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Functional *NPY* variation as a factor in stress resilience and alcohol consumption in rhesus macaques

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Abstract

Context—Neuropeptide Y (NPY) is important to countering stress and is involved in neuroadaptations that drive escalated alcohol drinking following repeated alcohol exposure in rodents. In humans, haplotype-driven diminution in *NPY* expression is predictive of amygdala response and emotional reactivity to stress. Genetic variation that affects the NPY system could impact resilience to stress and to developing addiction with continued alcohol use.

Objective—To determine whether functional *NPY* variation influences CSF NPY, 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 (rh*NPY*) and performed *in silico* analysis to identify functional variants. We performed gel shift assays for a −1002 T>G using nuclear extract from testes, brain and hypothalamus. Levels of NPY in CSF were measured by RIA, and mRNA levels were assessed in amygdala using RT-PCR. During infancy, animals were exposed to repeated social separation stress, and tested for individual differences in alcohol consumption as young adults. Animals were genotyped for −1002 T>G, and the effects of this variant on mRNA expression, CSF NPY, behavior arousal during stress, and ethanol consumption were assessed by ANOVA.

Results—The G allele altered binding of regulatory proteins in all nuclear extracts tested, and −1002 T>G resulted in lower levels of *NPY* expression in amygdala. Macaques exposed to adversity had lower CSF NPY 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 GxE interactions in humans.

Exposure to adversity is known to increase an individual's risk for developing stress-related conditions, such as anxiety, depression, and addictive disorders, including alcohol dependence [1, 2]. A number of studies have shown that genetic variants that increase stress reactivity interact with stressful life events to impart risk for these disorders [3, 4]. Functional genetic variation that reduces stress resiliency would be equally likely to moderate risk. The neuropeptide Y (NPY)

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system is one whose regulation mediates stress adaptation and is, therefore, a candidate system in which functional genetic variation may impact resilience. In response to protracted or repeated periods of stress exposure, NPY is released in key regions of the brain, a mechanism proposed to be important for countering the effects of stress [5]. Individuals who differ in the ability to recruit this system would be expected to differ in resilience and, therefore, vulnerability to stress-related disorders.

Studies indicate that stress exposure early in life is particularly likely to induce adult psychopathology [6]. The rhesus macaque model has led the way as a controlled experimental system that permits examination of how early adversity interacts with functional genetic variants to influence stress reactivity and alcohol consumption [7]. 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 [8, 9]. In addition to exhibiting these lifelong traits, peer-reared monkeys consume higher levels of alcohol [10, 11]. Whether *NPY* variation influences these phenotypes in stress-exposed 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 large part mediated through recruitment of corticotropin-releasing factor (CRF, or CRH) signaling within the amygdala complex [5]. Under these conditions, rodent studies have shown that both exogenous NPY administration and local over-expression of *Npy* within the amygdala not only reduce stress-responses, but also suppress excessive alcohol intake [12, 13]. Whether induced by genetic selection for alcohol preference [14, 15] or neuroadaptations encompassing stress circuitry [12], the emerging role of NPY is as a negative regulator of excessive alcohol consumption. It may be that NPY could also negatively regulate alcohol intake induced by other environmental stressors that recruit the CRF system. We predicted that *NPY* variation would modulate not only stress-reactivity but also voluntary 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 have been identified that are functionally equivalent to those in humans [16–19]. The existence of these variants and the demonstrated feasibility of modeling early life adversity in the rhesus macaque combine to provide a unique opportunity for studies of gene by environment (GxE) interactions that are likely to be relevant for humans [3, 7, 20]. Here, we examined whether rhesus *NPY* (rh*NPY*) variation influenced stress resiliency and voluntary alcohol consumption. We screened the rh*NPY* gene and regulatory regions for variation and investigated the functionality of a single nucleotide polymorphism, or SNP (rh*NPY* −1002T>G), located in a region that is orthologous to one demonstrated to be important for regulation of human *NPY* promoter activity [21]. Because of the role of the NPY system in stress and alcohol response, we examined whether −1002 T>G influenced both behavioral arousal during exposures to stress and voluntary alcohol consumption. Finally, because the NPY system becomes involved in neuroadaptations that drive escalated alcohol drinking, we also examined whether rh*NPY* −1002 T/G genotype differentially influenced alcohol intake over cycles of alcohol availability and deprivation.

METHODS

IDENTIFICATION OF *NPY* SEQUENCE VARIANTS

Genomic DNA was extracted from whole blood from rhesus macaques (*Macaca mulatta*) from the NIH Animal Center (NIHAC), and direct sequencing was performed using samples from 96 unrelated animals (pairwise Identity by Descent, or IBD ≤ 0.0125). We used primers designed from published human sequence and, subsequently, from rhesus sequence published on the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway) to sequence 2.5

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kB of the 5′ regulatory region, exons 1–4 (Exon 1= 5′UTR, Exon 2 = NPY, Exon 3 = CPON, Exon 4 = 3′UTR), 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, Inc., Foster City, CA). 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 [21, 22] and used web-based TFBS binding site prediction algorithms (TfSitescan, http://www.ifti.org/cgi-bin/ifti/Tfsitescan.pl [23] and TRANSFAC, http://www.cbrc.jp/research/db/TFSEARCH.html, [24]. Comparative genomic analyses across anthropoid primates (*H. sapiens, P. troglodytes, P. pygmaeus, M. mulatta, C. jacchus*) were performed using the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway).

ELECTROPHORETIC MOBILITY SHIFT ASSAY

Based on the identification of a putatively functional variant (−1002 T>G) within the rh*NPY* regulatory region, double-stranded oligonucleotides containing the T (5′-GCA AAT TAA TGT <u>T</u>CA TCG TTT TTA ACA TG -3′) and G (5′-GCA AAT TAA TGT <u>G</u>CA TCG TTT TTA ACA TG - 5′) alleles were used to perform gel shift assays using nuclear extract from human whole brain, the osteosarcoma cell line, MG-63 (both from ActivMotif, Carlsbad, CA) and from an immortalized glucocorticoid-treated hypothalamic cell line (IVB cells treated with 100 nM Dexamethasone)[25]. Assays were performed using the Gel Shift Assay System (Promega, Madison, WI) per manufacturer's instructions. After annealing complementary oligonucleotides (95°C 5 min, 25°C 30 min), double-stranded probes were [^{32}P]-ATP labeled using T4 kinase (Promega, Madison, WI) and purified using a Bio-Spin 30 chromatography column (Bio-Rad). Incorporation of radiolabel was > 1×10^5 cpm/ng DNA. Binding assays were performed using the Gel Shift Assay System (Promega, Madison, WI) per manufacturer instructions. Nuclear extracts (5 μg/assay) were incubated for 20 min with 1×10^5 cpm of each oligonucleotide probe. Competitor oligonucleotides were added at 10× the concentration of the labeled probes. Samples were immediately separated by electrophoresis (300 V for 20 min) at 4°C on a Novex 6% DNA retardation gel along with pre-stained protein molecular weight standards (Invitrogen, Carlsbad, CA). Each gel shift assay was performed in duplicate.

Acute stress regulates *NPY* expression in the hypothalamus, and the temporal dynamics of this regulation are similar to those observed in other regions of the brain [26]. Given that the −1002 T>G SNP disrupts a putative Glucocorticoid Response Element, or GRE, 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. In order to examine this, we performed gel super-shift assays using the an anti-GR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) with the GR-enriched MG-63 nuclear extract. Nuclear extract (1 μl) and antibody (1 μl) were pre-incubated for 30 min at 25°C prior to performance of the assay.

NPY mRNA QUANTIFICATION BY REAL-TIME PCR

RNA was extracted from rhesus amygdala using Trizol according to manufacturer's protocol (Invitrogen, UK). Prior to cDNA synthesis, RNA cleanup was performed using the RNeasy Mini Kit (Qiagen, USA), and RNA was treated with RQ1 RNase-free DNase (Promega, USA) following manufacturer's instructions. Total RNA quality and integrity were verified by OD measurements (260nm/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, CA, USA). RNA (100 ng) was then used for cDNA synthesis applying reverse transcription reagents (Applied Biosystems Inc., Foster City, CA, USA).

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NPY expression in amygdala samples (n=12) was assessed by Real-Time PCR. Applied Biosystems Assay # Rh02787751_m1 was used to detect *NPY* mRNA levels. β-actin expression was used as an endogenous reference (ABI #Hs99999903_m1). Samples were analyzed in quadruplicate on an ABI Prism 7900HT system with Taqman universal PCR master mix. The amplification conditions were 50°C for 2 min then 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The SDS 2.0 software (Applied Biosystems Inc., Foster City, CA, USA) was used to analyze and convert the expression data into cycle threshold values (Ct-values). Data are expressed as relative *NPY* mRNA levels normalized to the −1002T homozygote group.

EXPERIMENTAL ANIMALS: PHYSIOLOGIC AND BEHAVIOR ASSESSMENTS

Rearing—Rhesus macaque (*Macaca mulatta*) infants at NIHAC were randomly selected to be reared with their mothers or in a nursery by human caregivers. [10, 27, 28]. Mother-reared (MR) animals were reared in social groups composed of 8–14 females (about half of whom had same-aged infants) and two adult males. Peer-reared (PR) 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 terrycloth-covered, rocking surrogate. A bottle from which the infants would feed was fixed to the surrogate. At 37 days of age, PR infants were placed in a cage with three other age-mates with whom they had continuous contact. Mother-reared infants remained in their social group. At approximately 8 months of age, both PR and MR animals were placed together into 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 NIAAA and NICHD ACUC.

CSF Sampling and Radioimmunoassay—Cerebrospinal fluid (CSF) levels of NPY were assessed in order to determine whether rh*NPY*−1002 T>G was associated with differences in central NPY release. CSF samples were obtained from the cisterna magna using a 5 ml syringe with a 22 gauge needle (Becton Dickinson, Franklin Lakes, NJ) under ketamine anesthesia (15 mg/kg, IM). All samples were collected within 30 min of investigators entrance into the animal area. 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, CA). The between and within assay coefficients of variation for all tests were less than 10%.

Social Separation Stress—When the animals reached 6 months of age, they were subjected to four sequential, 4-day-long separations [28]. 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 one (Monday) of each separation week was designated as the “Acute” phase of separation. Days two through four (Tuesday through 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 nine behavioral observations were made, according to the following schedule (for behavior definitions, see Table S1). Three observations were made on day one- two immediately following separation and one at hour one (Acute). Two observations were made each day for days two, three, and four (Chronic). Each observation period was 300 s in duration. Behavioral data were collected by multiple observers, with inter-observer reliability of ≥ 85%.

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Alcohol Consumption—Nine cohorts of young adult macaques (age 3.5–5) were allowed to freely consume an aspartame-sweetened 8.4% (v/v) alcohol solution for one hour per day, 5 days a week in the home cage. This method consisted of three phases, which have previously been reported [29]: (1) *Spout Training*; (2) *Initial Alcohol Exposure*; and (*3*) *Experimental Period*. During the experimental phase, alcohol and vehicle were dispensed 5 days a week (Monday-Friday) from 1300 to 1400 while the animals were in their home-cage environment.

GENOTYPING

A portion of the rh*NPY* regulatory region (−1216 > −671) was amplified from 25ng of genomic DNA with flanking oligonucleotides (5-TGC TTT AAT TTC CCA ACA TGC; 5-GGA GAG TAC TTG AGG AAG GCT G) in 15-μL reactions using AmpliTaq Gold DNA Polymerase LD (Low DNA) kit from Applied Biosystems, Inc. (Foster City, CA). Amplifications were performed on a thermocycler (9700) (Applied Biosystems, Inc., Foster City, CA) with 1 cycle at 96°C for 5 minutes followed by 30 cycles of 94°C for 15 seconds, 60°C for 15 seconds, 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 both from Applied Biosystems, Inc. (Foster City, CA) per manufacturer's instructions. Cycle sequencing was performed with 1 cycle at 96°C for 1 minute followed by 40 cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 seconds, and a final 3-minute extension at 72°C. Genotypes were called by direct visualization of electropherograms using 4 Peaks (www.mekentosj.com).

STATISTICAL ANALYSES

We used archived datasets to examine the effects of *NPY* −1002 T>G on our phenotypes of interest. Behavioral scores during separation stress exposure were averaged for each phase (Acute and Chronic) across the four weeks of testing. As scores of behaviors relating to stress responding were inter-correlated, we performed factor analysis to reduce the dimensionality of the data. Factor analysis was then performed to yield behavioral factors relating to arousal to be used as dependent variables in ANOVA. Scores for each behavior were expressed as the mean frequency or duration of the behavior for the two testing conditions (Acute and Chronic Stress). 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 ("Arousal") were identified. Although NPY has not been linked to social attachment or stereotypies, it has been repeatedly demonstrated to influence levels of arousal. [30–32] To avoid uninformative repeat testing, we, therefore, focused on effects of rh*NPY* −1002 T>G genotype on arousal [30–32]. We performed two-way ANOVA on Acute and Chronic "Arousal", with genotype (T/T, T/G, G/G) and rearing condition (PR vs. MR) as nominal independent variables. Two-way ANOVA was also performed to assess effects of rearing condition and genotype on CSF levels of NPY and on voluntary alcohol consumption. Under a limited access schedule, we have determined that alcohol consumption is increased following a 3-day period of deprivation (from 0.3 g/kg/h to 1 g/kg/h, Figure S1), suggesting there to be an alcohol deprivation effect. In order to examine whether genotype interacted with periods of alcohol deprivation (5 days of 1 h access with 3 days of deprivation) to influence the pattern of alcohol consumption across time, we used a mixed design repeated measures ANOVA to examine the effects of genotype and rearing on alcohol consumption using data obtained on the first day of access (Monday) over the four 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 rh*NPY* variation, and not to general heritability of our traits of interest, we

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repeated our analyses using a set of three bi-allelic genetic markers with similar MAFs to the −1002 G allele (between 20 and 40%) [33, 34]. Similar effects of the other markers tested on our phenotypes of interest were not observed, supporting the argument that our current results are attributable to effects of *NPY* −1002T>G. We also excluded individuals carrying alleles known to predict our phenotypes of interest (ie, *rh5-HTT*-LPR *s* allele); as results were unchanged, these individuals (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 non-homogeneity of the data. In cases in which there was non-normality or inequality of variances, data were rank transformed and the analyses repeated. Analyses were performed using StatView 5.01 statistical software. Criterion for significance was set at $P \leq 0.05$.

RESULTS

IDENTIFICATION OF A FUNCTIONAL VARIANT IN THE rh*NPY* PROMOTER

We sequenced the rhesus macaque *NPY* 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 [21] predicted the loss of a GRE half site (Figure 1B). We found that the T>G SNP resulted in altered binding of regulatory proteins, with several bands increasing (MW of approx. 130, 210, and 260 kDa) and one of 180 kDa showing a relative decrease (Figure 1C). We also found that, in amygdala, the G allele resulted in decreased levels of *NPY* expression (Figure 1D, $F(1,9) = 24.4$, $P = 0.0008$). We performed gel super-shift assays using an anti-GR 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-GR antibody. The 180 kDa band (GR dimer) was preferentially bound in experiments performed with T allele oligonucleotides (Figure S2).

CSF NPY

There was a trend for an effect of rearing condition, with lower NPY levels among PR animals ($F(2, 66)= 2.68$, $P = 0.1$). There was no main effect for genotype ($F(2, 66) = 0.84$, $P = 0.44$). However, genotype interacted with rearing condition to predict CSF NPY ($F(2, 66) = 4.2$, $P < 0.02$). PR animals carrying the G allele (T/G or G/G) had lower CSF NPY levels than did PR T/T animals (Figure 2, Tukey-Kramer, $P < 0.05$). Among PR subjects, genotype accounted for 28 % of the variance.

BEHAVIORAL RESPONSES TO STRESS

Factor Analysis performed on behavioral measures taken during social separation generated three factors for each of the two phases of data collection. For the acute phase of stress, three factors were generated (“Separation Anxiety”, “Arousal”, and “Behavioral Pathology”) that accounted for 71.6% of the variance. The same three factors accounted for 77.6% of the variance for analysis performed on behaviors collected during chronic separation stress (Table S2).

During acute separation, there were main effects of rearing ($F(1, 96) = 6.4$, $P = 0.01$) and genotype ($F(2, 96) = 3.2$, $P = 0.04$) on “Arousal”, but no interaction. Post-hoc analyses demonstrated that PR infants exhibited higher levels of arousal than did MR infants and that those homozygous for the G allele had higher arousal scores than did those homozygous for the T allele (Figure 3A, Tukey-Kramer, $P < 0.05$). As with acute stress exposure, there was a main effect of rearing condition on “Arousal” ($F(1, 96) = 25.0$, $P < 0.0001$) during chronic separation, with PR animals exhibiting higher scores (Figure 3B, Tukey-Kramer, $P < 0.05$).

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There was no main effect for genotype. However, there was an interaction between rearing and genotype ($F(2, 96) = 4.2$, $P = 0.02$). Although PR T/T subjects responded no differently than MR animals, PR G allele carriers (T/G or G/G) exhibited higher levels of arousal (Figure 3B, Tukey-Kramer, $P < 0.05$). In both cases (acute and chronic stress), results remained the same following rank transformation of the data. Among PR 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 consumption in adolescent/adult subjects, with PR consuming more alcohol than MR subjects ($F(1, 85) = 16.5$, $P = 0.0001$). There was also an interaction between rearing and genotype ($F(2, 85) = 3.3$, $P = 0.04$), and this relationship remained following rank transformation of the data. Among monkeys reared in peer only groups, only those that were carriers of the G allele (T/G and G/G) consumed higher levels of alcohol than MR monkeys (Figure 4, Tukey-Kramer, $P < 0.05$). *NPY* −1002 T>G genotype accounted for 12.5% of the variance in alcohol consumption in the PR subjects.

When we examined the effects of rearing and genotype on alcohol consumption following periods of deprivation across the four weeks of testing, we found a main effect of rearing ($F(1, 204) = 12.5$, $P = 0.0007$), and both genotype × time ($F(6, 204) = 3.02$, $P < 0.008$) and rearing × genotype × time ($F(6, 204) = 2.2$, $P < 0.05$) interactions. While PR monkeys that were homozygous for the T allele showed a decline in drinking over time, there was an escalation in alcohol intake among PR monkeys with the T/G and G/G genotypes (Figure 5). There was no effect of genotype in MR monkeys. When we examined the effects of genotype, rearing and time over weeks of testing during the second through fifth days for the weekly sessions, the effects of genotype ($F(2,228) = 3.23$, $P = 0.04$), rearing ($F(1, 228) = 26.4$, $P < 0.0001$), and a genotype by rearing interaction ($F(2,228) = 4.9$, $P = 0.01$) were maintained, but there were not interactions of any of these factors with week of testing (Figure S3).

COMMENT

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 GxE interactionsis variation in genes encoding stress-responsive signaling molecules that may contribute to stress vulnerability or resiliency [7]. Perhaps most notable among the GxE 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 to influence stress reactivity and alcohol consumption, emphasizing the utility of this model for examining gene by environment interactions that translate to the human condition.

Because the NPY system is a key modulator of behavioral adaptation to stress, we screened the rh*NPY* gene for variants that might impact stress resilience, with the prediction that we would observe similar interactions. We identified a SNP (−1002 T>G) in the rh*NPY* regulatory region that predicts loss of a GRE half site. Glucocorticoids have long been known to regulate *NPY* expression [35], and this regulation may be important for NPY induction during periods of stress. We performed gel shift assays with nuclear extract derived from several GR-expressing cell lines[35, 36] and found that the G allele resulted in altered DNA-protein interactions with each nuclear extract tested. Among the bands that exhibited a relative decrease with the G allele (which, overall, showed increased TF binding) was one of 180 kDa, which was recognized by an anti-GR antibody. This suggests that the −1002 T>G SNP resulted in decreased preference for a functional GRE. We found that −1002 T>G predicted decreased *NPY* expression in amygdala, a brain region in which NPY release decreases anxious responding. Based on these functional differences, we predicted that this SNP would result in a decrease in NPY system

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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 PTSD [37–40]. There is also evidence that a gain-of-function variant resulting in a Leu7Pro substitution of the preproNPY signal peptide [40] may protect against depression, while markers on low expressing *NPY* haplotypes (−399 T>C) result in decreased levels NPY levels and up-regulated stress-responses [39, 41]. Here, we show that rhesus macaques exposed to adversity have lower CSF levels of NPY, but only as a function of the loss-of-function G allele. Consistent with this observation, G allele carriers are more aroused during both short-term and protracted exposures to stress. We postulate that a history of trauma and genotype may also interact to predict NPY levels in humans, and that individuals with low levels of *NPY* expression or who are unable to recruit 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 [42–44]. *Npy*-deficient mice consume more ethanol, while consumption is reduced in mice over-expressing *Npy* [44]. Moreover, the *Npy* gene maps to a quantitative trait locus underlying alcohol consumption in genetically selected alcohol preferring rats [45]. Based on these findings, a screen for functional variants was performed, identifying a marker (D4Mit7) that reduced brain expression of *Npy* in this line [46]. In humans, linkage to the chromosomal region containing the *NPY* gene has been demonstrated [47], and there have been associations of *NPY* variation with both alcohol consumption [40] and dependence [48, 49]. Other studies, however, have failed to replicate this association [50–52]. Of note, our present study did not find any effects of rh*NPY* −1002T>G on alcohol consumption in normally reared animals, even following repeated alcohol exposure. Instead, rh*NPY* −1002 T>G genotype increased alcohol consumption among those exposed to both early adversity and cycles of alcohol exposure. This suggests a high degree of stress loading may be required for the G allele to produce an effect, raising the possibility that human *NPY* variation could potentially increase risk for alcohol dependence moreso among individuals with especially traumatic life experiences 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 late-onset alcoholics [49] or samples highly represented by war veterans [48].

Dysregulation of the CRF system following repeated periods of alcohol exposure and deprivations contributes to the transition from reward- to relief- drinking [53, 5], and NPY signaling is a counter-regulatory process that buffers actions of CRF [5]. When we examined patterns of alcohol intake during repeat cycles of availability and deprivation, we found an interaction between *NPY* genotype and alcohol exposure, such that stress-exposed carriers of the G allele exhibit an escalation in alcohol intake. This is potentially indicative of 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 system would render individuals less resilient to stress and to addiction with continued alcohol use. Studies in humans have demonstrated there to be haplotypes that decrease *NPY* expression and predict stress-induced NPY release, amygdala response and stress resiliency [41]. However, whether *NPY* promoter variation moderates the risk for alcohol problems or interacts with life stress to moderate risk for stress-related disorders in humans has not been determined. Using an established primate model of adversity, we found that functional *NPY* variation influences CSF

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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 the NPY system as a treatment target in selected individuals. Our results also suggest *NPY* to be a candidate for examining gene by environment interactions in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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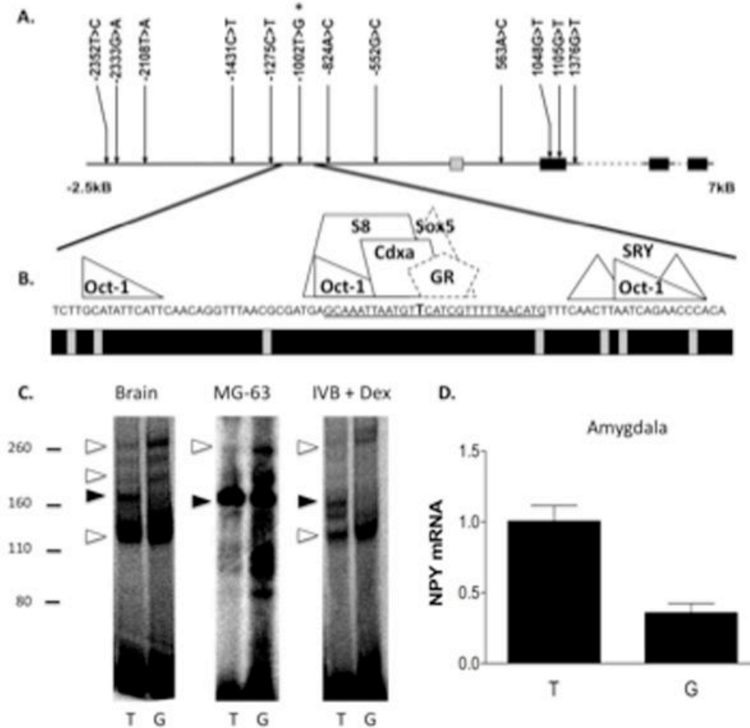

Figure 1. rh*NPY* −1002 T>G is present in a conserved portion of a *NPY* repressor and results in altered DNA-protein interactions and decreased amygdala *NPY* expression

A. Schematic of the NPY gene and regulatory region and SNPs detected by sequencing of genomic DNA. B. Region 40-bp up and downstream of the −1002 T>G SNP. The precise locations of the −1002 T>G SNP (in bold) and the oligonucleotide sequence used in the gel shift assays (underlined) are indicated. Predicted sites for transcription factor binding (above) and sequence conservation among primates (below, black = conserved) are shown. Binding sites in dashed lines (Sox5 and a preferred glucocorticoid response element half site, GR) were predicted to be disrupted by the −1002 T>G SNP. C. Gel shift assay results from experiments performed using nuclear extracts from Whole Brain, osteosarcoma cells (MG-63), and glucocorticoid-treated hypothalamic cells (IVB + Dex). Relative migrations of the protein molecular weight standards are shown to the left (kDa). Open arrows indicate bands that increase with G allele probes, closed arrows indicate that which shows a relative increase with T allele probes. D. *NPY* mRNA expression in amygdala as a function of the −1002 T>G allele ($P = 0.0008$; T/T, $N = 4$, G carrier, $N = 8$). *** $P < 0.001$.

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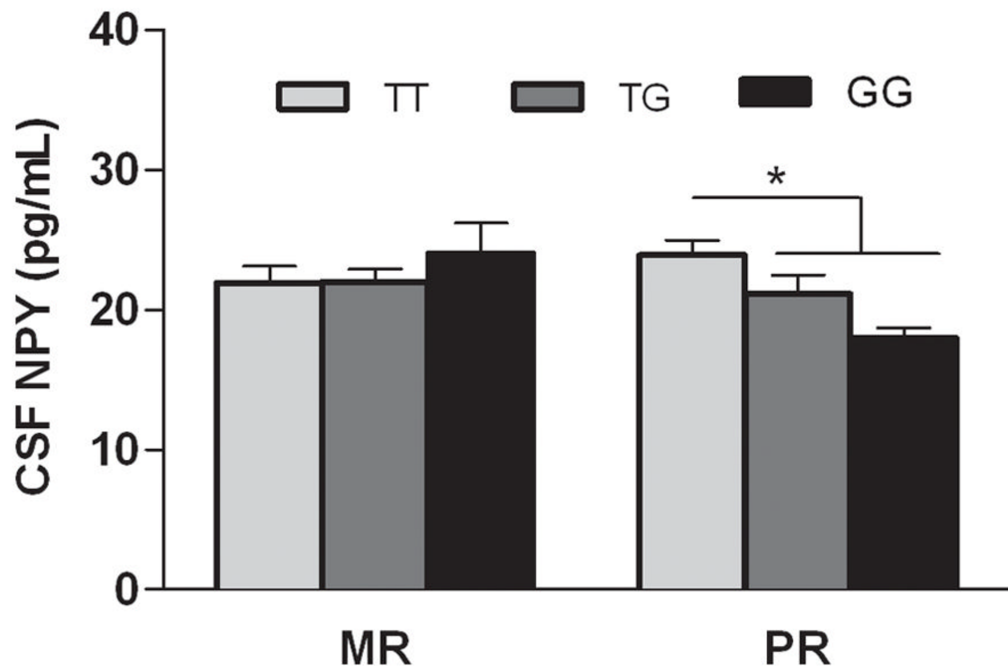

Figure 2. Interaction between rh*NPY* genotype (T/T, T/G, G/G) and early rearing history (MR, mother- reared, vs. PR, peer-reared) on CSF levels of NPY

There was an interaction between genotype and rearing (F(2, 66) = 4.2, $P < 0.02$). The G allele dose-dependently decreased levels of NPY measured in a cisternal CSF sample among stress-exposed monkeys (**Tukey-Kramer**, $P < 0.05$) (MR T/T = 17, MR T/G = 14, MR G/G = 4; PR T/T = 16, PR T/G = 13, PR G/G = 8). Genotype accounted for 28 % of the variance in PR subjects. Values shown are mean pg/ml ± SEM. * $P < 0.05$.

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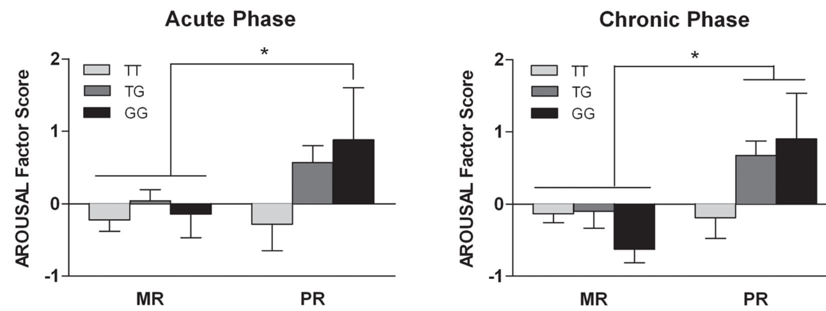

Figure 3. Interaction between rh*NPY* genotype (T/T, T/G, G/G) and early rearing history (MR, mother- reared, vs. PR, peer-reared) on arousal during periods of A. Acute (1 H) and B. Chronic (96 H) separation stress

During acute separation stress, there were main effects of both rearing ($F(1, 96) = 6.4$, $P = 0.01$) and genotype ($F(2, 96) = 3.2$, $P = 0.04$) on "Arousal", with genotype accounting for 7% of the variance. During chronic stress, there was an interaction between rearing and genotype ($F(2, 96) = 4.2$, $P = 0.02$). Although PR T/T subjects responded no differently than did MR animals, PR G allele carriers exhibited higher levels of arousal (T/G and G/G vs. T/T, Tukey-Kramer, $P < 0.05$), with genotype accounting for 10% of the variance in these subjects. (MR T/T = 35, MR T/G = 27, MR G/G = 10; PR T/T = 9, PR T/G = 15, PR G/G = 6). Values shown are "Arousal" factors scores ± SEM. * $P < 0.05$.

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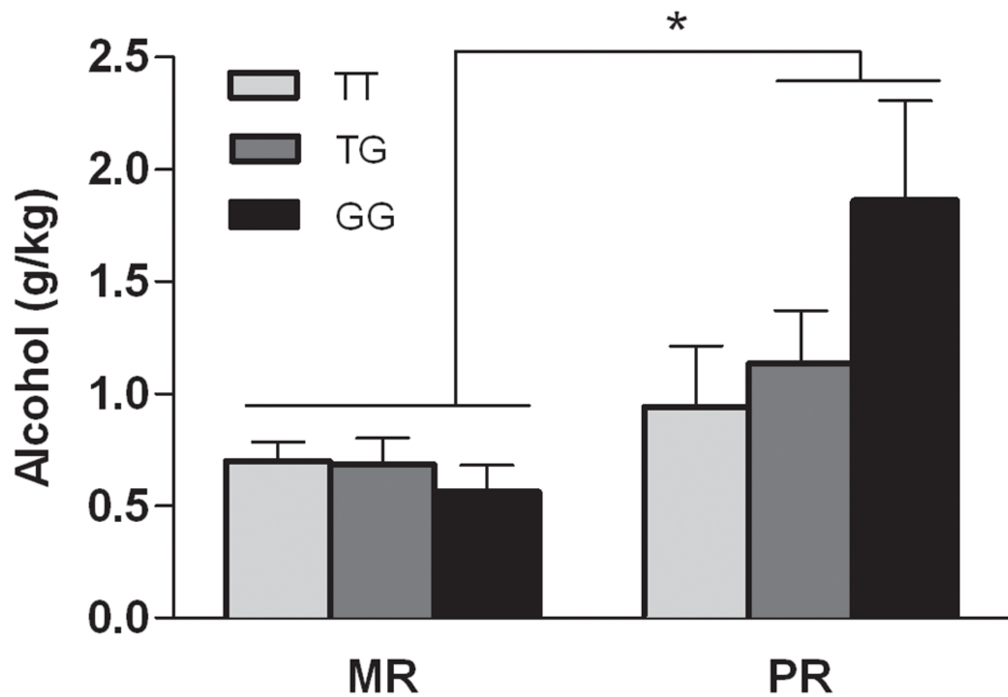

Figure 4. Interaction between rh*NPY* genotype (T/T, T/G, G/G) and early rearing history (MR, mother- reared, vs. PR, peer-reared) on levels of voluntary alcohol consumption (mean ± SEM)
There was an interaction between rearing condition and genotype on alcohol consumption (F (2, 85) = 3.3, *P* = 0.04). When given simultaneous access to alcohol (8.4% v/v) and sweetened vehicle in a limited access paradigm, PR monkeys who were carriers of the G allele consumed higher levels of alcohol than did non-stress-exposed (MR) subjects genotype (Tukey-Kramer, *P* < 0.05). Genotype accounted for 12.5% of the variance in PR monkeys. (MR T/T = 29, MR T/G = 25, MR G/G = 8; PR T/T = 10, PR T/G = 11, PR G/G = 8). Values shown are G/KG alcohol consumed in a 1-hour session (G/KG/H) ± SEM. * *P* < 0.05.

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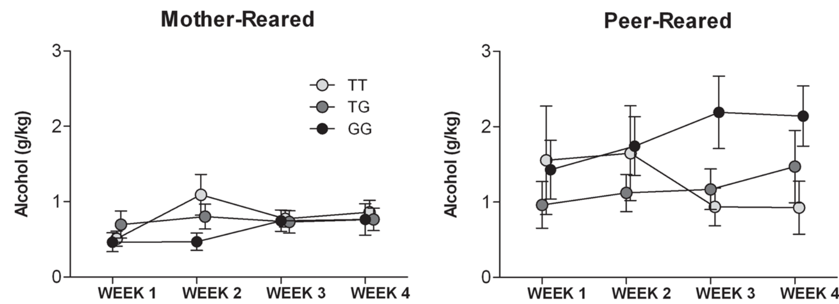

Figure 5. Interaction between rh*NPY* genotype (T/T, T/G, G/G) and early rearing history (MR, mother- reared, vs. PR, peer-reared) on alcohol consumption over repeated weeks of alcohol deprivation (WEEKS 1, 2, 3, and 4)

There were both genotype by time (F(6, 204) = 3.02, $P < 0.008$) and genotype by time by rearing time (F(6, 204) = 2.2, $P < 0.05$) interactions. In PR monkeys, alcohol intake decreased across over time in individuals with the T/T genotype, but an escalation in consumption was observed in those carrying G allele. There was no significant effect of genotype in MR monkeys. The interaction between time and genotype accounted for 19% of the variance in PR monkeys. (MR T/T = 22, MR T/G = 19, MR G/G = 7; PR T/T = 9, PR T/G = 10, PR G/G = 7). Values shown are G/KG alcohol consumed in a 1-hour session (G/KG/H) ± SEM following a 3-day period of deprivation.