Functional NPY Variation as a Factor in Stress Resilience and Alcohol Consumption in Rhesus Macaques

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Context: Neuropeptide Y (NPY) counters stress and is involved in neuroadaptations that drive escalated alcohol drinking in rodents. In humans, low NPY expression predicts amygdala response and emotional reactivity. Genetic variation that affects the NPY system could moderate stress resilience and susceptibility to alcohol dependence.

Objective: To determine whether functional NPY variation influences behavioral adaptation to stress and alcohol consumption in a nonhuman primate model of early adversity (peer rearing).

Design: We sequenced the rhesus macaque NPY locus (rhNPY) and performed in silico analysis to identify functional variants. We performed gel shift assays using nuclear extract from testes, brain, and hypothalamus. Levels of NPY in cerebrospinal fluid were measured by radioimmunoassay, and messenger RNA levels were assessed in the amygdala using real-time polymerase chain reaction. Animals were exposed to repeated social separation stress and tested for individual differences in alcohol consumption. Animals were genotyped for −1002 T→H11022 G, and the data were analyzed using analysis of variance.

Setting: National Institutes of Health Animal Center.

Subjects: Ninety-six rhesus macaques.

Main Outcome Measure: Behavior arousal during social separation stress and ethanol consumption.

Results: The G allele altered binding of regulatory proteins in all nuclear extracts tested, and −1002 T→H11022 G resulted in lower levels of NPY expression in the amygdala. Macaques exposed to adversity had lower cerebrospinal fluid NPY levels and exhibited higher levels of arousal during stress, but only as a function of the G allele. We also found that stress-exposed G allele carriers consumed more alcohol and exhibited an escalation in intake over cycles of alcohol availability and deprivation.

Conclusions: Our results suggest a role for NPY promoter variation in the susceptibility to alcohol use disorders and point to NPY as a candidate for examining gene × environment interactions in humans.

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Exposure to adversity is known to increase an individual’s risk of developing stress-related conditions, such as anxiety, depression, and addictive disorders, including alcohol dependence. A number of studies have shown that genetic variants that increase anxiety interact with stressful events to impart risk of these disorders. Functional genetic variation that reduces stress resiliency would be equally likely to moderate risk. The neuropeptide Y (NPY) system is one whose regulation mediates stress adaptation and is therefore a candidate system in which functional genetic variation may affect resilience. In response to protracted or repeated periods of stress, NPY is released in key regions of the brain, a mechanism proposed to be important for countering effects of stress. Individuals who differ in the ability to recruit this system would be expected to differ in resilience and thus vulnerability to stress-related disorders.

Studies indicate that stress exposure early in life is particularly likely to induce adult psychopathology. The rhesus macaque model has led the way as a controlled experimental system that permits examination of how early adversity in the form of maternal deprivation interacts with functional genetic variants to influence stress reactivity and alcohol consumption. Infants that are reared with age mates and not by their mothers (peer-reared), show evidence of harm avoidance, insecure attachment, and high levels of anxiety. In addition to exhibiting these lifelong traits, peer-reared monkeys consume...
higher levels of alcohol.\textsuperscript{10,11} Whether NPY variation influences these phenotypes in primates has not yet been demonstrated.

Prolonged exposure to alcohol leads to sensitization of behavioral stress responses and escalated alcohol intake. These neuroadaptations are in part mediated through recruitment of corticotropin-releasing hormone (CRH, or corticotropin-releasing factor [CRF]) signaling within the amygdala complex.\textsuperscript{3} Under these conditions, rodent studies have shown that both exogenous NPY administration and overexpression of Npy in the amygdala reduce stress responses and suppress excessive alcohol intake.\textsuperscript{12,13} Whether induced by genetic selection for alcohol preference\textsuperscript{14,15} or neuroadaptations encompassing stress circuitry,\textsuperscript{12} the emerging role of NPY is as a negative regulator of excessive alcohol consumption. It may be that NPY could negatively regulate alcohol intake induced by other environmental stressors that recruit the CRF system. We predicted that NPY variation would modulate stress reactivity and alcohol intake, particularly as a function of prior stress or alcohol exposure.

Functional variants in the macaque are of particular interest because several key mediators of stress responses, such as CRF, are differentially distributed between rodents and primates and also because several rhesus variants that are functionally equivalent to those in humans have been identified.\textsuperscript{16-18} The existence of these variants and the demonstrated feasibility of modeling early adversity in the rhesus macaque combine to provide a unique opportunity for studies of gene \times environment interactions that may be relevant for humans.\textsuperscript{3,7,20} Here, we examined whether rhesus NPY (rhNPY) variation influenced stress resiliency and voluntary alcohol consumption. We screened rhNPY and regulatory regions for variation and investigated the functionality of a single-nucleotide polymorphism (SNP; rhNPY \textminus1002 T\textsuperscript{G}), located in a region that is orthologous to one demonstrated to be important for regulation of human NPY promoter activity.\textsuperscript{21} Because of the role of the NPY system in stress and alcohol response, we examined whether \textminus1002 T\textsuperscript{G} influenced behavioral arousal during social separation stress and voluntary alcohol consumption. Finally, because the NPY system becomes involved in neuroadaptations that drive escalated alcohol drinking, we also examined whether rhNPY \textminus1002 T/G genotype differentially influenced alcohol intake over cycles of alcohol availability and deprivation.

\section*{METHODS}

\subsection*{IDENTIFICATION OF NPY SEQUENCE VARIANTS}

Genomic DNA was extracted from whole blood from rhesus macaques (\textit{Macaca mulatta}) from the National Institutes of Health Animal Center, and direct sequencing was performed using samples from 96 unrelated animals (pairwise identity by descent $\leq 0.0125$). We used primers designed from a published human sequence and rhesus sequence (http://genome.ucsc.edu/cgi-bin/hgGateway) to sequence 2.5 kilobases of the 5' regulatory region, exon 1 through 4 (exon 1 = 5' untranslated region, exon 2 = NPY, exon 3 = C-terminal flanking peptide of NPY, and exon 4 = 3' untranslated region), intron 1, and the exon-intron boundaries. Cycle sequencing was performed using the Big Dye Terminator, version 3.1, reaction in 96-well optical plates (Applied Biosystems, Foster City, California). Variants were detected by visualization of electropherograms generated by ABI Sequencing Analysis software.

To identify putatively functional variants, we examined regions containing consensus sites for factors known to regulate NPY transcription\textsuperscript{11,22} and used Web-based transcription factor binding site prediction algorithms (T5sitescan, http://www.ifit.org/cgi-bin/ftf/T5sitescan.pl\textsuperscript{23} and TFSEARCH, http://www.cbrp.jprresearch/dh/TFSEARCH.html\textsuperscript{24}). Comparative genomic analyses across anthropoid primates (\textit{Homo sapiens}, \textit{Pan troglodytes}, \textit{Pongo pygmaeus}, \textit{M musculus}, and \textit{Callithrix jacchus}) were performed using the University of California–Santa Cruz Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway).

\subsection*{ELECTROPHORETIC MOBILITY SHIFT ASSAY}

Based on the identification of a putatively functional variant (\textminus1002 T\textsuperscript{G} within the rhNPY regulatory region, double-stranded oligonucleotides containing the T (5'-GCA AAT TAA TGT TCA TGG TTT TTA ACA TG-3') and G (5'-GCA AAT TAA TGG TCA TGC TGT TTA ACA TG-5') alleles were used to perform gel shift assays using nuclear extract from the human whole brain, the osteosarcoma cell line, MG-63 (both from ActivMotif, Carlsbad, California), and from an immortalized glucocorticoid-treated hypothalamic cell line (IVB cells treated with 100 mM dexamethasone).\textsuperscript{25} Assays were performed using the Gel Shift Assay System (Promega, Madison, Wisconsin) according to the manufacturer's instructions. After annealing complementary oligonucleotides (at 95°C for 5 minutes and at 25°C for 30 minutes), double-stranded probes were labeled with [\textsuperscript{32P}]-adenosine triphosphate using T4 kinase (Promega) and purified using a Bio-Spin 30 chromatography column (Bio-Rad Laboratories, Hercules, California). Incorporation of the radiolabel was greater than 1 $\times$ 10\textsuperscript{5} cpm/ng DNA. Binding assays were performed using the Gel Shift Assay System (Promega) according to the manufacturer's instructions. Nuclear extracts (5 µg/assay) were incubated for 20 minutes with 1 $\times$ 10\textsuperscript{10} cpm of each oligonucleotide probe. Competitor oligonucleotides were added at 10 times the concentration of the labeled probes. Samples were immediately separated by electrophoresis (300 V for 20 minutes) at 4°C on a Novex 6% DNA retardation gel along with prestained protein molecular weight standards (Invitrogen, Carlsbad). Each gel shift assay was performed in duplicate.

Acute stress regulates Npy expression in the rat hypothalamus, and the temporal dynamics of this regulation are similar to those observed in other regions of the brain.\textsuperscript{26} Given that the \textminus1002 T\textsuperscript{G} SNP disrupts a putative glucocorticoid response element, we wanted to determine whether we would observe glucocorticoid-dependent differences in the patterns of DNA-protein interactions and whether these differed according to genotype. To examine this, we performed gel supershift assays using an anti–glucocorticoid receptor antibody (Santa Cruz Biotechnology, Santa Cruz, California) with the glucocorticoid receptor–enriched MG-63 nuclear extract. The nuclear extract (1 µL) and antibody (1 µL) were preincubated for 30 minutes at 25°C prior to performance of the assay.

\subsection*{NPY MESSENGER RNA QUANTIFICATION BY REAL-TIME POLYMERASE CHAIN REACTION}

RNA was extracted from rhesus amygdalae using Trizol according to the manufacturer's protocol (Invitrogen). Prior to complementary DNA synthesis, RNA cleanup was performed using the RNeasy Mini Kit (Qiagen, Germantown, Maryland),
and RNA was treated with RQ1 RNase-free DNase (Promega) following the manufacturer's instructions. Total RNA quality and integrity were verified by optical density measurements (260 nm/280 nm) and by measuring ribosomal 28S:18S ratios using RNA 6000 230 Nano Assay RNA chips run on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California). RNA (100 ng) was then used for complementary DNA synthesis, applying reverse-transcription reagents (Applied Biosystems). 

NPY expression in the amygdala (n = 12) was assessed by real-time polymerase chain reaction. Applied Biosystems assay Rh02787751_m1 was used to detect NPY messenger RNA. β-Actin expression was used as an endogenous reference (Applied Biosystems No. Hs99999903_m1). Samples were analyzed in quadruplicate on an ABI Prism 7900HT system with Taqman universal polymerase chain reaction master mix. The amplification conditions were 50°C for 2 minutes and then 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The SDS 2.0 software (Applied Biosystems) was used to analyze and convert the expression data into cycle threshold values (Ct values). Data are expressed as relative NPY messenger RNA levels normalized to the −1002T/T group.

**PHYSIOLOGIC AND BEHAVIOR ASSESSMENTS OF EXPERIMENTAL ANIMALS**

**Rearing**

Rhesus macaque (M mulatta) infants at the National Institutes of Health Animal Center were randomly selected to be reared with their mothers or in a nursery by human caregivers.10,27,28 Mother-reared animals were reared in social groups composed of 8 to 14 females (about half of which had same-aged infants) and 2 adult males. Peer-reared animals were separated from their mothers at birth and hand-reared in a neonatal nursery for the first 37 days of life. For the first 14 days, they were kept in an incubator and hand fed. From day 15 until day 37, they were placed alone in a nursery cage and provided a blanket and a terry cloth–covered rocking surrogate. A bottle from which the infants would feed was fixed to the surrogate. At 37 days of age, peer-reared infants were placed in a cage with 3 other age mates with whom they had continuous contact. Mother-reared infants remained in their social group. At approximately 8 months of age, both peer-reared and mother-reared animals were placed together in age-matched social groups and housed in large indoor-outdoor runs through late adolescence, at which point the cohorts were divided into same-sex groups. All procedures were approved by the National Institute on Alcohol Abuse and Alcoholism and The Eunice Kennedy Shriver National Institute of Child Health and Human Development Animal Care and Use Committee.

**Cerebrospinal Fluid Sampling and Radioimmunoassay**

Cerebrospinal fluid (CSF) levels of NPY were assessed in late adolescent and young adult animals prior to alcohol exposure to determine whether rhNPY −1002 T > G was associated with differences in central NPY release. The CSF samples were obtained from the cisterna cerebellomedullaris posterior using a 3-mL syringe with a 22-gauge needle while the animal was under ketamine anesthesia (15 mg/kg, intramuscularly). All samples were collected within 30 minutes of investigators' entering the area in which the animals were housed. The CSF samples were immediately aliquoted into polypropylene tubes, frozen in liquid nitrogen, and stored at −70°C until assay using a commercially available kit (Bachem/Peninsula Laboratories, San Carlos, California). The between- and within-assay coefficients of variation were less than 10%.

**Social Separation Stress**

Separation stress was used because of the known effects of separation in this highly social species and because we wanted to look at the effects of protracted stress, which would be impossible to achieve with, for example, immobilization. When animals reached 6 months of age, they were subjected to 4 sequential, 4-day separations.29 Subjects in the peer group were partitioned into individual sections of the home cage, which prevented the infants from seeing or touching one another. Mother-reared infants were separated from their mothers by removing the mother from the social group. Day 1 (Monday) of each separation week was designated as the acute phase of separation. Days 2 through 4 (Tuesday-Thursday) of each separation week were designated as chronic separation. Following each separation week, subjects were reunited with their attachment sources early on Friday morning and separated again at noon on Monday.

During each separation week, a total of 9 behavioral observations were made, according to the following schedule (for behavior definitions, see eTable 1, available at http://www.archgenpsychiatry.com). Three observations were made on day one: 2 immediately following separation and 1 at hour 1 (acute). Two observations were made each day for days 2 through 4 (chronic). Each observation period was 300 seconds. Behavioral data were collected by multiple observers, with an interobserver reliability of 85% or more.

**Alcohol Consumption**

Nine cohorts of young adult macaques (age, 3.5-5.0 years) were allowed to freely consume an aspartame-sweetened (8.4% vol/vol) alcohol solution for 1 hour per day, 5 days a week in the home cage. This method consisted of 3 phases, which have previously been reported29: (1) spout training, (2) initial alcohol exposure, and (3) an experimental period. During the experimental phase, the alcohol and vehicle were dispensed 5 days a week (Monday-Friday) from 1 to 2 PM while the animals were in their home cage environment.

**GENOTYPING**

A portion of the rhNPY regulatory region (−1216 > −671) was amplified from 25 ng of genomic DNA with flanking oligonucleotides (5′-TGC TTT TTT TAC CCA ACA TGC; 5′-GGA GAG TAC TTG AGG AAG GCT G) in 15-µL reactions using an AmpliTaq Gold DNA Polymerase LD (low DNA) kit from Applied Biosystems. Amplifications were performed on a thermocycler (9700; Applied Biosystems) with 1 cycle at 96°C for 5 minutes followed by 30 cycles at 94°C for 15 seconds, 60°C for 15 seconds, and 72°C for 30 seconds, and a final 3-minute extension at 72°C. Amplicons were sequenced using the Big Dye Terminator, version 3.1, kit and the 3100 Genetic Analyzer (Applied Biosystems). Genotypes were called by direct visualization of electropherograms using 4Peaks (http://www.mekentosj.com).

**STATISTICAL ANALYSIS**

We used archived data sets to examine the effects of NPY −1002 T > G on our phenotypes of interest. Most animals included in these data sets underwent other procedures prior to the time of social separation (primate neonatal neurobehavioral assessment,30,31 n = 88; developmental CSF sampling,32 n = 119). There were also subjects that underwent neuroimaging (positron-
emission tomography and single-photon emission computed tomography, n=20) or intravenous alcohol infusion (n=92) prior to alcohol testing.

Behavioral scores during separation stress exposure were averaged for each phase (acute and chronic) across the 4 weeks of testing. Scores for each behavior were expressed as the mean frequency or duration of the behavior for the 2 testing conditions (acute and chronic stress). As scores of behaviors relating to stress responding were intercorrelated, we performed factor analysis to reduce the dimensionality of the data. Separate factor analyses for both phases of separation were performed using principal components extraction and varimax orthogonal rotation. Factors indicative of high levels of attachment (separation anxiety), stereotypy (behavioral pathology), and arousal were identified. Although NPY has not been linked to social attachment or stereotypes, it has been repeatedly demonstrated to influence levels of arousal. To avoid uninformative, repeated testing, we therefore focused on effects of the rh-NPY –1002 T>G genotype on arousal. We performed 2-way analysis of variance on acute and chronic arousal, with genotype (T/T, T/G, and G/G) and rearing condition (peer-reared vs mother-reared) as nominal independent variables. Two-way analysis of variance was also performed to assess effects of rearing and genotype on CSF NPY and voluntary alcohol consumption. Under a limited access schedule, we have determined that alcohol consumption increases following a 3-day period of deprivation (0.3–1.0 g/kg) (Figure 1), suggesting there is an alcohol-deprivation effect. To examine whether genotype interacted with periods of alcohol deprivation (5 days of 1-hour access with 3 days of deprivation) to influence the pattern of alcohol consumption across time, we used a mixed-design repeated-measures analysis of variance to examine the effects of genotype and rearing on alcohol consumption using data obtained on the first day of access (Monday) during the 4 weeks of testing. All post hoc comparisons were made using the Tukey-Kramer method.

The frequency of the G allele was 37%, and genotype frequencies were in Hardy-Weinberg equilibrium. Although this is an outbred colony of macaques, to verify that our effects were attributable to effects of the rh-NPY –1002 T>G genotype and not to general heritability of our traits of interest, we repeated our analyses using a set of 3 biallelic genetic markers with similar minor allele frequencies to the –1002 G allele (15%–33% as carriers; OPRM1 C77G and SNPs in the DAT and CRH promoters). Similar effects of the other markers tested on phenotypes of interest were not observed, supporting the argument that our current results are attributable to effects of NPY –1002 T>G. We also excluded animals that carried alleles known to interact with early peer rearing to predict our phenotypes of interest (ie, rh-HTT-LPR s allele); as results were unchanged, these animals (n=20) were included in the final analyses. The Kolmogorov-Smirnov normality test and equality of variances F test were used to determine whether data deviated from normality and whether there was nonhomogeneity of the data. In cases in which there was nonnormality or inequality of variances, data were ranked transformed and the analyses repeated. Analyses were performed using StatView, version 5.01, statistical software. Significance was set at P<.05.

RESULTS

IDENTIFICATION OF A FUNCTIONAL VARIANT IN THE rhNPY PROMOTER

We sequenced the rhNPY gene, first intron, exon-intron boundaries, and 3’ and 5’ flanking regions and identified 12 polymorphic sites (Figure 1A). Variants were assigned positions relative to the transcription start site. In silico analysis indicated that a SNP (–1002 T>G) present in a region orthologous to one shown to be important to regulation of NPY transcriptional control predicted the loss of a glucocorticoid response element half-site (Figure 1B). We found that the T>G SNP resulted in altered binding of regulatory proteins, with several bands increasing (molecular weight of approximately 130, 210, and 260 kDa) and 1 of 180 kDa showing a relative decrease (Figure 1C). We also found that, in the amygdala, the G allele resulted in decreased levels of NPY expression (Figure 1D) (F1,98=24.4, P<.001). We performed gel supershift assays using an anti–glucocorticoid receptor antibody and found that the T allele showed a relative increase in the degree of binding of the 180-kDa (and 90-kDa) bands, both of which showed decreased motility with the addition of the anti–glucocorticoid receptor antibody. The 180-kDa band (glucocorticoid receptor dimer) was preferentially bound in experiments performed with T allele oligonucleotides (eFigure 2).

CSF NPY

There was a trend for an effect of rearing condition, with lower NPY levels among peer-reared animals (F2,96=2.68, P=.1). There was no main effect for genotype (F2,96=0.84, P=.44). However, genotype interacted with rearing condition to predict CSF NPY (F2,96=4.2, P=.02). Peer-reared animals carrying the G allele (T/G or G/G) had lower CSF NPY levels than did peer-reared T/T animals (Tukey-Kramer, P<.05) (Figure 2). Among peer-reared subjects, genotype accounted for 28% of the variance.

BEHAVIORAL RESPONSES TO STRESS

Factor analysis performed on behavioral measures recorded during social separation generated 3 factors for each of the 2 phases of data collection. For the acute phase of stress, 3 factors (separation anxiety, arousal, and behavioral pathology) accounted for 71.6% of the variance. The same 3 factors accounted for 77.6% of the variance for analysis performed on behaviors collected during chronic separation stress (eTable 2).

During acute separation, there were main effects of rearing (F1,96=6.4, P=.01) and genotype (F2,96=3.2, P=.04) on arousal, but no interaction. Post hoc analyses demonstrated that peer-reared infants exhibited higher levels of arousal than mother-reared infants and that those homozygous for the G allele had higher arousal scores than those homozygous for the T allele (Tukey-Kramer, P<.05) (Figure 3A). As with acute stress exposure, there was a main effect of rearing condition on arousal (F1,96=25.0, P<.001) during chronic separation, with peer-reared animals exhibiting higher scores (Tukey-Kramer, P<.05) (Figure 3B). There was no main effect for genotype. However, there was an interaction between rearing and genotype (F1,96=4.2, P=.02). Although peer-reared T/T subjects responded differently than mother-reared animals, peer-reared G allele carriers (T/G or G/G) exhibited higher levels of arousal (Tukey-Kramer, P<.05) (Figure 3B). In both cases (acute and chronic stress), results remained the same following rank transformation of the data. Among
peer-reared subjects, genotype accounted for 7% and 10% of the variance during acute and chronic stress exposure, respectively.

ALCOHOL CONSUMPTION

There was a main effect of rearing condition on alcohol, with peer-reared consuming more alcohol than mother-reared subjects ($F_{1,85}=16.5$, $P<.001$). There was also an interaction between rearing and genotype ($F_{2,85}=3.3$, $P=.04$), and this relationship remained following rank transformation of the data. Among peer-reared monkeys, only carriers of the G allele (T/G and G/G) consumed higher levels of alcohol than mother-reared monkeys (Tukey-Kramer, $P<.05$) (Figure 4). Genotype accounted for 12.5% of the variance in alcohol consumption in peer-reared subjects.

When we examined effects of rearing and genotype on alcohol consumption following periods of deprivation across the 4 weeks of testing, we found a main effect of rearing ($F_{1,204}=12.5$, $P<.001$). There were also different temporal courses of consumption during successive weeks as a function of genotype and rearing (genotype/time interaction, $F_{6,204}=3.02$, $P=.008$; rearing/genotype/time interaction, $F_{6,204}=2.2$, $P=.04$) (Figure 5). When we examined the effects of genotype, rearing, and time across weeks of testing during the second through fifth days for the weekly sessions, the effects of genotype ($F_{2,228}=3.23$, $P=.04$), rearing ($F_{1,228}=26.4$, $P<.001$), and genotype ×rearing interaction ($F_{2,228}=4.9$, $P=.01$) were main-
There is accumulating evidence that genetic and environmental factors interact to determine susceptibility to stress-related disorders later in life.\(^3\) Of particular interest for the study of gene × environment interactions is variation in genes encoding stress-responsive–signaling molecules that may contribute to stress vulnerability or resiliency.\(^1\) Perhaps most notable among the gene × environment studies are those examining interactions between life stress and the serotonin transporter-linked polymorphism (5-HTTLPR). We have previously demonstrated that a functionally similar variant in rhesus macaques, rh5-HTTLPR, interacts with early adversity in the form of peer-rearing to influence stress reactivity and alcohol consumption, emphasizing the utility of the peer-rearing model for examining gene × environment interactions that translate to the human condition.

Because the NPY system is a key mediator of behavioral adaptation to stress, we screened the rhNPY gene for variants that might affect stress resilience, with the prediction that we would observe similar interactions. We identified a SNP (−1002 T>G) in the rhNPY regulatory region that predicts loss of a glucocorticoid response element half-site. Glucocorticoids have long been known to regulate NPY expression,\(^4\) and this regulation may be important for stress-mediated NPY induction. We performed gel shift assays with nuclear extract derived from several glucocorticoid receptor–expressing cell lines\(^5\) and found that the G allele resulted in altered DNA-protein interactions. Among the bands that exhibited a relative decrease with the G allele (which, overall, showed increased transcription factor binding) was one measuring 180 kDa, which was recognized by an anti–glucocorticoid receptor antibody. This suggests that the −1002 T>G SNP resulted in decreased preference for a functional glucocorticoid response element. We found that −1002 T>G predicted decreased NPY expression in the amygdala, a brain region in which NPY release decreases anxious responding. Based on these functional differences, we predicted that this SNP would result in decreased NPY system activity and/or a failure to recruit the NPY system under stressful conditions, both of which could lead to reduced stress resiliency.

In humans, both genetic and environmental factors are suggested to influence NPY system function. Decreases in NPY levels are observed among subjects with treatment-refractory depression and posttraumatic stress disorder.\(^4\) There is also evidence that a gain-of-function variant resulting in a Leu7Pro substitution of the prepro-NPY signal peptide\(^5\) may protect against depression, while markers on low-expressing NPY haplotypes (−399 T>G) result in decreased NPY levels and upregulated stress responses.\(^6\) Herein, we showed that rhesus macaques exposed to adversity have lower CSF levels of NPY, but...
overexpressing NPY suggests more ethanol, while consumption is reduced in mice genetically selected alcohol-preferring rats.52 Based on these findings, a screen for functional variants was performed, and individuals with low levels of NPY expression or who are less capable of recruiting the NPY system in response to stress would be less stress resilient and therefore more vulnerable to stress-related disorders.

There is considerable evidence suggesting that NPY regulates alcohol consumption.49-51 Npy-deficient mice consume more ethanol, while consumption is reduced in mice overexpressing Npy.31 Moreover, Npy maps to a quantitative trait locus underlying alcohol consumption in genetically selected alcohol-preferring rats.32 Based on these findings, a screen for functional variants was performed, identifying a marker (D4Mit7) that reduced brain expression of Npy in this line.33 In humans, linkage to the chromosomal region containing NPY has been demonstrated,34 and there have been associations of NPY variation with both alcohol consumption57 and dependence.35,58 Other studies, however, have failed to replicate this association.57-59 Of note, our present study did not find any effects of rhNPY −1002 T > G on alcohol consumption in normally reared animals, even following repeated alcohol exposure. Instead, rhNPY −1002 T > G genotype increased alcohol consumption among those exposed to both early adversity and cycles of alcohol exposure. This suggests that a high degree of stress loading—or environmental stress/deprivation during critical developmental windows—may be required for the G allele to produce an effect, raising the possibility that human NPY variation could potentially increase risk of alcohol dependence more so among individuals with especially traumatic life experiences and early or high cumulative levels of stress exposure. In support of this argument, the only reports of a link between NPY variation and alcohol dependence have studied individuals with late-onset alcoholism56 or samples highly represented by war veterans.37

Dysregulation of the CRF system following repeated periods of alcohol exposure and deprivations contributes to the transition from reward to relief drinking.5,60 and NPY signaling is a counterregulatory process that buffers actions of CRF.3 When we examined patterns of alcohol intake during repeated cycles of availability and deprivations, we found an interaction between NPY genotype and alcohol exposure, such that stress-exposed G allele carriers exhibit a pattern of escalated alcohol intake. This is potentially indicative of a genotype-mediated inability to recruit NPY in response to induction of the CRF system among subjects consuming high levels of alcohol, suggesting that these subjects might more easily transition to the addicted state.

The NPY system is important to countering stress. We hypothesized that genetic variation that resulted in low
levels of NPY expression or a failure to recruit the NPY promoter variation influences CSF levels of NPY, behavioral arousal in response to stress, and alcohol consumption. Overall, this study suggests a role for NPY variation in the susceptibility to alcohol-related disorders and may further implicate this system as a treatment target in selected individuals. Our results also suggest NPY to be a candidate for examining gene × environment interactions in humans.

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