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ABSTRACT: Studies in humans and animals suggest a role for NPY in the mediation of behavioral stress responses. Here, we examined whether the NPY promoter variant rs16147 is functional for expression of NPY in a brain region relevant for behavioral control, anxiety and depression, the anterior cingulate cortex. *In silico* analysis of DNA structural profile changes produced by rs16147 variation suggests allelic differences in protein binding at the rs16147 site. This was confirmed by electrophoretic mobility shift assay, demonstrating that the rs16147-C site has strongly reduced affinity for a yet unknown factor compared to the T-allele. Analyzing 107 human post-mortem brain samples we show that allelic variation at rs16147 contributes to regulation of NPY mRNA and peptide levels in this region. Specifically, the C-allele leads to increased gene expression. In agreement with the molecular findings, rs16147 is associated with anxiety and depressive symptoms in 314 young adults via a gene x environment interaction with early childhood adversity, replicating the recent finding of rs16147-C as a risk factor for stress related psychopathology. Our results show the importance of rs16147 for regulation of NPY gene expression and brain function. ©2010 Wiley-Liss, Inc.

KEY WORDS: NPY, promoter variant, brain, gene expression, stress coping

INTRODUCTION

Dysregulation of stress responses is key to the pathophysiology of mood and anxiety disorders, common and disabling conditions that exhibit high levels of lifetime co-morbidity and have overlapping genetic underpinnings (Hettema et al., 2006). Neuropeptide Y (NPY, OMIM: 162640), a 36 amino acid peptide, is an established moderator of stress responses [reviewed in (Hokfelt et al., 1998;Heilig, 2004;Thorsell, 2008)]. Functional variation at the locus encoding NPY may therefore mediate individual differences in stress reactivity and resilience. Consequently, it was recently found that a five marker haplotype spanning the NPY promoter predicts central NPY mRNA levels, and is associated with brain responses to negative emotional stimuli (Zhou et al., 2008). A single promoter variant, rs16147, that was previously demonstrated to affect gene expression (Itokawa et al.,

2003;Buckland et al., 2005), accounted for about half of the variance in the haplotype analysis. Although compelling, these results remain controversial (Cotton et al., 2009).

Here, we carried out a multi-level candidate gene analysis to study the role of rs16147. First, using a novel bioinformatics tool for the quantitative evaluation of how the topography of genomic DNA (i.e. the shape of the backbone and grooves) varies throughout a genome (Greenbaum et al., 2007), altered interaction with DNA-binding proteins at rs16147 was predicted *in silico*, and subsequently confirmed *in vitro*.

We then tested the hypothesis that variation at rs16147 contributes to brain NPY expression in a set of samples drawn from the Human Brain Tissue Bank, Budapest. Samples in this collection were generally obtained after a short post mortem interval (PMI), which makes this sample highly suitable for gene expression studies (Dwivedi et al., 2006;Du et al., 1999). The effect of rs16147 on NPY expression was studied in the anterior cingulate cortex (ACC), a region with a critical role in the limbic-cortical circuit that modulates emotional behavior (Drevets et al., 2008). We found that the C-allele was associated with increased NPY gene expression in the ACC.

To evaluate whether the molecular findings have behavioral correlates, we turned to the Mannheim Study of Children at Risk, a longitudinal epidemiological cohort study following the outcome of early risk factors from infancy into adulthood (Laucht et al., 2000;Blomeyer et al., 2008). In this sample, we found an association of the rs16147 C-allele with measures of trait anxiety and depression at young adult age as a function of exposure to early childhood adversity factors.

MATERIALS AND METHODS

In silico NPY promoter analysis. A comprehensive library of hydroxyl radical cleavage profiles is available at dna.bu.edu/orchid and allows prediction of structural DNA profiles from sequence information *in silico* (Greenbaum et al., 2007). Standard transcription factor binding site analysis was done using an evolutionary sequence-constraint algorithm as implemented in the GENOMATIX portal (www.genomatix.de) using the functions MathInspector and SNPInspector with default settings (Cartharius et al., 2005).

Nuclear extract preparation and electrophoretic mobility shift assay. Human anterior cingulate cortex nuclear extracts were prepared using nuclear extract kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions and stored in aliquots at –80 ºC. Protein content was determined using a bicinchoninic acid colorimetric assay (Pierce, Rockford, IL). EMSA were performed on nuclear extracts (5 µg/assay) as described in (Barr et al., 2009) using double-stranded oligonucleotides containing either the T (5'- ACA GGA CTA CCA CCC ACT GGG TGC-3') or the C (5'- ACA GGA CTA CCG CCC ACT GGG TGC-3') allele. Briefly, after annealing complementary oligonucleotides (95°C 5 min, 25°C 30 min), double-stranded probes were [^{33}P]-ATP labeled using T4 kinase (Promega, Madison, WI) and purified using G-25 columns (GE Healthcare). Incorporation of radiolabel was > 1 x 10^5 cpm/ng DNA. Binding assays were performed using the Gel Shift Assay System (Promega, Madison, WI) according to the manufacturer instructions. Nuclear extracts (5 µg/assay) were incubated for 20 min with 1 x 10^5 cpm of each oligonucleotide probe. Competitor oligonucleotides were added at 10 and 50 pmole. Samples were immediately separated by electrophoresis (200 V for 20 min) on a Novex 6% DNA retardation gel (Invitrogen, Carlsbad, CA), after which gels were dried and bands visualized by autoradiography. Fuji BAS-5000 PhosphorImager plates (Fujifilm, Tokyo, Japan) were exposed to the hybridized sections, and PhosphorImager-generated digital images were analyzed using the manufacturer' image analysis software. Density values for each band are relative to the band generated by the T-allele oligonucleotide without competitor. Quality of nuclear extracts was assessed by western blot analysis demonstrating high abundance of the nuclear marker protein cyclin E2 in the nuclear fraction. Data are expressed as ratios of density values relative to the T-allele band generated without competitor. Extracts from three brains were analyzed and each gel shift assay was performed at least three times. For details, see *SI text*.

Postmortem sample. In all, 107 subjects from the Human Brain Tissue Bank, Budapest, were included into the study. The sample consists of 28 suicide completers (19 males/9 females) with established major depression as defined by DSM-IV criteria, and 79 controls (48 males/31 females) with no history of mental illness. Samples were obtained at autopsy at the Department of Forensic Medicine of the Semmelweis University Medical School (Budapest, Hungary) after written informed consent was obtained from next of kin. Consent was to consult the medical chart and to conduct neurochemical and/or biochemical analyses. The procedures were approved by the ethics committee of the Semmelweis University.

Medical, psychiatric and drug history of suicide subjects were obtained through chart review and interviews with the attending physician/psychiatrist and family members. In each instance a psychiatric diagnosis of major

depressive disorder was on record. The diagnoses were conducted and/or confirmed by experienced psychiatrists on the basis of DSM-IV criteria (American Psychiatric Association, 1994b). The major cause of death was hanging. Control subjects had never been treated for depression, and did not have a history of alcohol or drug abuse, according to interviews with family members and examination of medical records of the last ten years. Main causes of death in control subjects were acute cardiac arrest or traffic accident. There is no sex ratio difference between the groups but significant differences in age (mean age: 48 years, range 28-83, and 68 years, range 33-94, suicide vs. controls, respectively, t-test: $p > 0.001$) and postmortem interval (mean PMI: 4.6 hours, range 1-8, and 3.2 hours, range 0.5-8, suicide vs. controls, respectively, t-test: $p > 0.001$). These factors were therefore included as covariates into the statistical model (see below)

All samples are obtained within a narrow postmortem interval (PMI), ranging from 0.5 – 8 hours. PMI is considered one of the major factors determining sample degradation. For comparison, previous postmortem studies on NPY levels reported a mean PMI ranging from 17 – 30 hours (Widdowson et al., 1992;Ordway et al., 1995;Caberlotto and Hurd, 1999;Hashimoto et al., 2007), which may have contributed to some of the inconsistencies between reported findings. Another, more recently used marker of tissue degradation is brain pH, which is available only for a subset of samples, and therefore was not considered in the statistical model. The general short PMI makes extensive degradation of samples unlikely.

After removal from the skull, the brains were cut in six major pieces (four cortical lobes, basal ganglia-diencephalon and lower brain stem-cerebellum), rapidly frozen on dry ice, and stored at -70°C until microdissection (2 days to 2 months later). At the time of the dissection, the brain samples were sliced into 1-1.5 mm thick coronal sections at a temperature of 0-10°C. The *anterior cingulate cortex* (ACC, Brodmann area 24) was cut out of the sections by a fine microdissecting (Graefe's) knife or special microdissecting needles, immediately dorsal to the corpus callosum, as far posterior as the posterior edge of the frontal lobe. The samples were stored in airtight containers or plastic tubes at -80°C until further use.

Epidemiological sample. Participants were drawn from the Mannheim Study of Children at Risk, a longitudinal epidemiological cohort study following the outcome of early risk factors from infancy into adulthood (Laucht et al., 2000). The initial sample comprised 384 children born between 1986-88, of predominantly (>99.0%) European descent. Infants were recruited from two obstetric and six children's hospitals of the Rhine-Neckar Region of Germany and were included consecutively into the sample according to a two-factor design intended to enrich and control the status of the sample regarding obstetric and psychosocial risks (see *Supp. Table S1* and (Laucht et al., 1997). Only firstborn children with singleton births and German-speaking parents were enrolled in the study. Furthermore, children with severe physical disability, obvious genetic defects, or metabolic diseases were excluded. Of the initial sample of 384 participants, 18 (4.7%) were excluded because of severe disability (IQ or MQ<70 or neurological disorder), 36 (9.4%) were dropouts or had incomplete data, and 18 (4.7%) refused to participate in blood sampling. The final sample for which complete data were available consisted of 314 young adults (144 males, 170 females). Loss of subjects was not selective with regard to sex and obstetric or psychosocial risks. The study was approved by the ethics committee of the University of Heidelberg and written informed consent was obtained from all participants.

Measurement of early childhood adversity according to an "enriched" index as proposed by Rutter & Quinton (1977) was derived from a standardized parent interview conducted at the three-month assessment. The index assesses the presence or absence of 11 adverse family factors *(Supp. Table S1)*, covering characteristics of the parents, the partnership, and the family environment during a period of one year prior to birth (M=1.92, SD=2.06, range 0–7). Assessment of stability over a period of more than ten years revealed coefficients of about r=.70.

To assess psychiatric symptoms and obtain diagnoses for the period between age 15 and 19 years, i.e. between prior and current assessment, the Structured Clinical Interview for DSM IV (SCID, German version (American Psychiatric Association, 1994a;1997)) was administered to the 19-year-olds. The SCID is a widely used diagnostic interview, for which a considerable body of reliability and validity data has been published (Rogers, 2001). 24 of the young adults (7.6%) met criteria for any depressive disorder, and 20 (6.4%) met criteria for any anxiety disorder. Based on the shared common genetic factors underlying anxiety and depression, a broad phenotype was utilized for the present evaluation, defined as diagnosis of any anxiety or depressive disorder (N= 40, 12.7%). Symptoms of depression and trait anxiety at age 19 years were assessed by Beck's Depression Inventory (BDI, German version (Beck and Steer, 1987;1994)) and the State-Trait Anxiety Inventory (STAIT scale; German version (Spielberger et al., 1970;Laux et al., 1981)), respectively. Both self-report instruments have been used

extensively in clinical and epidemiological research and have excellent psychometric properties (Kimberly et al., 2000;Spielberger and Reheiser, 2004).

Genotyping and gene expression. Genomic DNA for the epidemiological sample was extracted from whole blood or saliva with the Qiamp (Qiagen, Chatsworth, California) kit. DNA and RNA from brain tissue were isolated using Trizol according to manufacturer's protocol (Invitrogen, UK).

DNA (50 ng/ul) was genotyped for rs14167 at the CIMH' Molecular Genetics Laboratory or at LCTS by a TaqMan allelic discrimination assay using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems Inc., Foster City, CA, USA) following the manufacturer's protocol. The distribution of genotypes did not deviate from Hardy-Weinberg equilibrium in any of the samples (epidemiological sample: TT=81, 25.8%, TC=155, 49.4%, and CC=78, 24.8% and *Supp. Table S2*; postmortem sample: TT = 33, 30.8%, TC = 43, 40.2%, and CC = 31, 28.9%). Ancestry scores in the post-mortem sample were estimated using a GoldenGate Assay (Illumina) 1,536-marker genotyping array as described (Enoch et al., 2006). The marker selection was based on the HapMap Project reference allele frequency (RAF) among European Caucasians, Asians, and Africans (Yoruban). The RAF difference is greater than 0.7 and the ratio larger than 10:1 between at least one pair of the continental populations and is balanced to distinguish between continental populations. The majority of the population is of European Caucasian ancestry. In 19 subjects (18% of population) European ancestry scores were below < .75 (*Supp. Figure S1*). At this threshold there is the possibility that the subjects could have had at least one second degree relative of non-European descent. To control for this heterogeneity European ancestry scores were included as covariates into the statistical analysis.

RNA samples underwent a cleanup step using the RNeasy Mini Kit (Qiagen, USA) and were then treated with RQ1 RNase-free DNase (Promega, USA) following manufacturer's instructions, to eliminate DNA contamination. All RNA samples had acceptable 260/280 ratios (1.8 - 2.1). RNA samples were then analyzed with an Agilent 2100 Bioanalyzer and the RNA integrity number (RIN) was calculated according to (Schroeder et al., 2006). 100 ng RNA was used for cDNA synthesis using reverse transcription reagents according to the manufacturer's protocol (Applied Biosystems Inc., Foster City, CA, USA).

Applied Biosystems Assay # Hs00173470_m was used to detect NPY mRNA levels and performed in quadruplicates for each sample on an ABI Prism 7900HT with TaqMan universal PCR master mix (Applied Biosystems) following the manufacturer's protocol. Expressed AluSx repeats were used for normalization of the mRNA fraction in the samples. Alu repeats are a class of short repeated sequences that are interspersed throughout the genome and are in part expressed as non-coding RNAs or as part of normal coding mRNAs. In preliminary experiments it was found that this single measure correlates well with three commonly used endogenous references assays, i.e. ACTB, PPIA, and 18S (Vandesompele et al., 2002).

The following primers were used for the AluSx assay (concentrations are given in parenthesis): forward (600 nM): 5'-TGGTGAAACCCCGTCTCTACTAA-3', reverse (600 nM): 5'-CCTCAGCCTCCCGAGTAGCT-3', probe (200 nM): 5'-AAAAATTAGTCGGGTGTGGTGACAGGCG-3'. 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). The data is presented as relative gene expression to endogenous AluSx expression levels (ΔCt). The mean cycle threshold (Ct) for AluSx was 21.87 ± 0.12 and 22.31 ± 0.19 in ACC and cerebellum , respectively. Ct values for AluSx did not show significant effects for any factor tested in the statistical analysis.

NPY radioimmunoassay. A competitive NPY radioimmunoassay (Phoenix Pharmaceuticals, Inc, Burlingame, CA) was employed according to the manufacturer's instructions. In short, after adding 0.5 M acetic acid brain tissue was homogenized and then boiled for 10 min. 10% of each sample was removed before the sample was boiled and used for protein determinations using BCA Protein Assay Kit (Pierce, Rockford, IL). The samples were then centrifuged at 1000 x g for 10 min and the supernatant fluid lyophilized. The samples were reconstituted in RIA buffer and mixed with rabbit anti-peptide serum and incubated at 4°C over night. ^{125}I-NPY at a concentration of 8000 cpm/100uL was added to the samples and again incubated at 4°C over night. Goat Anti-Rabbit IgG serum and Normal Rabbit Serum were added on the third day and all tubes were incubated at room temperature for 90 min. After centrifuging the samples at 4°C for 20 min, the supernatant was aspirated and the pellet counted using a gamma-counter and the result related to a standard curve.

Statistical analysis. Band intensities from gel shift experiments were normalized to the T-allele band without competitor and compared by 1-way ANOVA. For the post-mortem sample we used the generalized regression model module of STATISTICA 7.1 (StatSoft Inc, Tulsa, Oklahoma) assuming normal log distribution for

analyzing the effect of allele on NPY mRNA and peptide levels. Genotype (s16147 allele TT = 0, TC = 1, and CC = 2), age, sex, ancestry, cause of death, PMI, and RNA integrity number were used as predictors. In the epidemiological sample T-tests or analyses of variance and chi-square tests, respectively, were performed to test differences in scores and frequencies between sex and genotype groups. Genotypes for rs14167 were coded as TT = 0, TC = 1, and CC = 2. Linear and logistic regression analyses for continuous (BDI, STAIT) and categorical variables (DSM IV diagnosis), respectively, were conducted to examine whether NPY rs16147 genotype moderated the effect of environmental adversity (range 0-7) on outcome measures. For each analysis, sex was controlled for in the first step, the main effects of genotype and adversity were entered in the second step, followed by their interaction in the third step. Significant interactions were further examined using simple main effects analyses.

RESULTS

Besides the primary sequence, a feature that can influence transcription factor binding specificity is the backbone conformation of the DNA binding site (Joshi et al., 2007). In fact, topography-informed evolutionarily constrained DNA regions correlate better with functional non-coding elements than those predicted by primary sequence-constraint algorithms (Parker et al., 2009), likely because different DNA sequences may still be similar in structure, and thus may perform similar biological functions. The solvent accessible surface area of the DNA backbone is well reflected by its hydroxyl radical cleavage pattern (Balasubramanian et al., 1998). Strong differences in the predicted hydroxyl radical cleavage pattern were found among variants of the rs16147 SNP, predicting differences in protein binding affinity (*Fig. 1A*). The lower score of the T-allele reflects narrowing of the DNA backbone which can act as an electrostatic groove to enhance protein-DNA interaction (Joshi et al., 2007). Of the other two common genomic variants in the NPY promoter, rs17149106 did not affect DNA topology and rs3037354 could not be assessed by this tool because it is not an SNP but a deletion variant.

The *in silico* prediction of altered protein-DNA interaction at rs16147 was validated using electromobility shift assay on nuclear protein extracts from ACC and cerebellum incubated with oligonucleotides representing either the T- or the C-allele. Specificity of the protein-oligonucleotide interaction for the T-allele was demonstrated by reduced intensity of the shifted complex upon addition of increasing amounts of unlabeled oligonucleotide probe. Notably, the C-allele oligonucleotide showed only weak protein binding and did not compete as efficiently as the T-allele probe, suggesting that the C-variant decreases protein binding affinity (*Fig. 2B, C*). No differences in protein binding between the two brain regions were found. Similar to previous attempts (Itokawa et al., 2003;Buckland et al., 2004), we could not assign a conserved transcription factor binding site to rs16147. A classical evolutionary sequence-constraint algorithms did not find any known mammalian transcription factor binding sites within 20 bp up- and downstream of rs16147 (*Fig. 1D*).

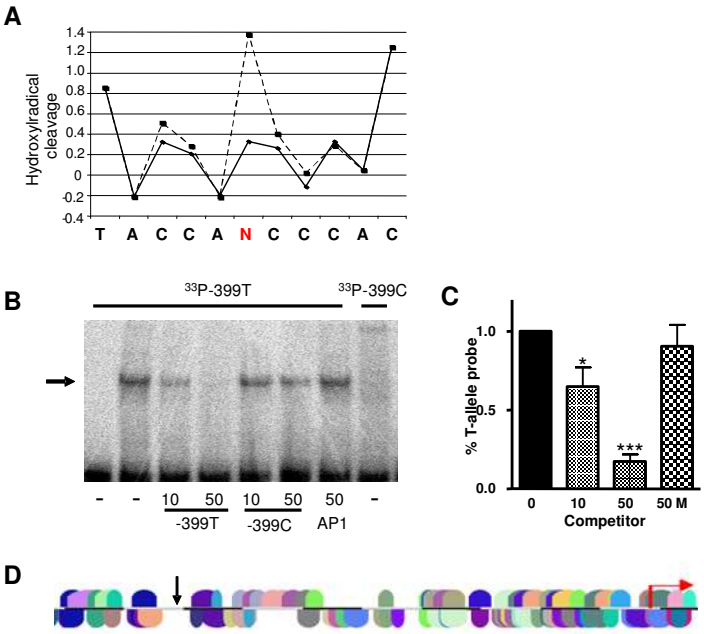


Figure 1: Altered DNA surface structure and DNA-protein interaction at rs16147. A) Allelic difference in DNA surface structure is shown at rs16147 by using predicted hydroxyl radical cleavage patterns (Greenbaum et al., 2007). Solid line = T-allele, dashed = C. **B)** Representative gel image of EMSA from ACC nuclear extracts show a single band (arrow) captured by T but not C allele-specific ^{33}P-labeled oligonucleotide probes (probe specificities shown above lanes). Specificity of this interaction is demonstrated by adding unlabeled T- or C-allele and AP1 oligonucleotides as competitors (10 or 50 pmole, shown below the lanes). No brain extract control is shown on the left lane. **C)** Bar graphs showing quantitative analysis of the EMSAs. Data represent density values relative to the band generated by the T-allele oligonucleotide (lane 2). 1-way ANOVA: * $p < 0.05$, *** $p < 0.001$ vs. the T-allele band without competitor. **D)** No transcription factor binding sites are predicted within 20 bp up- and downstream of rs16147 (arrow) by evolutionary sequence constraint analysis (www.genomatix.de). Binding motifs for mammalian transcription factors are shown up to -500 bp upstream of the NPY transcriptional start site (winkled arrow).

The effect of genotype on NPY gene expression was tested under the assumption of a co-dominant mode of action, because *cis*-acting promoter variants are expected to act independently from each other on their respective chromosomes. Generalized regression analysis was used to assess the effects of the following variables: age, sex, ancestry, cause of death, PMI, RNA quality and gene dose of rs16147 on NPY mRNA and peptide levels in the ACC *(Fig. 2)*. When controlling for all other factors, we found a significant effect of genotype, such that the C-allele confers higher NPY expression levels in the ACC. The effect of RNA quality on NPY mRNA levels is expected and reflects the fact that with increased sample degradation the amount of detectable NPY mRNA decreases. Notably, the cause of death (suicide vs. controls) does not seem to have any effect on NPY mRNA levels. To exclude the possibility that the suicide sample could skew the results via factors not addressed in the present model, we did a separate analysis on the control group only using the same model (excluding CoD as a factor). We found essentially the same genotype effect, but with weaker statistical significance as expected from the smaller sample size *(Supp. Table S3)*. Cerebellum was only available from 92 of the 107 subjects, but was included because Zhou et al (2008) reported expression data from this region. NPY levels in cerebellum are more than two orders of magnitude lower compared to ACC, and no evidence for alteration by rs16147 was found in this region (mean dCT: TT = 10.82 ± 0.21, TC = 10.83 ± 0.21, CC = 11.49 ± 0.23, computed at their covariate means).

Although stratification bias is not expected to exert strong effects on a functional SNP, we wanted to directly exclude a possible influence of genetic background. To this end, we compared the effect of genotype on NPY expression after splitting the population into subjects with > 75 % European AIM and the remaining subjects with increased proportion of non-European alleles (*Supp. Figure S1*). The effect of rs16147 on NPY expression is similar in both subpopulations (beta: -.208, $p < 0.05$ and -.488, $p < 0.01$, respectively) with no difference in allele frequencies between these groups.

Rs16147 also appears to modify NPY peptide levels in the ACC, although with greater variability *(Fig. 2)*. We found significant effects for age and PMI ($p < 0.05$ for both factors) and a trend toward significance for genotype ($p < 0.06$). Concordant with results for the mRNA levels, independent of diagnosis, higher amounts of NPY

peptide were associated with the C-allele (mean NPY peptide levels: TT = 0.141 ± 0.016 pmol/mg protein, TC = 0.186 ± 0.014, CC = 0.174 ± 0.16, computed at their covariate means). The effect of PMI reflects peptide degradation over time. The age effect is equivalent to about 25% decline in peptide levels over a 50 year period.

	ACC mRNA	ACC peptide	CB mRNA
Gene dose	-.224 (.087)**	.121 (.067)$^{+}$	.002 (.014)
Sex	-.011 (.084)	-.082 (.059)	-.002 (.013)
Age	.004 (.004)	-.010 (.003)*	-.001 (.001)
Ancestry	-.141 (.265)	.076 (.228)	-.033 (.050)
CoD	.035 (.093)	.004 (.066)	-.029 (.015)
PMI	.036 (.033)	-.067 (.027)*	-.001 (.006)
RIN	-.103 (.038)**	NA	.005 (.009)
Sex x CoD	-.064 (.079)	.039 (.058)	-.016 (.013)

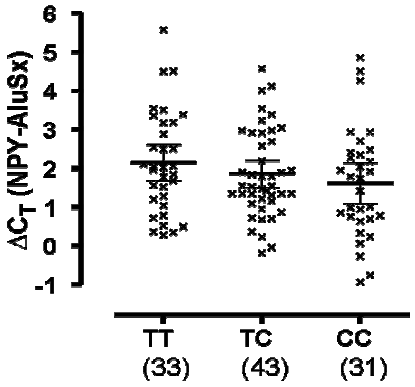


Figure 2: Generalized regression model for NPY mRNA and peptide expression in ACC. *Left:* Regression estimates and their standard errors are shown for the predictors used in the model. Genotype for rs16147 was coded as TT=0, TC=1, CC=2. C.o.D. = cause of death, PMI = post mortem interval, RIN = RNA integrity number. NA = not applicable. CB mRNA = mRNA from cerebellum.. $^{+}$p < .10, *p < .05, **p<.01. *Right*: Individual NPY mRNA expression values in ACC are shown, number of subjects is given in parenthesis. Lines mark the mean ± upper and lower 95% confidence interval. NPY mRNA levels are expressed as ΔC_t(NPY-AluSx) representing the difference to the endogenous AluSx control on a $\log_2$ scale. Higher values represent lower expression level.

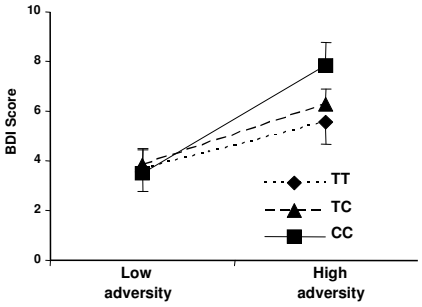
The effects of rs16147 genotype and psychosocial adversity on measures of depression and anxiety in young adults were tested in multiple regression models (summarized in *Fig 3A*). There was a significant effect of sex on measures of depression and anxiety, indicating higher BDI (p<.001) and STAIT scores (p<.002) as well as higher rates of DSM-IV diagnoses related to anxiety or depression (p<.002) among females than among males. Genotype groups did not differ significantly with regard to sex, age, IQ, or psychosocial adversity. There was a significant main effect of psychosocial adversity on all outcome measures, with higher scores and rates of depression or anxiety associated with increasing adversity. No significant main effect of rs16147 genotype was observed for these outcomes, but a significant genotype x adversity interaction emerged for the BDI, and a marginally significant interaction for the STAIT. Subsequent analysis revealed that the number of adversity factors present was associated with increasing BDI scores in carriers of the C allele (CC: beta=.393, p<.001; TC: beta=.264, p=0.001), but not among T homozygotes (beta=.129, p=.250).

To illustrate the interaction graphically, the continuous measure of psychosocial adversity was dichotomized into high and low adversity using a median split. The interaction is displayed in *Fig 3B*, indicating that, when exposed to elevated adversity, individuals scored higher on the BDI with increasing numbers of the C allele. Similar results were obtained for the STAIT score, demonstrating that the number of adversity factors present was associated with increasing scores among C carriers (CC: beta=.323, p=.004; TC: beta=.200, p=0.012), but not among T homozygotes (beta=.188, p=.851). No significant GxE was obtained between the rs16147 genotype and psychosocial adversity on the diagnosis of depression or anxiety.

A

	Any depressive or anxiety disorder [1]	BDI score [2]	STAIT score [2]
rs16147	.223 (.248)	.411 (.465)	.146 (.751)
Psychosocial adversity	.242 (.078)**	.798 (.160)***	.900 (.259)***
rs16147 x adversity	.014 (.113)	.453 (.228)*	.689 (.369) +

B



C

Item	Psychosocial adversity	%
1	Low education of a parent	18.2
2	Overcrowding	7.3
3	Parental psychiatric disorder	20.4
4	History of parental broken home or delinquency	18.5
5	Marital discord	11.1
6	Early parenthood	24.5
7	One-parent family	9.2
8	Unwanted pregnancy	13.1
9	Poor social integration and support of parents	4.8
10	Severe chronic difficulties	29.6
11	Poor coping skills of a parent	37.3

Figure 3: Gene x environment interaction at NPY rs16147. A) Multiple (linear and logistic) regression models testing the effects of NPY *rs16147* genotype, psychosocial adversity and their interaction on measures of depression and anxiety in young adults. Non-standardized regression coefficients b (SE) are shown for the main effects (second step), and for the interaction effects (third step) adjusted for sex; [1] coefficients from logistic regression; [2] coefficients from linear regression; +p < .10, *p < .05, **p<.01, ***p<.001. **B)** Mean BDI scores (SE), adjusted for sex, in young adults grouped by NPY rs16147 genotype and exposure to psychosocial adversity (divided by median split). **C)** Short Definition of the psychosocial adversity items (for detailed definition see *SI Text*. Numbers represent percentages of individuals exposed to the respective adversity condition. BDI = Beck Depression Inventory, STAIT = State-Trait Anxiety Inventory, T subscale

DISCUSSION

Our study supports allelic variation of the *NPY* gene as a genetic moderator of stress reactivity in humans. Expression analyses in human post-mortem brains show that the C-allele of the common NPY promoter variant rs16147 is associated with higher NPY expression levels in the ACC, a brain area of critical importance for affective processing (Drevets et al., 2008). A potential molecular mechanism is suggested by *in silico* and experimental findings of altered affinity to DNA binding proteins at rs16147. In agreement with previous observations (Zhou et al., 2008), we find that psychosocial adversity mediates a modest but significant association of rs16147-C with depressive and anxiety symptoms in an epidemiological sample of young adults.

Consistent evidence supports a role of rs16147 for transcriptional regulation of NPY expression (Itokawa et al., 2003;Buckland et al., 2004;Buckland et al., 2005;Shah et al., 2009). Tissue culture experiments with cells of neuronal and non-neuronal origin found strongly increased reporter gene expression under the control of C-variant carrying promoters compared to the T-allele (Buckland et al., 2005;Itokawa et al., 2003). The opposite allelic effect, i.e. decreased expression by the C-allele was found in immortalized lymphoblasts (Zhou et al., 2008). Furthermore, plasma levels of NPY are predicted by rs16147, but the direction of the effect seems to depend on the environmental conditions under which the sample was drawn. Thus, when blood samples were obtained under resting conditions reduced NPY plasma levels were found in rs16147 C-allele carriers (Zhou et al., 2008), while increased NPY plasma levels by rs16147-C were found in a large sample obtained under highly stressful, preoperative conditions (Shah et al., 2009).

In this light, the notable finding of opposing effects on NPY expression by rs16147 in ACC reported here and cerebellum (Zhou et al., 2008) appears as a reflection of the strong heterogeneity between brain regions and their respective mechanisms of transcriptional control. Indeed, we have repeatedly found in rodent brain that expression is a function of brain region and genetic background (Hansson et al., 2006;Sommer et al., 2006;Björk et al., 2008), making predictions of the directionality with which genetic variants will influence expression difficult.

Another important finding from the postmortem study is the age-related decline in NPY levels in the prefrontal cortex. A decline in plasma NPY levels was reported in normal elderly human subjects (Chiodera et al., 2000). Several studies in rodents supports a decline in brain NPY levels with advanced age (Vela et al., 2003;Hattiangady et al., 2005;Higuchi et al., 1988). Together these findings may point to lower NPY signalling in the elderly. This might contribute to memory loss, increased sensitivity to stress, loss of appetite and other aging related

pathologies. Finally, we did not find an association between NPY expression and suicide. This result is consistent with a previous findings of unaltered NPY mRNA levels in *post mortem* samples from prefrontal cortex of suicide victims (Caberlotto and Hurd, 1999). Completed suicide is a highly heterogeneous category, and these findings are therefore unsurprising.

Extensive preclinical studies and recent clinical investigations suggest NPY to be involved in regulation of stress responses (Heilig, 2004;Thorsell, 2008;Yehuda et al., 2006;Hou et al., 2006;Czermak et al., 2008;Nikisch and Mathé, 2008). The potential role of genetic variation within the NPY promoter region for these responses and the risk to develop psychiatric disorder is currently under debate (Zhou et al., 2008;Mottagui-Tabar et al., 2005;Itokawa et al., 2003;Heilig et al., 2004;Cotton et al., 2009;Inoue et al., 2009;Zill et al., 2008;Hall et al., 2007;Duan et al., 2005;Lindberg et al., 2006). Three common variants (rs16147, rs17149106, and rs3037354), located within 1000 base pairs upstream of the transcriptional start site have been studied, individually or as haplotypes, for their contribution to the risk of developing schizophrenia, major depression and alcoholism. A cardiovascular study identified and confirmed genome-wide significance for rs16147 as a risk allele for early onset arteriosclerosis (Shah et al., 2009), but the results of psychiatric case-control studies are inconclusive (Mottagui-Tabar et al., 2005;Itokawa et al., 2003;Heilig et al., 2004;Inoue et al., 2009;Zill et al., 2008;Hall et al., 2007;Duan et al., 2005;Lindberg et al., 2006). Using an alternative strategy, Zhou et al investigated the contribution of these variants to stress coping and emotionality by assessing intermediate phenotypes, including regional brain responses to emotional stimuli and pain. They found in independent populations of healthy subjects a convergent pattern of correlation between stress-related intermediate phenotypes and NPY gene expression estimates predicted by diplotypes mostly reporting on rs16147 (Zhou et al., 2008). They also found modest association with harm avoidance. A later study failed to replicate the association with anxious personality traits, but used a different measure, neuroticism, which makes comparison difficult (Cotton et al., 2009). In fact, however, despite this discrepancy, there was a trend toward significance in this study when diplotype-predicted NPY mRNA levels were used in the analysis. Thus, together with our present findings, these three studies in essence support the view that an analysis of continuous intermediate phenotypes, such as regional brain responses or mRNA levels, is more powerful for detecting the effects of genetic variation compared to more distal behavioral phenotypes, which may also pose more of a challenge to ascertain with high reliability (Meyer-Lindenberg and Weinberger, 2006).

Our results from the epidemiological sample are in agreement with and extend the findings of Zhou et al. (2008) by demonstrating that the impact of NPY genotype on negative emotional symptoms appears to be conditional on stress exposure. In individuals homozygous for the C allele of rs16147, exposure to early childhood adversity was significantly associated with higher scores of depression and anxiety, while no such relationship was found in individuals homozygous for the T-allele. Our findings would therefore seem to add to a growing body of literature that links genetic variation to individual differences in stress responses (Uher and McGuffin, 2008). However, the GxE findings obtained should be considered with caution regarding the sample size and the number of statistical tests performed. It should be emphasized that the GxE we observed would not hold up to stringent correction for multiple testing and thus should be regarded as preliminary. As our sample was not large enough to permit internal replication in split samples, our findings will require replication in independent cohorts. Effects of genetic variation were most robustly detected for the biological intermediate phenotype, mRNA expression, and a gene x environment effect on emotional outcomes was demonstrated for dimensional measures of psychopathology (e.g. BDI) but not for categorical measures (diagnoses). The lack of effect of rs16147-C on clinical diagnoses of depression and anxiety is likely related to the lower power to detect an association with a dichotomous phenotype, but also to the fact that, in this young adult group, psychiatric symptoms are still emerging. Given the strong effect of high adversity on psychopathology, it can be expected that the prevalence of depressive and anxiety disorders will increase with age in the sample, and that at later time points a main effect of rs16147 will become detectable.

Much effort is currently undertaken to identify common genetic variants and understand their function. These may be maintained in the population because they confer a fitness advantage under some conditions in the evolutionary history of our species, but may carry some of the genetic risk to common diseases (Lander, 1996) and contribute to the high prevalence of complex psychiatric disorders in modern society (Freudenberg et al., 2007). Our present findings support the view that rs16147 contributes to NPY mRNA levels in a critical brain region for emotional behaviors, and interacts with measures of adversity in a manner that may contribute to stress-related behavioral outcomes. We also demonstrate that a biologically informed intermediate phenotype strategy is a valid and powerful approach to assess the function of common variants in the human brain.

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Supporting Online Materials

Table S1: Definition of the psychosocial adversity items

	Item	*Definition*	**Total**
1	Low educational level of a parent	Parent without completed school education or without skilled job training	18.2
2	Overcrowding	More than 1.0 person per room or size of housing < 50 m^2	7.3
3	Parental psychiatric disorder	Moderate to severe axis I or II disorder according to DSM-III-R criteria (interviewer rating, kappa = .98)	20.4
4	History of parental broken home or delinquency	Institutional care of a parent / more than two changes of parental figures until the age of 18 or history of parental delinquency	18.5
5	Marital discord	Low quality of partnership in two out of three areas (harmony, communication, emotional warmth) (interviewer rating, kappa = 1.00)	11.1
6	Early parenthood	Age of a parent < 18 years at child birth or relationship between parents lasting less than 6 months at time of conception	24.5
7	One-parent family	At child birth	9.2
8	Unwanted pregnancy	An abortion was seriously considered	13.1
9	Poor social integration and support of parents	Lack of friends and lack of help in child care (interviewer rating, kappa = .71)	4.8
10	Severe chronic difficulties	Affecting a parent lasting more than one year, such as long-term unemployment, chronic disease, or troubled family relationships (interviewer rating, kappa = .93)	29.6
11	Poor coping skills of a parent	Inadequate coping with stressful life events of the past year, such as denial of obvious problems, withdrawal, or resignation (interviewer rating, kappa = .67)	37.3

Note: numbers represent percentages of individuals in each group exposed to the respective adversity condition

Table S2: Socio-demographic and clinical characteristics of the epidemiological sample grouped by NPY genotype

	TT (n=81)	TC (n=155)	CC (n=78)	*p*
Sex (male): N (%)	41 (50.6)	72 (46.5)	32 (41.0)	.477
Age	19.2 (0.3)	19.2 (0.4)	19.3 (0.3)	.126
IQ [a]	104.2 (15.0)	102.7 (14.0)	104.1 (15.1)	.686
Psychosocial adversity	1.74 (1.96)	1.86 (2.10)	2.21 (2.08)	.304

Data are given as means, standard deviation in parenthesis. [a] nonverbal IQ assessed at age 11 years

Table S3: Generalized regression model for NPY mRNA, control subjects only

	ACC mRNA
Gene dose	-.213 (.098)*
Sex	-.074 (.078)
Age	.004 (.005)
Ancestry	-.398 (.317)
PMI	.071 (.039)
RIN	-.082 (.048)

Regression estimates and their standard errors are shown for the predictors used in the model. Genotype for rs16147 was coded as TT=0, TC=1, CC=2. PMI = post mortem interval, RIN = RNA integrity number. *p < .05

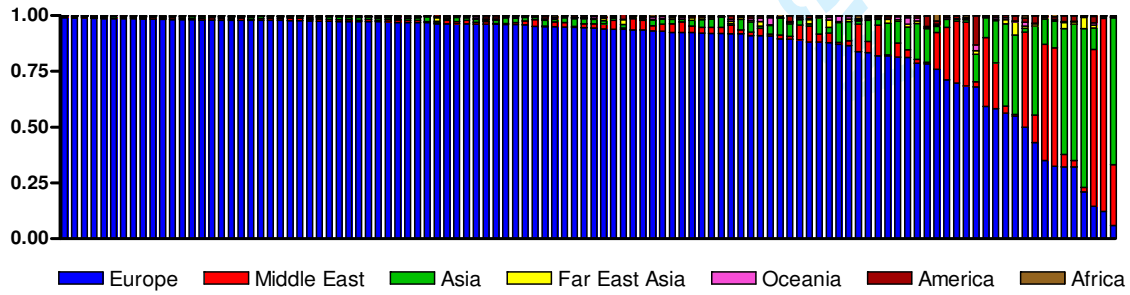

Figure S1. Distribution of ancestral informative markers (AIMs) within the population of postmortem samples. Ancestry was estimated using 186 AIMs with a seven factor solution and using the CEPH population samples to anchor the analysis as described in Enoch et al (2006). .