed: 20628038]

Carr LG, Foroud T, Bice P, Gobbett T, Ivashina J, Edenberg H, Lumeng L, Li TK. A quantitative trait locus for alcohol consumption in selectively bred rat lines. Alcohol Clin. Exp. Res. 1998; 22:884–887. [PubMed: 9660316]

Carr LG, Habegger K, Spence JP, Liu L, Lumeng L, Foroud T. Development of congenic rat strains for alcohol consumption derived from the alcohol-preferring and nonpreferring rats. Behav. Genet. 2006; 36:285–290. [PubMed: 16470346]

Carr LG, Kimpel MW, Liang T, McClintick JN, McCall K, Morse M, Edenberg HJ. Identification of candidate genes for alcohol preference by expression profiling of congenic rat strains. Alcohol Clin. Exp. Res. 2007; 31:1089–1098. [PubMed: 17451403]

Cathomen T, Joung JK. Zinc-finger nucleases: the next generation emerges. Mol. Ther. 2008; 16:1200–1207. [PubMed: 18545224]

Clark JT, Kalra PS, Crowley WR, Kalra SP. Neuropeptide Y and human pancreatic polypeptide stimulate feeding behavior in rats. Endocrinology. 1984; 115:427–429. [PubMed: 6547387]

Corvino V, Marchese E, Giannetti S, Lattanzi W, Bonvissuto D, Biamonte F, Mongiovi AM, Michetti F, Geloso MC. The neuroprotective and neurogenic effects of neuropeptide Y administration in an animal model of hippocampal neurodegeneration and temporal lobe epilepsy induced by trimethyltin. J. Neurochem. 2012; 122:415–426. [PubMed: 22537092]

Croce N, Gelfo F, Ciotti MT, Federici G, Caltagirone C, Bernardini S, Angelucci F. NPY modulates miR-30a-5p and BDNF in opposite direction in an *in vitro* model of Alzheimer disease: a possible role in neuroprotection? Mol. Cell. Biochem. 2013; 376:189–195. [PubMed: 23358924]

Egan MF, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, Zaitsev E, Gold B, Goldman D, Dean M, Lu B, Weinberger DR. The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell. 2003; 112:257–269. [PubMed: 12553913]

Ehlers CL, Chaplin RI, Wall TL, Lumeng L, Li TK, Owens MJ, Nemeroff CB. Corticotropin releasing factor (CRF): studies in alcohol preferring and non-preferring rats. Psychopharmacology (Berl.). 1992; 106:359–364. [PubMed: 1570383]

Ehlers CL, Li TK, Lumeng L, Hwang BH, Somes C, Jimenez P, Mathe AA. Neuropeptide Y levels in ethanol-naive alcohol-preferring and nonpreferring rats and in Wistar rats after ethanol exposure. Alcohol Clin. Exp. Res. 1998; 22:1778–1782. [PubMed: 9835294]

Fuzesi T, Wittmann G, Liposits Z, Lechan RM, Fekete C. Contribution of noradrenergic and adrenergic cell groups of the brainstem and agouti-related protein-synthesizing neurons of the arcuate nucleus to neuropeptide-y inner-vation of corticotropin-releasing hormone neurons in hypothalamic paraventricular nucleus of the rat. Endocrinology. 2007; 148:5442–5450. [PubMed: 17690163]

Geurts AM, Cost GJ, Freyvert Y, Zeitler B, Miller JC, Choi VM, Jenkins SS, Wood A, Cui X, Meng X, Vincent A, Lam S, Michalkiewicz M, Schilling R, Foeckler J, Kalloway S, Weiler H, Menoret S, Anegon I, Davis GD, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Jacob HJ, Buelow R. Knockout rats via embryo microinjection of zinc-finger nucleases. Science. 2009; 325:433. [PubMed: 19628861]

Gilpin NW, Misra K, Herman MA, Cruz MT, Koob GF, Roberto M. Neuropeptide Y opposes alcohol effects on gamma-aminobutyric acid release in amygdala and blocks the transition to alcohol dependence. Biol. Psychiatry. 2011; 69:1091–1099. [PubMed: 21459365]

Gilpin NW, Richardson HN, Koob GF. Effects of CRF1-receptor and opioid-receptor antagonists on dependence-induced increases in alcohol drinking by alcohol-preferring (P) rats. Alcohol Clin. Exp. Res. 2008a; 32:1535–1542. [PubMed: 18631323]

Gilpin NW, Stewart RB, Badia-Elder NE. Neuropeptide Y administration into the amygdala suppresses ethanol drinking in alcohol-preferring (P) rats following multiple deprivations. Pharmacol. Biochem. Behav. 2008b; 90:470–474. [PubMed: 18499241]

Gilpin NW, Stewart RB, Badia-Elder NE. Neuropeptide Y suppresses ethanol drinking in ethanol-abstinent, but not non-ethanol-abstinent, Wistar rats. Alcohol. 2008c; 42:541–551. [PubMed: 18835592]

Gilpin NW, Stewart RB, Murphy JM, Badia-Elder NE. Sensitized effects of neuropeptide Y on multiple ingestive behaviors in P rats following ethanol abstinence. Pharmacol. Biochem. Behav. 2005; 81:740–749. [PubMed: 16023707]

Goldstone AP, Unmehopa UA, Bloom SR, Swaab DF. Hypothalamic NPY and agouti-related protein are increased in human illness but not in Prader-Willi syndrome and other obese subjects. J. Clin. Endocrinol. Metab. 2002; 87:927–937. [PubMed: 11836343]

Heilig M, McLeod S, Brot M, Heinrichs SC, Menzaghi F, Koob GF, Britton KT. Anxiolytic-like action of neuropeptide Y: mediation by Y1 receptors in amygdala, and dissociation from food intake effects. Neuropsychopharmacology. 1993; 8:357–363. [PubMed: 8099792]

Heilig M, Soderpalm B, Engel JA, Widerlov E. Centrally administered neuropeptide Y (NPY) produces anxiolytic-like effects in animal anxiety models. Psychopharmacology (Berl.). 1989; 98:524–529. [PubMed: 2570434]

Heilig M, Thorsell A. Brain neuropeptide Y (NPY) in stress and alcohol dependence. Rev. Neurosci. 2002; 13:85–94. [PubMed: 12013027]

Horger BA, Iyasere CA, Berhow MT, Messer CJ, Nestler EJ, Taylor JR. Enhancement of locomotor activity and conditioned reward to cocaine by brain-derived neurotrophic factor. J. Neurosci. 1999; 19:4110–4122. [PubMed: 10234039]

Jeanblanc J, He DY, Carnicella S, Kharazia V, Janak PH, Ron D. Endogenous BDNF in the dorsolateral striatum gates alcohol drinking. J. Neurosci. 2009; 29:13494–13502. [PubMed: 19864562]

Jolicoeur FB, Michaud JN, Rivest R, Menard D, Gaudin D, Fournier A, St-Pierre S. Neurobehavioral profile of neuropeptide Y. Brain Res. Bull. 1991; 26:265–268. [PubMed: 2012986]

Kash TL, Winder DG. Neuropeptide Y and corticotropin-releasing factor bi-directionally modulate inhibitory synaptic transmission in the bed nucleus of the stria terminalis. Neuropharmacology. 2006; 51:1013–1022. [PubMed: 16904135]

Kask A, Harro J, von Horsten S, Redrobe JP, Dumont Y, Quirion R. The neurocircuitry and receptor subtypes mediating anxiolytic-like effects of neuropeptide Y. Neurosci. Biobehav. Rev. 2002; 26:259–283. [PubMed: 12034130]

Krashes MJ, Koda S, Ye C, Rogan SC, Adams AC, Cusher DS, Maratos-Flier E, Roth BL, Lowell BB. Rapid, reversible activation of AgRP neurons drives feeding behavior in mice. J. Clin. Invest. 2011; 121:1424–1428. [PubMed: 21364278]

Krashes MJ, Shah BP, Koda S, Lowell BB. Rapid versus delayed stimulation of feeding by the endogenously released AgRP neuron mediators GABA, NPY, and AgRP. Cell Metab. 2013; 18:588–595. [PubMed: 24093681]

Leising ML, Bertholomey ML, Stewart RB, Badia-Elder NE. The NPY Y2 antagonist BIIE0246 blocks the expression of an alcohol deprivation effect in alcohol preferring (P) rats. Alcohol. Clin. Exp. Res. 2009; 33:95A. [PubMed: 18945220]

Liang T, Kimpel MW, McClintick JN, Skillman AR, McCall K, Edenberg HJ, Carr LG. Candidate genes for alcohol preference identified by expression profiling in alcohol-preferring and -nonpreferring reciprocal congenic rats. Genome Biol. 2010; 11:R11. [PubMed: 20128895]

Lin S, Boey D, Herzog H. NPY and Y receptors: lessons from transgenic and knockout models. Neuropeptides. 2004; 38:189–200. [PubMed: 15337371]

Lindblom J, Wikberg JE, Bergstrom L. Alcohol-preferring AA rats show a derangement in their central melanocortin signalling system. Pharmacol. Biochem. Behav. 2002; 72:491–496. [PubMed: 11900824]

Lowery EG, Spanos M, Navarro M, Lyons AM, Hodge CW, Thiele TE. CRF-1 antagonist and CRF-2 agonist decrease binge-like ethanol drinking in C57BL/6J mice independent of the HPA axis. Neuropsychopharmacology. 2010; 35:1241–1252. [PubMed: 20130533]

Lu L, Dempsey J, Liu SY, Bossert JM, Shaham Y. A single infusion of brain-derived neurotrophic factor into the ventral tegmental area induces long-lasting potentiation of cocaine seeking after withdrawal. J. Neurosci. 2004; 24:1604–1611. [PubMed: 14973246]

Maeder ML, Thibodeau-Beganny S, Osiak A, Wright DA, Anthony RM, Eichtinger M, Jiang T, Foley JE, Winfrey RJ, Townsend JA, Unger-Wallace E, Sander JD, Muller-Lerch F, Fu F, Pearlberg J, Gobel C, Dassie JP, Pruett-Miller SM, Porteus MH, Sgroi DC, Iafrate AJ, Dobbs D, McCray PB Jr. Cathomen T, Voytas DF, Joung JK. Rapid “open-source” engineering of customized zinc-finger nucleases for highly efficient gene modification. Mol. Cell. 2008; 31:294–301. [PubMed: 18657511]

McBride WJ, Rodd ZA, Bell RL, Lumeng L, Li TK. The alcohol-preferring (P) and high-alcohol-drinking (HAD) ratseanimal models of alcoholism. Alcohol. 2014; 48:209–215. [PubMed: 24268381]

Moonat S, Sakharkar AJ, Zhang H, Pandey SC. The role of amygdaloid brain-derived neurotrophic factor, activity-regulated cytoskeleton-associated protein and dendritic spines in anxiety and alcoholism. Addict Biol. 2011; 16:238–250. [PubMed: 21182574]

Morales-Medina JC, Dumont Y, Quirion R. A possible role of neuropeptide Y in depression and stress. Brain Res. 2010; 1314:194–205. [PubMed: 19782662]

Murphy JM, Stewart RB, Bell RL, Badia-Elder NE, Carr LG, McBride WJ, Lumeng L, Li TK. Phenotypic and genotypic characterization of the Indiana University rat lines selectively bred for high and low alcohol preference. Behav. Genet. 2002; 32:363–388. [PubMed: 12405517]

Olling JD, Ulrichsen J, Haugbol S, Glenthoj B, Hemmingsen R, Woldbye DP. Decreased gene expression of neuropeptide Y and its receptors in hippocampal regions during ethanol withdrawal in rats. Neurosci. Lett. 2007; 424:160–164. [PubMed: 17723274]

Olney JJ, Sprow GM, Navarro M, Thiele TE. The protective effects of the melanocortin receptor (MCR) agonist, melanotan-II (MTII), against binge-like ethanol drinking are facilitated by deletion of the MC3 receptor in mice. Neuropeptides. 2014; 48:47–51. [PubMed: 24290566]

Palmiter RD, Erickson JC, Hollopeter G, Baraban SC, Schwartz MW. Life without neuropeptide Y. Recent Prog. Horm. Res. 1998; 53:163–199. [PubMed: 9769708]

Pandey SC, Zhang H, Roy A, Xu T. Deficits in amygdaloid cAMP-responsive element-binding protein signaling play a role in genetic predisposition to anxiety and alcoholism. J. Clin. Invest. 2005; 115:2762–2773. [PubMed: 16200210]

Parker LM, Kumar NN, Lonergan T, McMullan S, Goodchild AK. Distribution and neurochemical characterization of neurons in the rat ventrolateral medulla activated by glucoprivation. Brain Struct. Funct. 2015; 220:117–134. [PubMed: 24100548]

Parker, S. Neuropeptide Y: Molecular Structure, Role in Food Intake and Direct/Indirect Effect. Nova Science Publishers; Hauppauge, NY.: 2013.

Patrylo PR, van den Pol AN, Spencer DD, Williamson A. NPY inhibits glutamatergic excitation in the epileptic human dentate gyrus. J. Neurophysiol. 1999; 82:478–483. [PubMed: 10400974]

Pecina M, Martinez-Jauand M, Love T, Heffernan J, Montoya P, Hodgkinson C, Stohler CS, Goldman D, Zubieta JK. Valence-specific effects of *BDNF* Val66Met polymorphism on dopaminergic stress and reward processing in humans. J. Neurosci. 2014; 34:5874–5881. [PubMed: 24760847]

Pleil KE, Rinker JA, Lowery-Gionta EG, Mazzone CM, McCall NM, Kendra AM, Olson DP, Lowell BB, Grant KA, Thiele TE, Kash TL. NPY signaling inhibits extended amygdala CRF neurons to suppress binge alcohol drinking. Nat. Neurosci. 2015; 18:545–552. [PubMed: 25751534]

Ploj K, Roman E, Kask A, Hyytia P, Schioth HB, Wikberg JE, Nylander I. Effects of melanocortin receptor ligands on ethanol intake and opioid peptide levels in alcohol-preferring AA rats. Brain Res. Bull. 2002; 59:97–104. [PubMed: 12379439]

Polidori C, Geary N, Massi M. Effect of the melanocortin receptor stimulation or inhibition on ethanol intake in alcohol-preferring rats. Peptides. 2006; 27:144–149. [PubMed: 16198024]

Racz I, Schurmann B, Karpushova A, Reuter M, Cichon S, Montag C, Furst R, Schutz C, Franke PE, Strohmaier J, Wienker TF, Terenius L, Osby U, Gunnar A, Maier W, Bilkei-Gorzo A, Nothen M, Zimmer A. The opioid peptides enkephalin and beta-endorphin in alcohol dependence. Biol. Psychiatry. 2008; 64:989–997. [PubMed: 18589403]

Roberto M, Gilpin NW, Siggins GR. The central amygdala and alcohol: role of gamma-aminobutyric acid, glutamate, and neuropeptides. Cold Spring Harb. Perspect. Med. 2012; 2:a012195. [PubMed: 23085848]

Romano S, D'Angelillo A, D'Arrigo P, Staibano S, Greco A, Brunetti A, Scalvenzi M, Bisogni R, Scala I, Romano MF. FKBP51 increases the tumour-promoter potential of TGF-beta. Clin. Transl. Med. 2014; 3:1. [PubMed: 24460977]

Roseboom PH, Nanda SA, Fox AS, Oler JA, Shackman AJ, Shelton SE, Davidson RJ, Kalin NH. Neuropeptide Y receptor gene expression in the primate amygdala predicts anxious temperament and brain metabolism. Biol. Psychiatry. 2014; 76:850–857. [PubMed: 24342924]

Ryabinin AE, Tsoory MM, Kozicz T, Thiele TE, Neufeld-Cohen A, Chen A, Lowery-Gionta EG, Giardino WJ, Kaur S. Urocortins: CRF's siblings and their potential role in anxiety, depression and alcohol drinking behavior. Alcohol. 2012; 46:349–357. [PubMed: 22444954]

Sajdyk TJ, Fitz SD, Shekhar A. The role of neuropeptide Y in the amygdala on corticotropin-releasing factor receptor-mediated behavioral stress responses in the rat. Stress. 2006; 9:21–28. [PubMed: 16753930]

Sajdyk TJ, Shekhar A, Gehlert DR. Interactions between NPY and CRF in the amygdala to regulate emotionality. Neuropeptides. 2004; 38:225–234. [PubMed: 15337374]

Scharfman HE, MacLusky NJ. Estrogen-growth factor interactions and their contributions to neurological disorders. Headache. 2008; 48(Suppl. 2):S77–S89. [PubMed: 18700946]

Schroeder JP, Olive F, Koenig H, Hodge CW. Intra-amygdala infusion of the NPY Y1 receptor antagonist BIBP 3226 attenuates operant ethanol self-administration. Alcohol Clin. Exp. Res. 2003; 27:1884–1891. [PubMed: 14691375]

Smialowska M, Wieronska JM, Szewczyk B, Obuchowicz E. Effect of some iono- and metabotropic glutamatergic ligands on neuropeptide Y immunore-activity in the rat hippocampus. Pol. J. Pharmacol. 2002; 54:723–726. [PubMed: 12866732]

Sorensen G, Lindberg C, Wortwein G, Bolwig TG, Woldbye DP. Differential roles for neuropeptide Y Y1 and Y5 receptors in anxiety and sedation. J. Neurosci. Res. 2004; 77:723–729. [PubMed: 15352219]

Spence JP, Lai D, Shekhar A, Carr LG, Foroud T, Liang T. Quantitative trait locus for body weight identified on rat chromosome 4 in inbred alcohol-preferring and -nonpreferring rats: potential implications for neuropeptide Y and corticotrophin releasing hormone 2. Alcohol. 2013; 47:63–67. [PubMed: 23312492]

Spence JP, Liang T, Habegger K, Carr LG. Effect of polymorphism on expression of the neuropeptide Y gene in inbred alcohol-preferring and -non-preferring rats. Neuroscience. 2005; 131:871–876. [PubMed: 15749341]

Tasan RO, Nguyen NK, Weger S, Sartori SB, Singewald N, Heilbronn R, Herzog H, Sperk G. The central and basolateral amygdala are critical sites of neuropeptide Y/Y2 receptor-mediated regulation of anxiety and depression. J. Neurosci. 2010; 30:6282–6290. [PubMed: 20445054]

Teixeira L, Sousa DM, Nunes AF, Sousa MM, Herzog H, Lamghari M. NPY revealed as a critical modulator of osteoblast function *in vitro*: new insights into the role of Y1 and Y2 receptors. J. Cell. Biochem. 2009; 107:908–916. [PubMed: 19459152]

Thiele TE, Badia-Elder NE. A role for neuropeptide Y in alcohol intake control: evidence from human and animal research. Physiol. Behav. 2003; 79:95–101. [PubMed: 12818714]

Thiele TE, Marsh DJ, Ste Marie L, Bernstein IL, Palmiter RD. Ethanol consumption and resistance are inversely related to neuropeptide Y levels. Nature. 1998; 396:366–369. [PubMed: 9845072]

Thiele TE, Miura GI, Marsh DJ, Bernstein IL, Palmiter RD. Neurobiological responses to ethanol in mutant mice lacking neuropeptide Y or the Y5 receptor. Pharmacol. Biochem. Behav. 2000; 67:683–691. [PubMed: 11166058]

Thorsell A. Central neuropeptide Y in anxiety- and stress-related behavior and in ethanol intake. Ann. N. Y. Acad. Sci. 2008; 1148:136–140. [PubMed: 19120101]

Thorsell A, Repunte-Canonigo V, O'Dell LE, Chen SA, King AR, Lekic D, Koob GF, Sanna PP. Viral vector-induced amygdala NPY overexpression reverses increased alcohol intake caused by repeated deprivations in Wistar rats. Brain. 2007; 130:1330–1337. [PubMed: 17405766]

Thorsell A, Slawecki CJ, Ehlers CL. Effects of neuropeptide Y and corticotropin-releasing factor on ethanol intake in Wistar rats: interaction with chronic ethanol exposure. Behav. Brain Res. 2005a; 161:133–140. [PubMed: 15904720]

Thorsell A, Slawecki CJ, Ehlers CL. Effects of neuropeptide Y on appetitive and consummatory behaviors associated with alcohol drinking in wistar rats with a history of ethanol exposure. Alcohol Clin. Exp. Res. 2005b; 29:584–590. [PubMed: 15834223]

Topel H. Beta-endorphin genetics in the etiology of alcoholism. Alcohol. 1988; 5:159–165. [PubMed: 2969250]

Wahlestedt C, Karoum F, Jaskiw G, Wyatt RJ, Larhammar D, Ekman R, Reis DJ. Cocaine-induced reduction of brain neuropeptide Y synthesis dependent on medial prefrontal cortex. Proc. Natl. Acad. Sci. U. S. A. 1991; 88:2078–2082. [PubMed: 2006146]

Wetherill L, Schuckit MA, Hesselbrock V, Xuei X, Liang T, Dick DM, Kramer J, Nurnberger JI Jr. Tischfield JA, Porjesz B, Edenberg HJ, Foroud T. Neuropeptide Y receptor genes are associated with alcohol dependence, alcohol withdrawal phenotypes, and cocaine dependence. Alcohol Clin. Exp. Res. 2008; 32:2031–2040. [PubMed: 18828811]

Widdowson PS, Ordway GA, Halaris AE. Reduced neuropeptide Y concentrations in suicide brain. J. Neurochem. 1992; 59:73–80. [PubMed: 1613514]

Widerlov E, Lindstrom LH, Wahlestedt C, Ekman R. Neuropeptide Y and peptide YY as possible cerebrospinal fluid markers for major depression and schizophrenia, respectively. J. Psychiatr. Res. 1988; 22:69–79. [PubMed: 3397912]

Woldbye DP, Madsen TM, Larsen PJ, Mikkelsen JD, Bolwig TG. Neuropeptide Y inhibits hippocampal seizures and wet dog shakes. Brain Res. 1996; 737:162–168. [PubMed: 8930362]

Wu G, Feder A, Wegener G, Bailey C, Saxena S, Charney D, Mathe AA. Central functions of neuropeptide Y in mood and anxiety disorders. Expert Opin. Ther. Targets. 2011; 15:1317–1331. [PubMed: 21995655]

Yan QS, Feng MJ, Yan SE. Different expression of brain-derived neurotrophic factor in the nucleus accumbens of alcohol-preferring (P) and -non-preferring (NP) rats. Brain Res. 2005; 1035:215–218. [PubMed: 15722062]

Yong W, Spence JP, Eskay R, Fitz SD, Damadzic R, Lai D, Foroud T, Carr LG, Shekhar A, Chester JA, Heilig M, Liang T. Alcohol-preferring rats show decreased corticotropin-releasing hormone-2 receptor expression and differences in HPA activation compared to alcohol-nonpreferring rats. Alcohol Clin. Exp. Res. 2014; 38:1275–1283. [PubMed: 24611993]

Yoshimura M, Hagimoto M, Matsuura T, Ohkubo J, Ohno M, Maruyama T, Ishikura T, Hashimoto H, Kakuma T, Yoshimatsu H, Terawaki K, Uezono Y, Toyohira Y, Yanagihara N, Ueta Y. Effects of food deprivation on the hypothalamic feeding-regulating peptides gene expressions in serotonin depleted rats. J. Physiol. Sci. 2014; 64:97–104. [PubMed: 24162946]

Yoshimura R, Ito K, Endo Y. Differentiation/maturation of neuropeptide Y neurons in the corpus callosum is promoted by brain-derived neurotrophic factor in mouse brain slice cultures. Neurosci. Lett. 2009; 450:262–265. [PubMed: 19103259]

Zarjevski N, Cusin I, Vettor R, Rohner-Jeanrenaud F, Jeanrenaud B. Chronic intracerebroventricular neuropeptide-Y administration to normal rats mimics hormonal and metabolic changes of obesity. Endocrinology. 1993; 133:1753–1758. [PubMed: 8404618]

Zhang H, Sakharkar AJ, Shi G, Ugale R, Prakash A, Pandey SC. Neuropeptide Y signaling in the central nucleus of amygdala regulates alcohol-drinking and anxiety-like behaviors of alcohol-preferring rats. Alcohol Clin. Exp. Res. 2010; 34:451–461. [PubMed: 20028368]

Zhou Y, Colombo G, Niikura K, Carai MA, Femenia T, Garcia-Gutierrez MS, Manzanares J, Ho A, Gessa GL, Kreek MJ. Voluntary alcohol drinking enhances proopiomelanocortin gene expression in nucleus accumbens shell and hypothalamus of Sardinian alcohol-preferring rats. Alcohol Clin. Exp. Res. 2013a; 37(Suppl. 1):E131–E140. [PubMed: 22724395]

Zhou Z, Karlsson C, Liang T, Xiong W, Kimura M, Tapocik JD, Yuan Q, Barbier E, Feng A, Flanigan M, Augier E, Enoch MA, Hodgkinson CA, Shen PH, Lovinger DM, Edenberg HJ, Heilig M, Goldman D. Loss of metabotropic glutamate receptor 2 escalates alcohol consumption. Proc. Natl. Acad. Sci. U. S. A. 2013b; 110:16963–16968. [PubMed: 24082084]

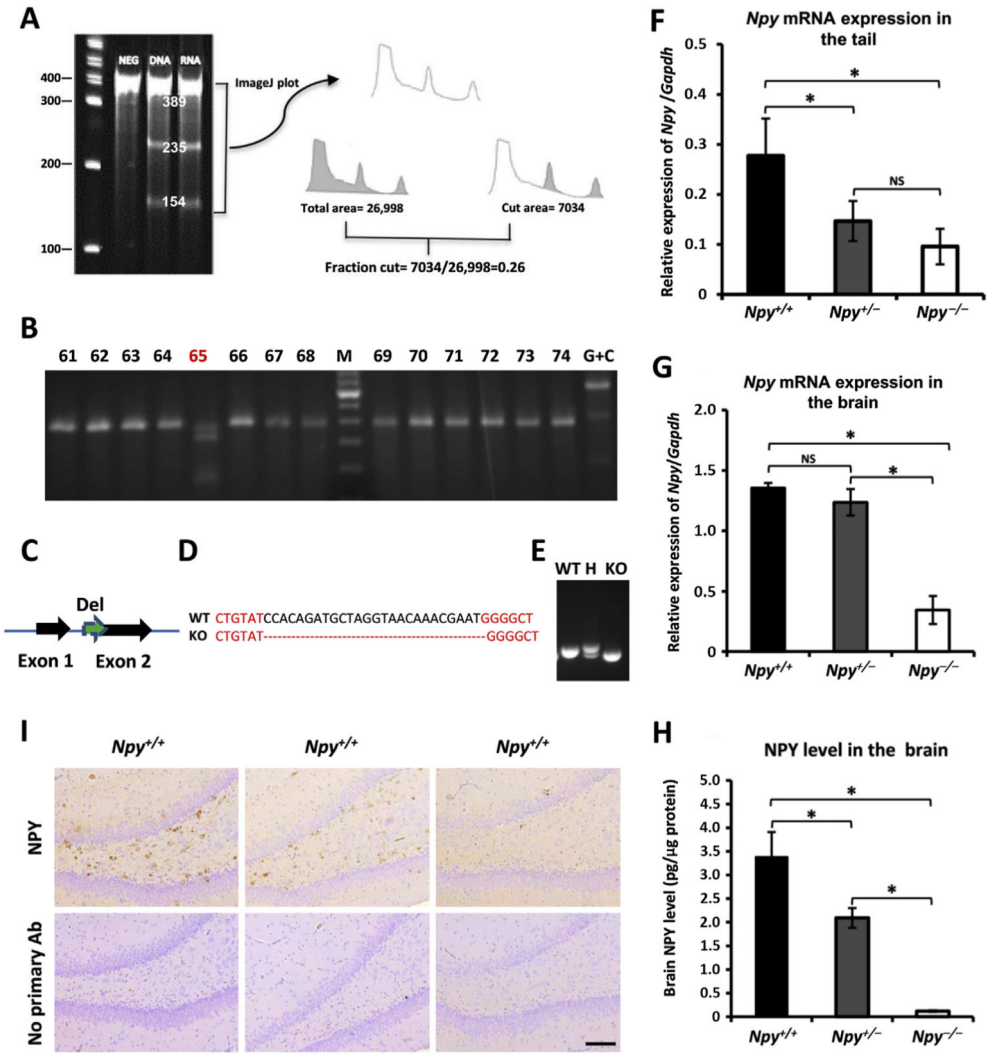

Fig. 1.

Successful creation of *Npy* KO rats. **A**: *In vitro* ZFN activity assay. Rat C6 cells were transfected and harvested one day after transfection. The pre-designed ZFNs were validated by Surveyor nuclease cleavage analysis, in which endonuclease *CEL*-I is able to cut both strands of a DNA heteroduplex. *CEL*-I digestions were performed and samples were run on a 10% PAGE-TBE gel. Design #6 has the highest ZFN activity (14%). The fraction cut ratio was calculated based on the relative intensity of the DNA bands (389 bp, 235 bp and 154 bp) using ImageJ software. NEG: negative control. **B**: Animal #65 was a KO rat that was confirmed by Surveyor nuclease cleavage assay. G + C lane is positive control of the assay. **C**: Location of the deletion on the *Npy* gene. **D**: A 26-bp deletion was confirmed by sequencing, which includes 6 bp in intron 1 and 20 bp in exon 2. Multiple KO sequences aligned with $Npy^{+/+}$ (WT). **E**: PCR analyses of WT ($Npy^{+/+}$), H (heterozygous, $Npy^{+/-}$), and KO ($Npy^{-/-}$) genotypes. **F**: *Npy* mRNA expression in the tail of rats with different genotypes. **G**: *Npy* mRNA expression in the brain of rats with different genotypes. **H**: NPY peptide level in the brain. **I**: Immunohistochemical staining indicated no expression of NPY

in the hippocampus of $Npy^{-/-}$ rats and medium level of NPY expression was observed in $Npy^{+/-}$ compared to $Npy^{+/+}$. Scale bar = 100 μm. *, $P < 0.05$; NS, not significant.

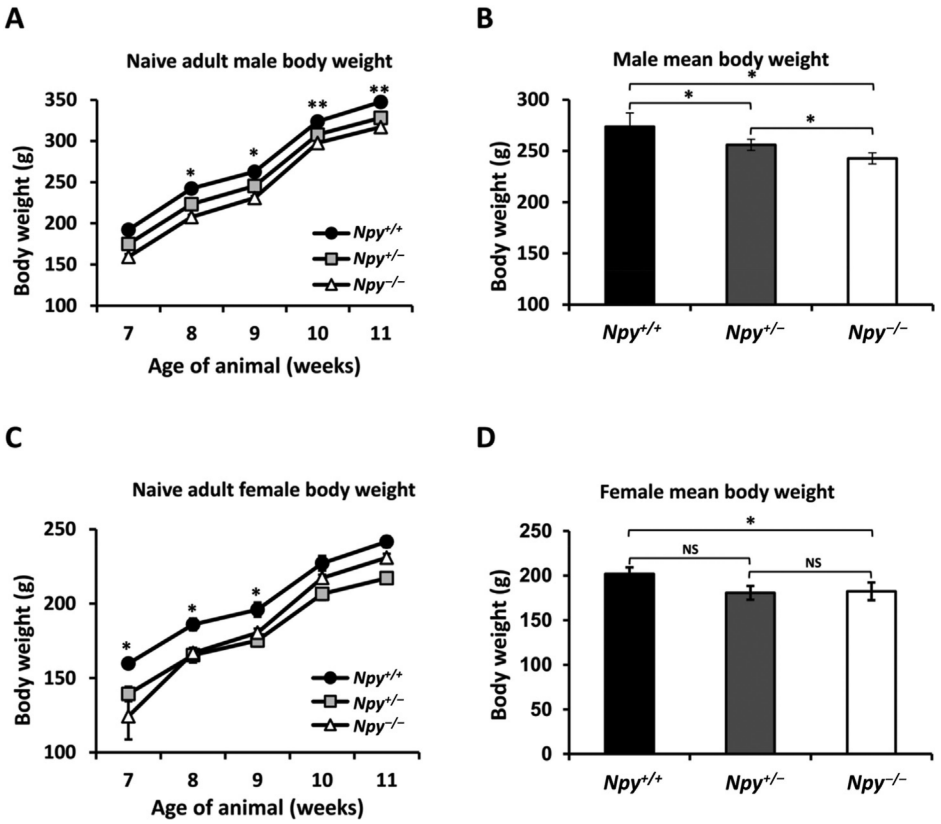

Fig. 2.

Naive adult animal body weight analysis. In male rats, significant body weight differences were observed between $Npy^{+/+}$ ($n = 8$), $Npy^{+/-}$ ($n = 5$), and $Npy^{-/-}$ ($n = 8$) genotypes at multiple time points (**A**) and also average body weight (mean ± SEM) of all five weeks are shown (**B**). In female rats ($Npy^{+/+}$, $n = 3$; $Npy^{+/-}$, $n = 3$; $Npy^{-/-}$, $n = 5$), no body weight difference was found between $Npy^{-/-}$ and $Npy^{+/-}$ rats at any time point, but significant decrease of body weight of $Npy^{-/-}$ and $Npy^{+/-}$ was observed at 7, 8, and 9 weeks when compared to $Npy^{+/+}$ rats (**C**), and average body weight (mean ± SEM) of all five weeks are shown (**D**). *, $P < 0.05$; **$P < 0.01$; NS, not significant.

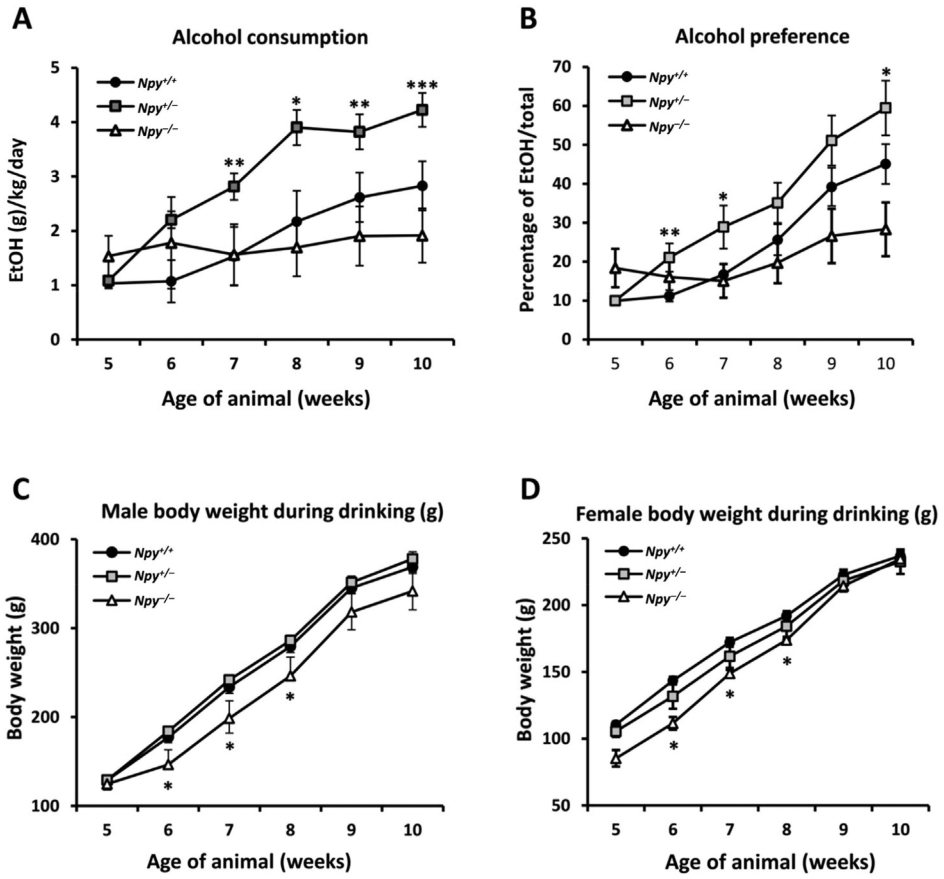

Fig. 3.

Alcohol consumption analysis. Both alcohol consumption (**A**) and alcohol preference (**B**) were significantly increased in heterozygous ($Npy^{+/-}$, n = 6 m and 7 f) animals when compared to homozygous KO ($Npy^{-/-}$, n = 16 m and 11 f) and WT ($Npy^{+/+}$, n = 9 m and 2 f) animals, when tested with intermittent access to 5% EtOH, 10% EtOH, and water. Body weight during drinking sessions was plotted in both male (**C**) and female (**D**) rats. Significant body weight decreases were observed at 6, 7, and 8 weeks old between KO ($Npy^{-/-}$) and WT ($Npy^{+/+}$) of both male and female rats. No difference between Npy KO and heterozygous ($Npy^{+/-}$) was observed at any time point. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

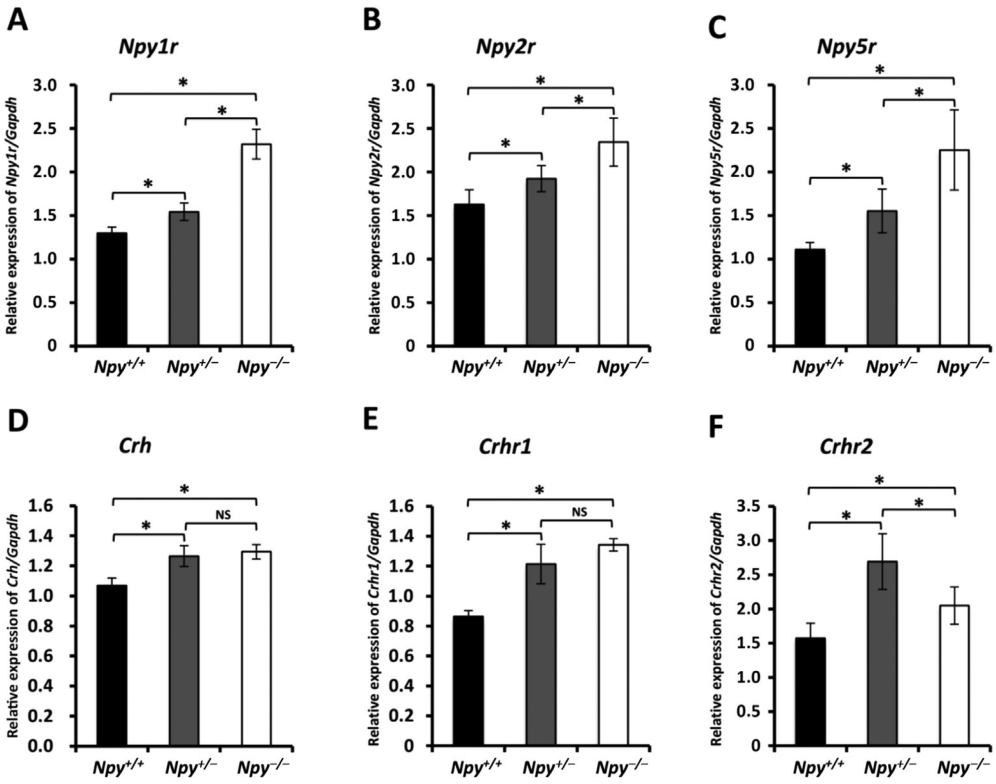

Fig. 4.

Npy function-related gene expression in the brain of *Npy* KO rats. Significant increase of *Npy1r*, *Npy2r*, *Npy5r*, *Crh*, *Crhr1*, and *Crhr2* expressions was observed in the brain of both $Npy^{+/-}$ and $Npy^{-/-}$ animals when compared to $Npy^{+/+}$ rats, with *Crhr2* expressed highest in the heterozygous animals. *, $P < 0.05$; NS, not significant.

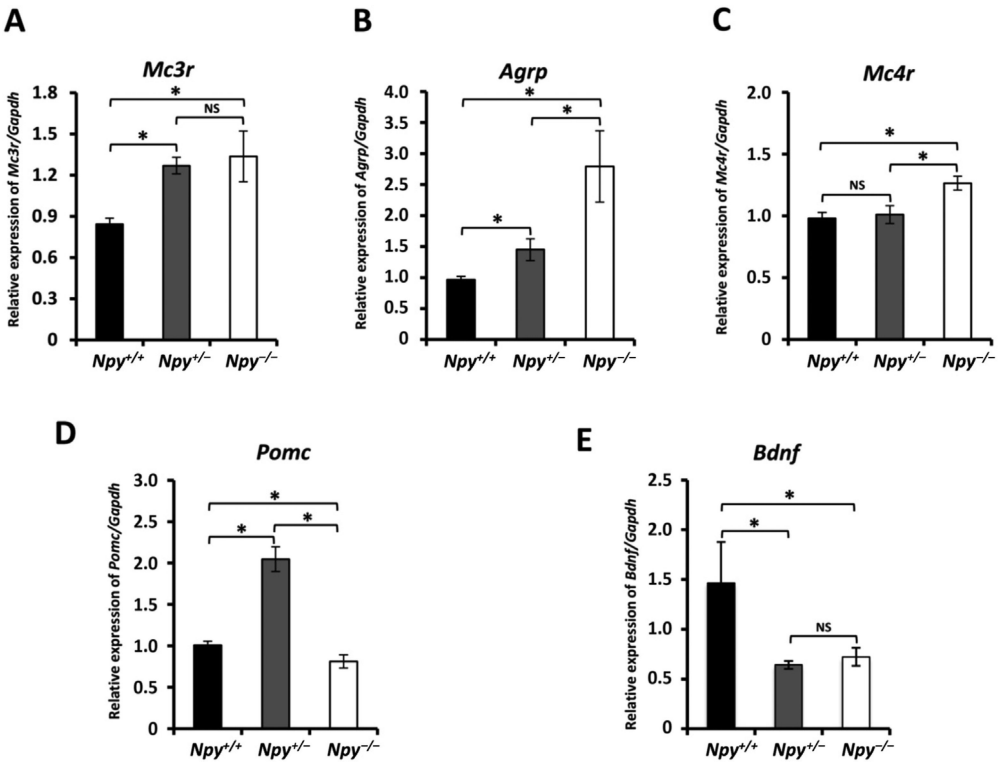

Fig. 5.

Expression analysis of melanocortin receptors (*Mc3/4r*), their antagonist (*Agrp*), *Pomc* and *Bdnf*. Significant increase of *Mc3r* and *Agrp* mRNA expressions was shown in the brain of both $Npy^{+/-}$ and $Npy^{-/-}$ animals when compared to $Npy^{+/+}$ rats (**A** and **B**). A significant increase of *Mc4r* was observed in KO rats only (**C**). *Pomc* was increased in heterozygous animals but decreased in KO rats (**D**). A significant decrease of *Bdnf* mRNA expression was shown in the brain of both $Npy^{+/-}$ and $Npy^{-/-}$ animals when compared to $Npy^{+/+}$ (**E**). *, $P < 0.05$; NS, not significant.

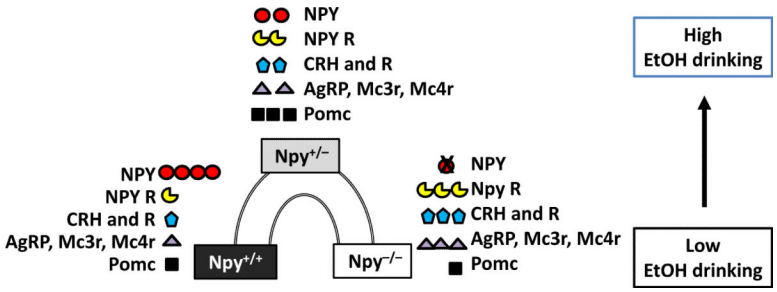

Fig. 6.

Hypothesized inverted U-shaped relationship between gene expression level and alcohol drinking behavior. At both ends of the inverted U-shaped relationship, the WT and KO animals showed low alcohol consumption. When *Npy* expression was completely abrogated, other gene expression probably began to play a larger role in determining alcohol drinking behavior in the KO rats. Combination of moderate expression of some genes, high expression of *Pomc*, and low expression of *Bdnf* may contribute to high alcohol consumption in heterozygous animal. NPY, neuropeptide Y; NPY R, NPY receptor; CRH, corticotropin-releasing hormone; CRH R, CRH receptor; AgRP, agouti-related peptide; Mc3r/Mc4r, melanocortin receptor 3/4; Pomc, proopiomelanocortin.