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

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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* expression 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.

Key words: Knockout rat; Alcohol drinking behavior; NPY and receptors; CRH and receptors; Melanocortin and receptors; BDNF.

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 x iNP F₂ 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 neuropeptide 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 had previously been 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; Gilpin et al., 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 neuropeptide 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-

56 Elder et al., 2001; Thiele and Badia-Elder, 2003; Thorsell et al., 2005a, b; Gilpin et al., 2008c) and over-
57 expression of *Npy* in the amygdala suppressed increased alcohol intake of animals after repeated
58 alcohol deprivation (Thorsell et al., 2007). In agreement with studies in mice, our findings with P and NP
59 rats indicate that NPY levels are inversely related to free-choice alcohol drinking (Thiele et al., 1998;
60 Spence et al., 2005). These studies suggest that low NPY expression is a risk factor for high alcohol
61 use; however, previous pharmacological experiments may have overlooked the effect of endogenous
62 NPY. Thus, the development of an *Npy* knockout (KO) animal is an important advance for the research
63 community.

64 NPY also functionally interacts with corticotropin releasing hormone (CRH) (Kash and Winder,
65 2006; Sajdyk et al., 2006) and decreases γ -aminobutyric acid (GABA)-ergic signaling in the central
66 amygdala and bed nucleus of the stria terminalis (BNST) (Kash and Winder, 2006; Gilpin et al., 2011;
67 Roberto et al., 2012). Additionally, NPY modulates catecholaminergic (Fuzesi et al., 2007),
68 dopaminergic (Romano et al., 2014), glutamatergic (Patrylo et al., 1999; Smialowska et al., 2002), and
69 serotonergic neuronal activities (Yoshimura et al., 2014) within the mesocorticolimbic and extended
70 amygdala reward circuits. Together, these studies indicate that *Npy* is a critical gene for various
71 neurobiological functions including addiction.

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

81 consume less ethanol. Thus, any increase in alcohol consumption resulting from *Npy* elimination
82 cannot be attributed to a predisposition to high alcohol consumption.

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

94

95 **RESULTS**

96 **Creation of *Npy* KO rat**

97 To test the efficiency of the 16 designed ZFN constructs targeting the rat *Npy* gene (Table S1),
98 constructs were transfected into rat C6 cells. Surveyor nuclease analysis showed that design #6 had
99 the highest ZFN activity and therefore was used for the single-cell embryo injection (Fig. 1A). After
100 multiple attempts, a ZFN-induced KO rat (#65) was successfully created. *NPY* KO was confirmed by
101 Surveyor assay (Fig. 1B). The sequenced result indicated a ZFN-induced 26-bp deletion at the targeted
102 site, including 6 bp in intron 1 and 20 bp in exon 2 (Fig. 1C). The sequencing alignment depicts the
103 difference in the *Npy* gene deletion region between the WT and KO (Fig. 1D). Genotyping was
104 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–1H). 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 hippocampus 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.

***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 naïve animals. We observed lower body weights 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 weights when compared to *Npy*^{+/-} rats, but no difference was found between *Npy*^{-/-} and *Npy*^{+/+} rats (Fig. 2C), with mean body weights 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).

***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*^{+/-}

129 rats at 7–10 weeks compared to *Npy*^{-/-} or WT rats. This test was initiated during early adolescence
130 postnatal day (PND) 30–35 (i.e., 5 week old rats) (Fig. 3A). Alcohol preference significantly increased in
131 6, 7, and 10-week-old *Npy*^{+/-} rats ($P < 0.05$), with trends of increases at 8 and 9 weeks as well (Fig. 3B).
132 During alcohol testing session, body weights were significantly lower in 6, 7, and 8 weeks old male and
133 female *Npy*^{-/-} rats when compared to *Npy*^{+/-} and WT littermates (Fig. 3C and D). Voluntary alcohol
134 consumption in adulthood also showed the same trend, with *Npy*^{+/-} rats drinking more alcohol than the
135 other two genotypes (Fig. S2).

136 ***Npy*^{-/-} rats show no alteration in anxiety-like behavior**

137 ICV infusion of NPY can abolish the normal preference for the closed arm in the EPM test
138 (Heilig et al., 1989), can reduce the level of punishment response in the Geller-Seifterpunished
139 responding test (Heilig et al., 1993), and can inhibit or attenuate the fear-potentiated startle response
140 (Broqua et al., 1995). These studies demonstrate *Npy* has a dose-dependent anxiolytic effect. We
141 applied the EPM test, which involves exploration of a plus-shaped maze consisting of two open and two
142 closed arms, where decreased time in the open arms is considered an indicator of elevated anxiety.
143 When compared to *Npy*^{-/-} and WT rats, *Npy*^{+/-} rats spent a longer time in the open arms (Fig. S3A).
144 However, no statistically significant difference was observed in either duration or percentage of time
145 spent in the open arm when compared to total time of arm entries (Fig. S3A and S3C). Likewise, there
146 was no observed significant difference in open arm entries and percentage of open arm entries to total
147 arm entries (Fig. S3B and S3D). Taken as a whole, the results indicate no difference in anxiety-like
148 behavior between genotypes.

149 ***Npy* elimination affects multiple neuropeptide systems**

150 Genes known to interact with *Npy* (*Npy1r*, *Npy2r*, *Npy5r*, *Crh*, *Crhr1*, *Crhr2*, *Pomc*, *Mc3r*, *Mc4r*,
151 *Agrp*, and *Bdnf*) were selected for gene expression measurement. Alcohol use disorder is a complex
152 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* expression in the whole brain relative to WT rats (Fig. 4A-4C), 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-4F) 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–5C). 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). Brain-derived neurotrophic factor (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).

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

203 receptor activity. Similarly, expressions of *Crh* and its receptors (*Crhr1* and *Crhr2*) were up-regulated
204 following *Npy* reduction (+/-) and deletion (-/-). These results are consistent with previous studies
205 indicating that the central actions of NPY and CRH have opposing functions in the regulation of
206 emotional and reward-seeking behaviors (Sajdyk et al., 2004; Sajdyk et al., 2006; Pleil et al., 2015).
207 However, *Crhr2* levels were significantly higher in *Npy*^{+/-} than both *Npy*^{-/-} and WT control rats. The
208 increases in ethanol consumption run counter to previous work, which indicated that activation of *Crhr2*
209 decreases binge-like ethanol drinking (Lowery et al., 2010; Ryabinin et al., 2012) and that NP rats
210 possess innately higher *Crhr2* expression in the brain (Yong et al., 2014). Studying the homeostatic
211 balance between multiple neuropeptide systems will further reveal the systematic adaptations
212 mediating alcohol-drinking behavior. On the other hand, higher expression of *Pomc* in the heterozygous
213 animals appears to be consistent with a previous report that Sardinian alcohol-preferring (sP) rats have
214 higher *Pomc* expression in specific brain regions (Zhou et al., 2013a). POMC is a polypeptide hormone
215 precursor. Prior to alcohol consumption, sP rats had higher basal hypothalamic *Pomc* mRNA
216 expression than Sardinian alcohol-nonpreferring (sNP) rats, and this expression increased following
217 alcohol consumption. (Zhou et al., 2013a). Manipulation of the MC receptor using the melanocortin 3/4
218 receptor M 3/4(R) agonist MTII decreases alcohol intake in chronic ethanol-drinking Alko alcohol-
219 accepting (AA) rats (Ploj et al., 2002; Polidori et al., 2006). High expression of *Mc3r* and *Mc4r* in *Npy*^{-/-}
220 rats may play a role in their lower alcohol consumption. This *Npy* KO rat model provides an opportunity
221 to study *Npy* expression level-dependent effects on other neuropeptide systems and how these
222 neuropeptide systems interact to control alcohol consumption and other behaviors.

223 Previous studies have indicated that NPY and BDNF interact to modulate alcohol addiction. For
224 example, it was found that lower BDNF expression is associated with higher alcohol consumption
225 (Pandey et al., 2005; Yan et al., 2005; Jeanblanc et al., 2009); and in the P rat, higher alcohol
226 consumption is consistent with its lower BDNF level (Moonat et al., 2011). Other studies have
227 demonstrated a positive association between BDNF and NPY CNS expression levels (Scharfman and

228 MacLusky, 2008; Yoshimura et al., 2009; Corvino et al., 2012; Croce et al., 2013). In the present study,
229 both *Npy*^{+/-} and *Npy*^{-/-} rats displayed dramatically reduced *Bdnf* expression levels (Fig. 5E). This
230 suggests that deficits in NPY signaling and decreased BDNF appear to be important elements
231 underlying molecular processes mediating alcoholism. However, unexpectedly only *Npy*^{+/-} rats exhibited
232 the higher alcohol drinking phenotype, which indicates that low *Bdnf* alone is insufficient to promote
233 alcohol consumption. Importantly, this is the first evidence that the total or partial loss of NPY
234 significantly reduces the expression of *Bdnf* in a KO model.

235   The relative expressions of *Npy*, *AgRP*, and *Pomc* were also investigated in relation to body
236 weight differences in the *Npy*^{-/-}, *Npy*^{+/-}, and WT rats. *Npy* and *AgRP* are co-localized in the
237 hypothalamus and function as orexigenic peptides (Krashes et al., 2011; Krashes et al., 2013; Parker,
238 2013). However, some clinical studies have found that hypothalamic expression levels of *Agrp* and *Npy*
239 are negatively correlated with body weight change, with higher *Agrp* expression level associated with
240 lower body weights (Alkemade et al., 2012). NPY immunoreactivity is lower in obese subjects
241 (Goldstone et al., 2002; Alkemade et al., 2012), while AgRP immunoreactivity shows either no change
242 (Goldstone et al., 2002) or a U-shaped correlation with BMI (Alkemade et al., 2012). These mixed
243 findings, along with the present results, indicate that multiple neuropeptide effects must be considered
244 when investigating consumptive behaviors or their consequences. We observed that *Npy*^{-/-} rats with
245 higher *Agrp* and lower *Pomc* (Fig. 5B and 5D) expression have lower body weights. Previous studies
246 have shown that activation of AgRP neurons promotes feeding, while stimulation of POMC neurons
247 reduces food intake and body weight (Aponte et al., 2011). In the present study, *AgRP* and *Pomc*
248 expression appear to counter each other in *Npy*^{-/-} and *Npy*^{+/-} animals. Mabe *Npy* dose was sufficient to
249 maintain body weight. Additionally, this result implies that altered body weight in the KO rats is
250 independent of food intake or alcohol preference. Thus, other possible mechanisms of energy
251 utilization/balance should be considered (Parker et al., 2015).

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

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

266 MATERIALS AND METHODS

267 Animals

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

272 ZFN design and testing in cell lines

273 ZFN constructs were co-designed by Sigma-Aldrich (USA) and the authors. In brief, the
274 constructs consisted of sequence-generated zinc-finger proteins that specifically bind to targeted

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

283 **Creation of *Npy* KO rats**

284 Background iNP rats were developed by selective breeding for low alcohol preference, followed
285 by inbreeding for more than 15 generations (Carr et al., 2006). Embryo collection and RNA injection
286 were performed as described previously (Geurts et al., 2009). Genomic DNA isolated from all F1 rats
287 was used to sequence areas around the ZFN targeted region using four primers (Table S2, *Npy*-ZFN
288 primers). Primers were designed based on the *Npy* genomic sequence accessed from the Ensembl
289 Genome Browser (accession # ENSRNOG00000009768). These primers were utilized to amplify two
290 overlapping DNA fragments that encompassed a 2-kb region including the promoter, exon 1, intron 1,
291 and exon 2.

292 **Food intake and body weight measurement**

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

298 **Free-choice alcohol drinking protocol**

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

304 **EPM test**

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

311 **Gene expression**

312 Whole brains were extracted, snap frozen in dry ice-bathed isopentane, transferred on dry ice,
313 and stored at -80°C until RNA isolation. RNA was isolated using TRIzol (Life Technologies, USA) and
314 purified using the RNeasy Mini Kit (Qiagen, USA). Using the ABI PRISM 7300 Sequence Detection
315 System (Life Technologies), the relative mRNA expression levels were normalized to *Gapdh*. The used
316 primers are listed in Table S2. Mixed model ANOVA were used and statistical significance was set at *P*
317 < 0.05.

318 **ELISA and immunohistochemical staining**

319 Npy levels were measured using the EZRMNPY-27K ELISA kit following the manufacturer's
320 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 slides and dried by incubating the slides at 55°C. After the slides 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 slides were then incubated overnight at 4°C with the mouse anti-NPY primary antibody (2 µg/mL, SAB1404138, Sigma-Aldrich,), and incubated with Polink-2 plus Polymer HRP Detection System following the manufacturer's instructions (Zhongshan Goldenbridge Biotechnology). Finally, slides were visualized with DAB chromagen (Zhongshan Goldenbridge 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.

333 **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 $P < 0.05$.

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353 Figure Legend

354 Fig. 1. Successful creation of *Npy* KO rats.

355 **A:** *In vitro* ZFN activity assay. Rat C6 cells were transfected and harvested one day after transfection.
356 The pre-designed ZFNs were validated by Surveyor nuclease cleavage analysis, in which
357 endonuclease *CEL-I* is able to cut both strands of a DNA heteroduplex. *CEL-I* digestions were
358 performed and samples were run on a 10% PAGE-TBE gel. Design #6 has the highest ZFN activity
359 (14%). The fraction cut ratio was calculated based on the relative intensity of the DNA bands (389 bp,
360 235 bp and 154 bp) using ImageJ software. NEG: negative control. **B:** Animal #65 was a KO rat that
361 was confirmed by Surveyor nuclease cleavage assay. G+C lane is positive control of the assay. **C:**
362 Location of the deletion on the *Npy* gene. **D:** A 26-bp deletion was confirmed by sequencing, which
363 includes 6 bp in intron 1 and 20 bp in exon 2. Multiple KO sequences aligned with *Npy*^{+/+} (WT). **E:** PCR
364 analyses of WT (*Npy*^{+/+}), H (heterozygous, *Npy*^{+/-}), and KO (*Npy*^{-/-}) genotypes. **F:** *Npy* mRNA
365 expression in the tail of rats with different genotypes. **G:** *Npy* mRNA expression in the brain of rats with
366 different genotypes. **H:** NPY peptide level in the brain. **I:** Immunohistochemical staining indicated no
367 expression of NPY in the hippocampus of *Npy*^{-/-} rats and medium level of NPY expression was
368 observed in *Npy*^{+/-} compared to *Npy*^{+/+}. Scale bar = 100 μ m. *, $P < 0.05$; NS, not significant.

369 Fig. 2. Naive adult animal body weight analysis.

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

377 Fig. 3. Alcohol consumption analysis.

378 Both alcohol consumption (**A**) and alcohol preference (**B**) were significantly increased in heterozygous
379 ($Npy^{+/-}$, $n = 6$ m/7 f) animals when compared to homozygous KO ($Npy^{-/-}$, $n = 16$ m/11 f) and WT ($Npy^{+/+}$,
380 $n = 9$ m/2 f) animals, when tested with intermittent access to 5% EtOH, 10% EtOH, and water. Body
381 weight during drinking sessions was plotted in both male (**C**) and female (**D**) rats. Significant body
382 weight decreases were observed at 6, 7, and 8 weeks old between KO ($Npy^{-/-}$) and WT ($Npy^{+/+}$) of both
383 male and female rats. No difference between Npy KO and heterozygous ($Npy^{+/-}$) was observed at any
384 time point. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

385 Fig. 4. Npy function-related gene expression in the brain of Npy KO rats.

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

389 Fig. 5. Expression analysis of melanocortin receptors ($Mcr3/4$), their antagonist ($Agrp$), $Pomc$ and $Bdnf$

390 Significant increase of $Mcr3$ and $Agrp$ mRNA expressions was shown in the brain of both $Npy^{+/-}$ and
391 $Npy^{-/-}$ animals when compared to $Npy^{+/+}$ rats (**A** and **B**). A significant increase of $Mc4r$ was observed in
392 KO rats only (**C**). $Pomc$ was increased in heterozygous animals but decreased in KO rats (**D**). A
393 significant decrease of $Bdnf$ mRNA expression was shown in the brain of both $Npy^{+/-}$ and $Npy^{-/-}$ animals
394 when compared to $Npy^{+/+}$ (**E**). *, $P < 0.05$; NS, not significant.

395 Fig. 6. Hypothesized inverted U-shaped relationship between gene expression level and alcohol
396 drinking behavior.

397 At both ends of the inverted U-shaped relationship, the WT and KO animals showed low alcohol
398 consumption. When Npy expression was completely abrogated, other gene expression levels probably
399 began to play a larger role in determining alcohol drinking behavior in the KO rats. Combination of

400 moderation level expression of some genes, high expression of *Pomc*, and low expression of *Bdnf* may
401 contribute to high alcohol consumption in heterozygous animal. NPY, neuropeptide Y; Npy R, NPY
402 receptor; CRH, corticotropin-releasing hormone; CRH R, CRH receptor; AgRP, agouti-related peptide;
403 Mc3r/Mc4r, melanocortin receptor 3/4; *Pomc*, proopiomelanocortin.

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666

Figure1-5

Figure 1

A **B** **C** **D**

Figure2

Figure 3

Figure 4

A **B** **C** **D** **E**

Figure 5

Figure 6

Figure 6