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Genetic Modulation of Plasma NPY Stress Response is Suppressed in Substance Abuse: Association with Clinical Outcomes

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Abstract

Background—Neuropeptide Y (NPY) is involved in stress regulation. Genetic variations predict plasma NPY and neural correlates of emotion and stress. We examined whether the functional *NPY* haplotype modulates stress-induced NPY and anxiety responses, and if plasma NPY stress responses are associated with substance dependence outcomes.

Methods—Thirty-seven treatment-engaged, abstinent substance dependent patients (SD) and 28 controls (HC) characterized on *NPY* diplotypes (HH: high expression; HLLL: intermediate/low expression) were exposed to stress, alcohol/drug cues and neutral relaxing cues, using individualized guided imagery, in a 3-session laboratory experiment. Plasma NPY, heart rate and anxiety were assessed. Patients were *prospectively* followed for 90-days post-treatment to assess relapse outcomes.

Results—HH individuals showed significantly lower stress-induced NPY with greater heart rate and anxiety ratings, while the HLLL group showed the reverse pattern of NPY, anxiety and heart rate responses. This differential genetic modulation of NPY stress response was suppressed in the SD group, who showed no stress-related increases in NPY and higher heart rate and greater anxiety, regardless of diplotype. Lower NPY predicted subsequent higher number of days and greater amounts of post-treatment drug use.

Conclusion—These preliminary findings are the first to document chronic drug abuse influences on *NPY* diplotype expression where *NPY* diplotype modulation of stress-related plasma NPY, heart rate and anxiety responses was absent in the substance abuse sample. The finding that lower

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stress-related NPY is predictive of greater relapse severity provides support for therapeutic development of neuropeptide Y targets in the treatment of substance use disorders.

Keywords

Neuropepetide Y; Functional Haplotype; Stress; Substance Use Disorders

Introduction

Converging evidence from animal and human studies suggests that neuropeptide Y (NPY) regulates stress response and has anxiolytic-like action (Broqua *et al.* 1995; Heilig *et al.* 1989). Animal studies show central effects of NPY in the amygdala and cortical regions that are important in stress regulation (Heilig 2004; Karl *et al.* 2008a; Sajdyk *et al.* 2008; Thorsell *et al.* 1999; Thorsell *et al.* 2000a). Human studies indicate basal NPY levels from cerebrospinal fluid (CSF) and plasma NPY are significantly decreased in patients with major depression (Heilig *et al.* 2004), patients with suicide attempts (Westrin *et al.* 1999) and survivors of military combat with post-traumatic stress disorder (Rasmusson *et al.* 2000). These results suggest that plasma NPY may contribute to the pathophysiology of major depression and stress related disorders (Heilig 2004). However, no previous research has examined the effects of *NPY* genetic variation on peripheral NPY levels during acute stress manipulation in healthy control (HC) and substance dependent (SD) patients, and examined its effects on subsequent alcohol and drug use behaviors.

Stress mechanisms increase addiction vulnerability and also significantly increase addiction relapse risk. Chronic alcohol and drug abuse are associated with altered functioning of stress and reward pathways (Koob 2008; Sinha 2008). Recovering alcohol and cocaine dependent individuals present with altered autonomic responses, poor sympathetic and parasympathetic balance, high levels of anxiety and greater alcohol/drug craving and compulsive drug seeking as compared to healthy individuals (Fox *et al.* 2008; Koob *et al.* 2004; Sinha 2009; Sinha et al., 2009). Furthermore, high levels of anxiety and drug craving and altered hypothalamic-pituitary- adrenal (HPA) responses to stress contribute to increased relapse risk in addiction (Majewska 2002; Sinha 2007; Walter *et al.* 2006). There is growing data from animal studies that NPY levels are highly sensitive to acute and chronic ethanol administration and NPY administration suppresses ethanol drinking in ethanol abstinent rats (Gilpin *et al.* 2008). Infusion of NPY attenuates ethanol withdrawal symptoms in rats (Woldbye *et al.* 2002) and NPY gene expression is decreased during alcohol withdrawal in rats (Olling *et al.* 2007). In human study, plasma NPY level was negatively correlated with heroin craving and anxiety in the first post-treatment heroin dependent population (Shi et al, 2009). Interestingly, a recent study reported that NPY injections increased cocaine seeking behavior and heroin self-administration behavior (Maric *et al.* 2008a; Maric *et al.* 2008b). Together, these findings suggest that high levels of anxiety and autonomic dysregulation in substance abusing individuals could be associated with dysfunctional NPY responses, which may, in turn, contribute to addiction relapse and poor substance abuse outcomes.

Growing evidence indicates that genetic and environmental interaction contributes to individual differences in stress responses. Genetic variation confers significant risk for stress-related disorders such as addiction (Goldman 1995; Goldman *et al.* 2005; Kreek *et al.* 2005). A functional *NPY* polymorphism (*Leu7Pro*) has been directly studied for association with prevalence of substance dependence with inconsistent findings (Lappalainen *et al.* 2002; Mottagui-Tabar *et al.* 2005; Zhu *et al.* 2003; Zill *et al.* 2008). However, we recently reported that the functional *NPY* haplotype (combination of genetic variations) predicted both *NPY* mRNA expression in human postmortem brain tissue and lymphoblasts and plasma NPY levels, and it was inversely associated with high trait anxiety and amygdala

activity in response to threatening stimuli or a pain/stress challenge (Zhou *et al.* 2008). Variation in this functional NPY haplotype has also been recently associated with brain response to stress and increased risk of major depression (Mickey et al., 2011). In healthy controls, individuals with low-expression NPY haplotype reported more negative effects under a painful stress condition. Furthermore, low-expression NPY haplotype presented higher frequency in major depression subjected than healthy controls (Mickey et al, 2011). These data suggest that genetic variation in this functional NPY haplotype modulates baseline peripheral NPY and neural responses to stressful and aversive situations. Some individuals with low-expression NPY haplotype are more vulnerable to stress and with greater risk of stress related pathological conditions. As discussed above, stress is associated with increased vulnerability to addiction (Sinha, 2008) and increased risk of relapse. Central injection NPY reduces alcohol intake in animals and plasma NPY level is associated with higher anxiety and craving in post-treatment heroin addiction in human (Shi et al, 2009). Thus, we hypothesize that NPY plays a role in modulating dysfunctional stress responses and autonomic arousal associated with addictive disorders. We further hypothesized that dysfunctional NPY response in addicted individuals would be predictive of greater relapse severity and poor clinical outcomes.

Therefore, using the well-validated guided imagery method to provoke emotional stress and drug craving in controls and patient samples (see Sinha, 2009 for review), we examined whether plasma NPY and anxiety responses are modulated by the functional *NPY* haplotype in an experiment involving exposure to stress, alcohol/drug cue and neutral imagery on consecutive days in recently abstinent, inpatient treatment engaged substance dependent (SD) individuals and in healthy controls (HC). The SD patients were also *prospectively* followed to assess post-treatment drug use behaviors after discharge from inpatient treatment. We hypothesized that stress-induced plasma NPY and anxiety responses will be influenced by the *NPY* diplotype in controls (HC), but that this normal variation in stress-related NPY will be altered in the addicted sample, and the level of alteration will affect subsequent substance use and relapse severity.

Method

Participants

Treatment-seeking individuals between the ages of 21–50 (n = 37, male/female 24/13) meeting DSM-IV criteria for current substance dependence (SD) as assessed by the Structured Clinical Interview for DSM-IV (SCID-I, First et al., 1995) conducted by trained masters level research associate staff were included in the study. These addicted patients were admitted to the Clinical Neuroscience Research Unit (CNRU) of the Connecticut Mental Health Center (CMHC) at Yale University for 5 weeks of inpatient treatment and research participation. Urine and breathalyzer testing were conducted regularly to ensure continued abstinence. All patients participated in specialized substance abuse treatment for 4 weeks prior to the laboratory sessions. Healthy individuals in the 21–50 age range (n = 28, male/female 15/13) who were light social drinkers (up to 6 drink weekly) and did not meet current or lifetime DSM-IV criteria for any substance use disorders (as per the SCID-I) were recruited from the community via local advertisements. In addition, controls and addicted individuals on medications for current medical or psychiatric problems, including those receiving medications for current mood and anxiety disorders, were excluded from the study. However, because of high comorbidity of lifetime mood disorders with addiction and increased current mood and anxiety symptoms in substance dependence during early abstinence, patients who met lifetime DSM-IV criteria of anxiety and mood disorders were not excluded from the study. Substance abusing individuals with current or past opioid abuse were also excluded. The study was approved by the Human Investigation Committee of the Yale University School of Medicine.

During the first two weeks of the inpatient stay, SD patients underwent an initial medical evaluation and completed demographic, psychiatric, drug use and psychosocial assessments. Subjects then completed preparation for individualized guided imagery (see description below) in week 3. To control for any residual alcohol or drug withdrawal/abstinence effects, the laboratory experiment was only conducted in week 5 of their inpatient stay (as described in previous reports (Fox *et al.* 2008; Sinha 2007; 2009). Healthy control subjects completed demographic, diagnostic, and alcohol/drug-related assessments in two to three assessment appointments and were then admitted for a 3-day hospital stay to the Yale General Clinical Research Center (GCRC) at Yale-New Haven Hospital for participation in the experimental study procedures as the SD patients.

Procedures and measurements

Imagery Script Development Procedures—Prior to the laboratory sessions, individualized guided imagery scripts for stressful situations, alcohol/drug-related stimuli and neutral relaxing states were developed using well-established and previously validated procedures (Fox *et al.* 2007; Sinha 2009; Sinha *et al.* 1992; Sinha *et al.* 2008; Sinha and O'Malley 1999; Sinha and Parsons 1996; Sinha *et al.* 2003). Stress imagery scripts were developed from subjects' descriptions of recent personal stressful events that were experienced as "most stressful", which was determined by having the subjects rate their distress level for each stressful situation on a 10-point Likert scale where "10=the most stress they felt recently in their life". Only situations rated as 8 or above were accepted as appropriate for script development such as divorce and loss of family members. Alcohol/drug cue scripts were developed based on situations that included alcohol or drug-related stimuli and resulted in subsequent alcohol/drug use (e.g. buying alcohol or drugs, being at a bar, watching others drink alcohol or use drugs). Alcohol/drug-related situations that occurred in the context of negative affect or psychological distress were not allowed. Neutral scripts were developed from the participants' individual experiences of commonly experienced neutral-relaxing situations, such as a summer day relaxing at the beach or a fall day reading at the park. Details of each elicited situation were described using the Scene Construction Questionnaires, and a script was developed for each of the three types of situations described, based on methods developed by Lang and colleagues (Lang *et al.* 1980) and further adapted in our previous work with healthy and addicted samples (Fox *et al.* 2007; Fox *et al.* 2008; Sinha 2007; 2009; Sinha *et al.* 1992; Sinha *et al.* 2008; Sinha and O'Malley 1999; Sinha and Parsons 1996; Sinha *et al.* 2003). The stress, drug cue and neutral relaxing script was audiotaped for presentation in the laboratory sessions. The stress, drug cue and neutral relaxing imagery conditions were randomized and counterbalanced across subjects to account for any possible order effects. Only one script was presented per day and in each laboratory session and research staff conducting the experiment were blind to the imagery condition on each day.

Habituation and Imagery Training Session—On a day prior to the laboratory sessions, subjects were brought into the testing room where they were acclimatized to specific aspects of the study procedures, such as intravenous (IV) insertion, and training in completing the subjective rating forms and specific training in relaxation and imagery procedures (see Sinha 2009 for review).

Laboratory Sessions—On each day of the experiment, all subjects were allowed an initial smoke break at 7:30 AM to prevent potential nicotine abstinence symptoms from affecting the experiment. A heparin-treated catheter was inserted by the research nurse in order to periodically obtain repeated blood samples during the laboratory sessions. A pulse sensor was also attached to the subject's index figure. At 9:00 AM, subjects were provided headphones and the audiotape presented the instructions for the imagery procedure and the

script for guided imagery. After imagery, subjects remained in the testing room for an additional 75 minutes for repeated measurements to examine recovery from the imagery exposure. After the last assessment, the subject was disconnected from the apparatus.

Plasma NPY measurement—To assess plasma NPY levels, 4 ml of whole blood were collected at two baseline timepoints (−20 and −5), immediately following imagery (0 timepoint) and at +15, +30, +45, +60 and +75 minutes after imagery for all three experimental sessions. Within 30 minutes of collection, the blood was centrifuged at 4°C and 2 ml of plasma were pooled, aliquoted and stored at −80°C until shipment to the Hauger laboratory at the University of California, San Diego for analysis.

Plasma NPY were measured using our previously well-characterized double-antibody radioimmunoassay using ^{125}I-NPY as the tracer (Allen *et al.* 1991) (Rasmusson *et al.* 1998). Plasma samples are prepared by completing an acid ethanol extraction (recovery ~70%). After lyophilized extracts are reconstituted in assay buffer, NPY is detected using a highly sensitive and specific NPY antibody (Allen *et al.* 1991). The NPY assay working range is 19.5 to 1250 pg/ml, and the assay sensitivity is ~15 pg/ml. The assay intra- and inter-assay coefficients of variation are ~4% and ~9%, respectively. To more carefully account for variability in the plasma NPY levels across days and timepoints, the NPY data were normalized to standardized z scores and these were used in the final analysis.

Heart rate—A pulse sensor was attached to the subject's finger and connected to the Dinamap Monitor to provide a continuous measure of pulse. Heart rate was averaged for the 5 minutes prior to imagery (as a baseline measurement), during the 5-minute imagery period (imagery time-point) and then at each of the +15, +30, +45, +60 and +75 minutes after imagery.

Subjective Anxiety—The anxiety subscale from the Differential Emotions Scale-Revised short form (DES-R; Izard, 1972) was used to assess subjective anxiety at each time point before and after exposure to stress, drug/alcohol cues and neutral imagery. The anxiety subscale is made up of 5 adjectives describing distress, anxiety and arousal (tense, anxious, jittery, aroused, nervous). Participants rate on a 5-point scale the extent to which each word describes the way they feel at the present moment. Assessments were made at the same time point as described above.

Prospective Follow-up of Substance Abuse Post-inpatient Treatment

All SD patients participated in three face-to-face follow-up interviews at day 14, 30 and 90 days following discharge from the inpatient treatment research, where they provided urine and breath alcohol samples and a detailed daily assessment of alcohol and cocaine use using the Time-line Follow-back method on the Substance Use Calendar (SUC). This is a well-established and reliable instrument for assessing self-reported alcohol and drug use outcome measures in treatment studies (Scheurich *et al.* 2005). As in our previous studies (Paliwal *et al.* 2008; Sinha *et al.* 2006), the urine and the SUC data were matched for corroboration, and number of days of alcohol and drug use (*frequency of days of using alcohol or cocaine or both*) and the average weekly and total number of alcohol drinks and total number of grams of cocaine (0.1 gram, dime bags) consumed (*quantity sum*) in the 90-day follow-up period was calculated as measures of alcohol and drug use.

NPY SNP genotyping and diplotype identification

All participants were genotyped as per procedures described previously (Zhou *et al.* 2008). Briefly, a total of six SNPs (rs3037354; rs17149106; rs16147; rs16139; rs5573; rs5574) from the *NPY* gene were genotyped in this study population by using 5′-nuclease assay.

Our previous study found that one haplotype block defined by six SNPs showed strong pairwise linkage disequilibrium in a Finnish population (Zhou *et al.* 2008). The haplotype configurations are: H1 Ins/G/C/T/A/T; H2: Del/G/T/T/G/C; H3: Ins/G/T/T/G/C; H4: Ins/G/C/T/A/C; H5: Ins/T/T/C/G/C. Three common haplotypes (H1, H2, H3) were previously found to predict NPY mRNA expression in lymphoblasts among 47 healthy Finnish individuals, which represented six common diplotypes: H1/H1, H1/H2, H1/H3, H2/H2, H2/H3, H3/H3 (Zhou *et al.* 2008). Individual diplotype was determined based on haplotype configuration on each chromosome. On the basis of lymphoblast NPY mRNA levels, the expression value for each haplotype was calculated by regression analysis. Diplotypes were clustered into three NPY expression groups: predicted high NPY expression (HH) including diplotype H2/H3 and H2/H2, predicted intermediate expression (HL) including diplotype H1/H3, H3/H3 and H1/H2 and predicted low expression (LL) including diplotype H1/H1.

Diplotype distributions for the 65 subjects were HH: 20; HL: 35, and LL: 10. As in previous studies (Hariri et al., 2002; Pezwas et al., 2005; Colzato et al., 2010), low frequency of expression (LL) in one group (HC: LL=2; SD: LL=8) were combined with the intermediate (HL) expression group for statistical analyses (HLLL Group: SD=25; HC=20; HH Group: SD=12; HC=8).

Statistical Analysis

Chi-square analysis and t-tests were conducted to assess both demographic and individual characteristics and diplotype frequency differences in the SD and HC groups. Significant differences between groups and/or diplotype on any of these variables led to the inclusion of the measure as a covariate in all final analyses.

Linear Mixed Effect (LME; Laird & Ware, 1982) models were implemented to analyze the data, using the SAS software package (Version 9, 2006; SAS Institute, Cary, NC). Between-subjects factors of Group 2 (SD vs. HC), predictive NPY Expression group (HH vs. HLLL) and within-subjects factors of Condition 3 (neutral, stress, drug/alcohol cue) and Time-point (varying levels) were the Fixed effects while Subject was the Random effect in the models predicting plasma NPY (z-NPY), heart rate and anxiety responses. In the case of baseline differences for any particular measure, the LME models included the baseline values as a covariate. Otherwise, baseline timepoints were included in the LME model to ensure baseline variation was accounted for in the zNPY, heart rate and subjective anxiety responses to stress, drug/alcohol cue and neutral imagery. To illustrate the findings, baseline adjusted means across the timepoints in each condition are presented in figures. Finally, the association between z-NPY and number of days of alcohol/drug use and amounts of drug used post-discharge was examined using multiple regression analyses.

Results

Demographic and Individual Characteristics of Participants

There were no significant differences on race, gender and prevalence of lifetime mood and anxiety disorders across the SD and HC groups and by *NPY* genotype. However, the SD groups were older, somewhat less educated, and included more cigarette smokers than the HC groups (p's <0.05). *All analyses presented below included age, years of education and cigarette smoking as covariates*.

Baseline Differences—No baseline differences between conditions were seen for plasma NPY, heart rate and subjective anxiety in the control and the substance abusing group. This indicates that the randomizing and counterbalancing of the condition order across the 3 days of testing was successful.

Plasma NPY

There were no significant differences between groups on baseline NPY levels. In experimental stress, cue and neutral provocation, a significant 2-way (Diplotype X Condition) ($F[2,107]=12.28$, $p<0.0001$; *effect size* ***f=.48***) and a significant 3-way (Diplotype X Group X Condition) interaction ($F[2,107]=4.03$, $p<0.02$; *effect size* ***f=.28***) was found. The Diplotype X Condition interaction resulted from the HH group showing significant reduction in plasma NPY in stress versus the neutral condition ($p=0.0023$), while the HLLL group showed significant increase in NPY during stress versus the neutral condition ($p<0.0001$). There were no significant differences in plasma NPY in cue versus neutral condition in either HH or HLLL diplotype groups.

However, the Diplotype X Group X Condition interactions revealed that the differential effect of NPY in stress condition by diplotype was only significant in the controls (HC) and not present in the SD group (Figure 1a, 1b). Thus, in the HC group, the HH individuals showed significantly lower plasma NPY during stress compared to neutral condition ($p=0.035$), while the HLLL individuals showed significantly higher NPY in comparison of stress and neutral ($p<0.0001$) conditions (Figure 1a, 1b). The HLLL group also showed greater NPY in stress versus the cue ($p<0.0009$), as well as cue compared to neutral condition ($p < 0.01$). The SD individuals presented NPY reduction in HH group (0.02) and no NPY up-regulation in HLLL groups with stress provocation compared to the neutral condition (Figure 1a, shown with averaged NPY level across all timepoints in each condition). A summary of plasma NPY changes in stress condition across all time points is presented in the supplement figure 1. As a follow-up to these analyses, stress relative to neutral change in NPY was computed for the HH and HLLL diplotypes in the HC and the SD groups and as shown in figure 1b, the HLLL diplotype showed significant higher level of plasma NPY in stress than the HH diplotype individuals in the HC group but this difference was not present in the SD group (Figure 1b). All addicted subjects regardless of *NPY* diplotype showed no significant changes of plasma NPY in alcohol/drug cue compared with neutral condition.

Heart Rate

As expected based on previous studies ((Fox *et al.* 2007; Ingjaldsson *et al.* 2003; Sinha 2009), there was an overall main effect of Group ($F[1,59]=7.14$, $p<0.001$) and Condition ($F[2,122]=5.38$, $p<0.006$), and Group X Condition interaction ($F[2,122]=15.08$, $p<0.0001$). These data indicated greater basal heart rate in the SD vs the HC group ($p=0.0035$), higher heart rate responses in the stress relative to the neutral condition, but only in the HC group ($p=0.0047$) and not in the SD group ($p=0.3927$).

More importantly, there was a significant 2-way (Diplotype X Condition) ($F[2,122]=6.63$, $p<0.002$; *effect size* ***f=.33***) and a significant 3-way (Diplotype X Group X Condition) interaction ($F[2,122]=5.55$, $p<0.005$; *effect size* ***f=.30***) (see Fig 2a, 2b showing averaged timepoints in each condition). The findings indicated that the HH individuals showed increased heart rate in response to stress relative to neutral ($p=0.0204$) and relative to the cue condition ($p<0.0001$), but no such increases were observed in the HLLL group ($p=0.56$ stress vs neutral; $p= 0.77$ stress vs cue), and higher overall heart rate was seen in the SD versus HC group ($p<0.001$), irrespective of diplotype group and condition (also see supplemental figure 2).

Subjective Anxiety

As expected, an overall main effect of Condition ($F[2,122]=16.08$, $p<0.0001$), and Group X Condition interaction ($F[2,122]=11.07$, $p<0.0001$) was observed, indicating greater overall anxiety in the stress ($p <0.0001$) and cue ($p < 0.0001$) versus neutral and in the stress versus

cue (p<0.001) conditions. The Group X Condition interaction resulted from significant higher anxiety during stress versus neutral in both the SD and HC group (p's <0.0001), but significantly increased anxiety during alcohol/drug cue exposure only in the SD group (p<0.0001) but not in the HC group (p=0.58) (see Figure 3a, shown averaged across timepoints in each condition). More interestingly, a significant 2-way Diplotype X Condition interaction (F[2,122]=5.25, p=0.0065; *effect size* ***f*=.30**) was also observed, resulting from the HH diplotype group showing greater anxiety ratings relative to the HLLL diplotype group during stress (p=0.03), but no differences in anxiety ratings by diplotype in the neutral and the cue conditions (Fig 3b, also see supplemental figure 3).

Association between Plasma NPY and Post-treatment Alcohol/Drug Use

Plasma NPY during stress, alcohol/drug cue and neutral conditions were not associated with baseline levels of alcohol and cocaine use. However, lower plasma NPY during both the stress and the alcohol/drug cue condition was negatively associated with higher number of days of alcohol and/or drug consumed post-treatment (Stress: R^2=0.18, $\underline{r}$ = −0.42, p<0.02; Cue: R^2=0.11, $\underline{r}$ = −0.33, p<0.08) and higher total amounts of alcohol and drug consumed during the 90-day follow-up period (Stress: R^2=0.40, $\underline{r}$ = −0.63, p<0.0003; Cue: R^2=0.26, $\underline{r}$ = −0.51, p<0.006).

Discussion

The current findings demonstrate that genetic variations in *NPY* haplotype which predicts NPY mRNA expression modulated stress-induced plasma NPY levels, heart rate arousal and subjective anxiety. While on the basis of our previous work (Sinha *et al.*, 2003; Fox *et al.*, 2008; Chaplin *et al.*, 2008; Sinha *et al.*, 2009), emotional stress exposure significantly increased heart rate and anxiety levels relative to the alcohol cue and the neutral imagery condition, current data indicate genetic variation in *NPY* haplotype predicted individual differences in these responses. Differential plasma NPY and anxiety responses to stress provocation were observed in healthy controls with the HH vs HLLL *NPY* variants, but not in the recovering addicted patients. Stress-induced NPY release was low, while anxiety and heart rate responses were increased in the addicted individuals, particularly in those with the HLLL genotype compared to HLLL controls. This suppressed NPY response to stress was also predictive of greater alcohol/drug use in the subsequent post-treatment period.

The current results are consistent with previous animal studies revealing that pro-NPY mRNA and NPY peptide levels typically increase in the amygdala of laboratory rats in response to restraint stress (Krysiak *et al.* 1999; Thorsell *et al.* 1999). In rhesus macaques, a genetic variant on the promoter region of *NPY* resulted in lower mRNA expression in the amygdala and lower cerebrospinal fluid NPY level. These rhesus macaques exhibited higher level of arousal during stress exposure (Lindell et al., 2010). Furthermore, while mice with a deletion of the *NPY* gene exhibit higher anxiety and increased alcohol consumption (Karl et al., 2008) (Thiele *et al.* 1998), transgenic rats with NPY over expression have a remarkably decreased reaction to anxiogenic-like stimuli and fail to develop fear-induced behavioral suppression (Thorsell et al., 2000). Relevant to the present study, NPY-overexpressing transgenic mice display hypersensitivity to alcohol intoxication and reduced alcohol intake (Thiele *et al.* 1998). Although the above basic science findings indicate central NPY effects and there is no evidence of similar central effects in humans, the current findings extend this previous preclinical literature, and are the first in humans to show functional genetic variations in *NPY haplotype* (i.e., HH vs HLLL) influences individual differences in peripheral NPY stress response and behavioral adaptation to stress in healthy subjects. On the other hand, suppressed NPY levels during stress in recovering addicted patients raise the possibility that epigenetic mechanisms regulating *NPY* gene expression could play a role in

stress dysregulation of plasma NPY and anxiety responses in addicted individuals, which in turn significantly impacts subsequent alcohol/drug abuse.

Significantly lower stress-induced NPY release with greater heart rate responses and higher anxiety were observed in individuals with the diplotype predicting higher mRNA expression (HH), while those with the NPY diplotype predicting low-moderate mRNA expression (HLLL) showed up-regulated plasma NPY responses to stress, with lower heart rate and subjective anxiety responses during stress relative to the neutral condition. The genetic influence on the inverse response in plasma NPY on the one hand, and heart rate and anxiety on the other, indicates that the regulatory role of peripheral NPY stress responses is modulated by variation in the *NPY* haplotype. The results suggest that the anti-anxiety effect of plasma NPY could function in an inverted "U" manner, where individuals with a HH diplotype, known to have higher basal plasma NPY levels (Zhou *et al.* 2008), show lower peripheral NPY reactivity to stress, while individuals with HLLL diplotype, known to have lower basal plasma NPY (Zhou *et al.*, 2008), show increased NPY reactivity to stress stimuli. In this way, normal genetic variation in *NPY* mRNA expression could modulate plasma NPY regulation during stress and contribute to individual differences in stress adaptation in healthy controls.

The same functional NPY haplotype was previously linked to higher amygdala and hippocampus activation in fMRI responses to negative emotional faces in individuals with lower NPY expression diplotype (LL) than in those with HH diplotype. Lower NPY expression diplotype also showed higher anxiety traits as measured by TPQ. Although these findings may appear to be inconsistent with the current data, there are key differences between the previous and current study in methodology and measures representing anxiety or distress. For example, the Zhou et al (2008) study included data from post-mortem brains, a large epidemiological sample, smaller samples for basal plasma NPY, and assessed brain responses in limbic regions associated with emotions and stress, while the current study examined peripheral NPY and self reported anxiety and heart rate responses. A one-to-one correspondence in central and peripheral NPY action cannot be assumed. Furthermore, our findings indicate that peripheral NPY, which is co-released with norepinephrine from the sympathetic nerve terminals during stress and arousal (Gehlert 2004), plays a role in modulating subjective anxiety and heart rate. One possible mechanism for this pattern of responses is the sympathetic arousal during stress along with activation of NPY receptor signaling in the arcuate nucleus, which projects to the hypothalamus and influences neurotransmission in lower and mid-brain limbic regions (Chronwall *et al.* 1985) involved in emotional arousal and regulation, thereby exerting central anxiolytic effects via autonomic pathways. Despite the differences in methods and measures, together, the Zhou et al paper and the current study indicate that genetic variants in the functional *NPY* haplotype modulate stress responses via regulation of both central and peripheral NPY pathways.

A significant finding of clinical relevance is that the addicted patients showed suppressed NPY in the stress and alcohol/drug cue conditions, regardless of *NPY* diplotype. This was accompanied by enhanced stress- and cue-induced anxiety responses, with elevated basal heart rate and blunted stress and cue related heart rate reactivity in the addicted patients as compared to controls. Chronic alcohol-related autonomic dysregulation has been previously documented (Bar *et al.* 2006; Fox *et al.* 2008; Paliwal *et al.* 2008; Pandey *et al.* 2008; Sinha 2009), and these changes co-occur with increased anxiety and drug craving in recently abstinent, treatment engaged alcohol and cocaine dependent individuals (Fox *et al.* 2008; Sinha et al., 2009). Suppression of *NPY* diplotype expression in the addicted patients raises the possibility that stress and/or alcohol related epigenetic mechanisms may be involved in NPY stress dysregulation. Substance abusing patients report higher cumulative stress and adversity, show reciprocal effects of stress and chronic alcohol/drug use (Sinha, 2008), and

stress and drug-related epigenetic changes have been documented in a number of addiction relevant signaling pathways (Renthal & Nestler, 2008). Some evidence also suggests chronic alcohol-related epigenetic changes involving DNA methylation and histone modification-induced chromatin remodeling in several signaling systems, including significant effects in decreasing central *NPY* expression, which in turn, is associated with increased alcohol intake and higher anxiety like behaviors in alcohol dependent animals (Pandey *et al.* 2004; Pandey *et al.* 2008). Thus, it is possible that stress and/or alcohol/drug-related decreases in NPY gene expression contributes to the autonomic dysregulation and suppressed *NPY* diplotype effects, resulting in lower plasma NPY, which in turn predicts higher levels of subsequent alcohol and drug intake as observed in the current study. These data suggest that increasing NPY signaling and NPY levels could decrease substance use in addicted samples, thereby lending support to preclinical research indicating that therapeutic strategies that selectively target Y1, Y2 and/or Y5 receptors and activate NPY signaling may be effective in the addiction treatment (Pandey *et al.* 2008; Prakash *et al.* 2008).

As discussed earlier, association studies of the functional *NPY Leu7Pro* (rs16139) variants with alcohol/cocaine dependence in epidemiological samples have been inconsistent, with positive association in some studies (Kauhanen *et al.*, 2000; Lappalainen *et al.*, 2002; Mottagui-Tabar *et al.*, 2005) but not in the others (Zhu *et al.*, 2003; Hu *et al.* 2005; Mottagui-Tabar *et al.*, 2005; Zill *et al.*, 2008). Limited sample size, small gene effect size, and population stratification may contribute to these inconsistent findings in epidemiologically based case-control populations. On the other hand, the current study used a well-defined stress provocation procedure to induce stress responses and assess it as an intermediate endophenotype to examine the effects of the *NPY* functional haplotype (different from the one previously studied), and showed significant effects of *NPY* haplotype and environmental influences of alcohol and drug use history on plasma NPY responses and their association with future clinical outcomes. These data suggest that using stress-related endophenotypes may provide greater sensitivity to assess functional *NPY* gene expression changes in substance abuse.

This study was limited by the small sample size of control and addicted patients in each diplotype group, and thus the reported findings should be considered preliminary. However, it is important to note that the *effect sizes* for the significant Diplotype X Condition and Diplotype X Group X condition interaction effects for plasma NPY, heart rate and anxiety were in the medium (<.25) to large (<.40) range (Cohen, 1988), indicating that there was adequate power to detect differences in the two diplotypes across the conditions and the HC and SD groups, as hypothesized. Despite this drawback, this is the first study in humans to document functional *NPY* haplotype influences on stress-related plasma NPY, heart rate and subjective responses in controls, and show suppressed genetic influences on plasma NPY stress responses in recently abstinent, recovering addicted patients. Furthermore, lower plasma NPY stress response was also associated with subsequent poor clinical outcomes, providing preliminary support for developing NPY targets in the treatment of substance use disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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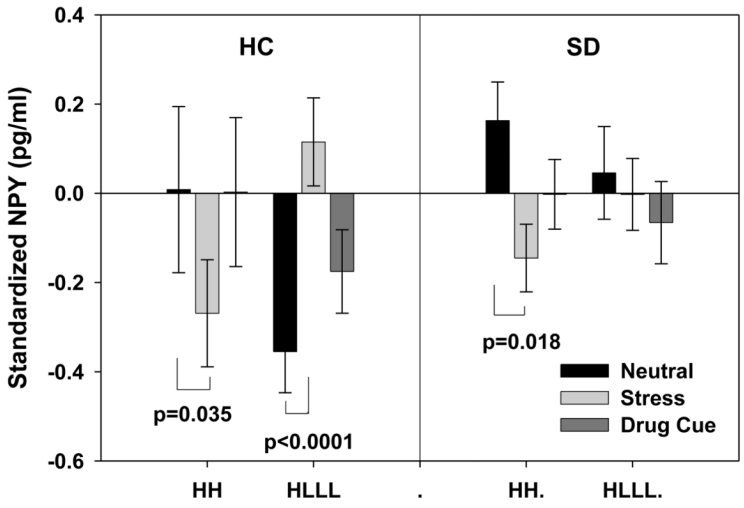
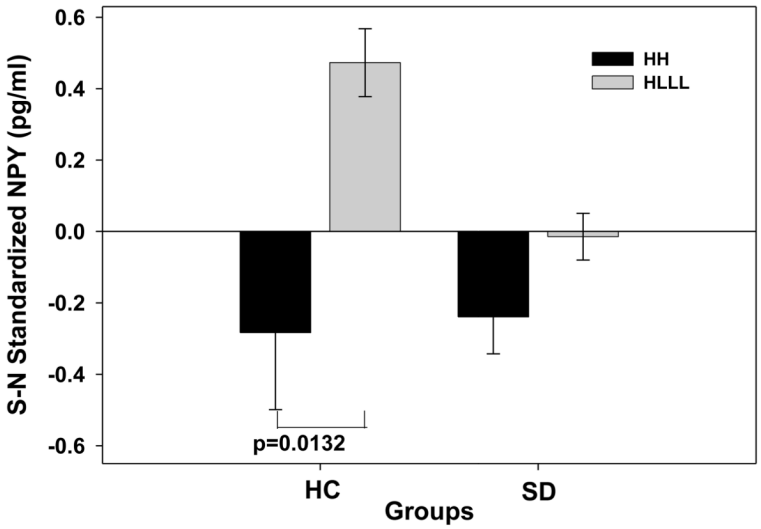


1a.

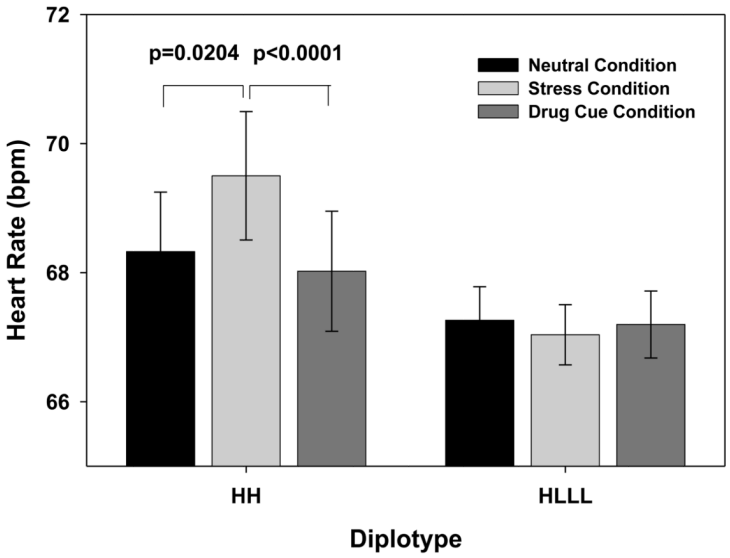


1b

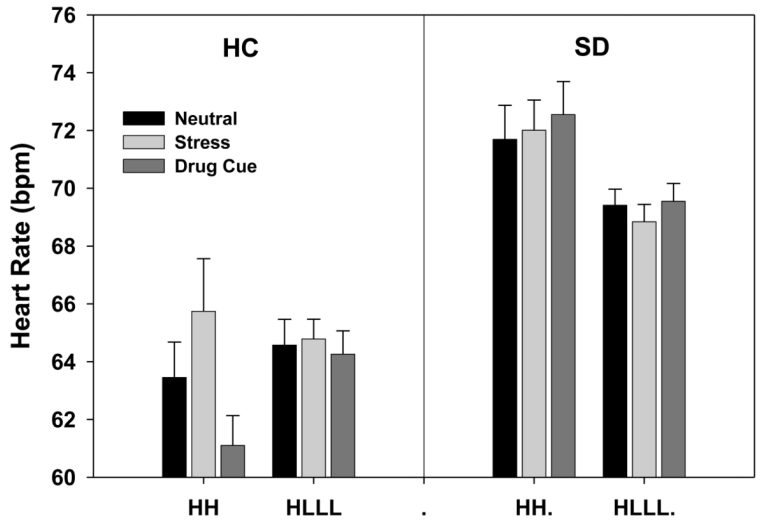
Figure 1.

(1a) Baseline adjusted mean and SE for plasma NPY for the SD and HC groups with the HH and HLLL diplotypes (averaged across time points in each condition). The significant effect of Diplotype x Condition x Group (p=0.0206) is shown. In HC group, HH individuals showed reduced NPY (p=0.035) while HLLL individuals showed increased plasma NPY (p<0.0001) in the stress relative to neutral condition. In the SD group, HH individuals showed significantly lower NPY (p=0.0176), while HLLL individuals showed no differences in plasma NPY (p=0.703) during stress relative to neutral condition. No plasma NPY difference between diplotypes for either group was observed in the cue versus neutral conditions.

(1b) Baseline adjusted plasma NPY changes in stress (S) relative to the neutral (N) condition expressed as a subtraction in response values for neutral from the stress condition (S-N NPY) in SD and HC groups for the HH and HLLL diplotypes is shown. Relative NPY increases in the HLLL subjects but decreases in the HH subjects in the HC group ($p<0.0132$) is shown, but no such effect of diplotype on NPY stress response is seen in the SD group.



2a

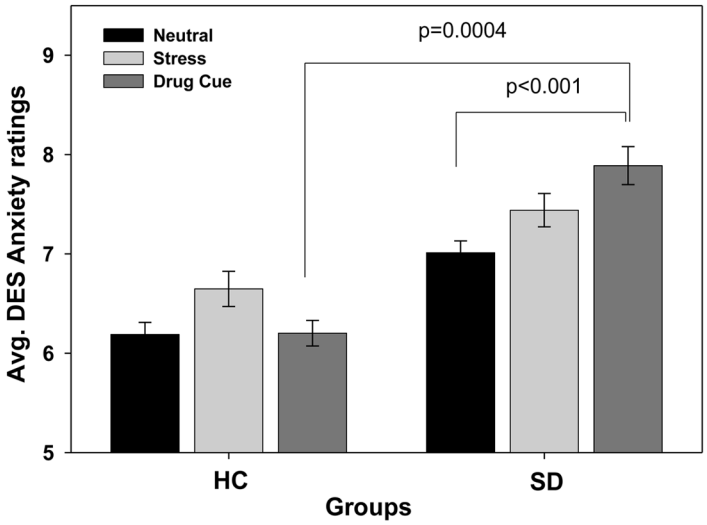


2b

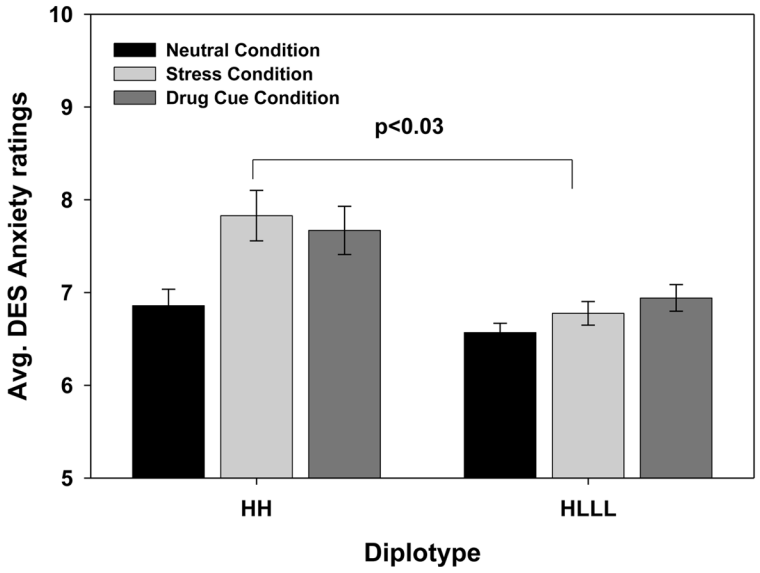
Figure 2.

Baseline adjusted mean heart rate response (and SE) during stress, drug cue and neutral conditions for the HH and HLLL diplotypes and the HC and SD groups are presented (averaged across time points in each condition). **(2a)** A significant diplotype X condition ($p<0.002$) effect is shown with increased heart rate during stress relative to neutral ($p=0.0204$) and relative to the drug cue condition ($p<0.0001$) in the HH group, but no stress-related increases in heart rate were seen in the HLLL individuals.

(2b) Higher overall heart rate was observed in the SD versus the HC group ($p<.0.001$). A diplotype X group X condition interaction ($p<0.005$) was seen due to HH and not the HLLL individuals showing stress related increases in heart rate, as shown in 4a, but this difference by diplotype was only present in the HC and not in the SD group, who showed no condition related increases in heart rate.



3a.



3b:

Figure 3.

Baseline adjusted mean and SE for subjective anxiety ratings during the stress, drug cue and neutral conditions (averaged across time points in each condition). **(3a)** Significantly higher anxiety ratings were observed in the stress ($p<0.0001$) and drug cue ($p<0.0001$) relative to the neutral and in the stress relative to cue ($p<0.001$) conditions. A significant Group X Condition effect ($p<0.0001$) was observed with both HC and SD groups showing higher stress-related anxiety relative to neutral condition ($p's<0.001$), but significantly higher anxiety during drug cue exposure in the SD and not in the HC group ($p<0.0004$). In cue relative to neutral, anxiety level was significantly increased in SD group ($p<0.001$) but not in the HC group ($p=0.582$).

(3b). A significant Diplotype X Condition effect ($p=0.0065$) was observed with greater anxiety ratings in the stress (HH: $p<0.0001$; HLLL: $p<.07$) and in cue (HH: $p<0.0004$; HLLL: $p<.007$) versus to the neutral conditions, but the HH individuals showed significantly higher stress-related anxiety than the HLLL individuals ($p<0.03$), and no differences between diplotypes in the neutral and cue conditions.

Table 1

Demographic and clinical characteristics of the substance dependent (SD) and healthy control (HC) subjects

Subject Variable	SD/HH (N=12)	SD/HLLL (N=25)	HC/HH (N=8)	HC/HLLL (N=20)
Race				
Caucasian	4 (33.3%)	17 (68%)	6 (75%)	11 (55%)
African American	6 (50%)	6(24%)	1(12.5%)	6(30%)
Hispanic	0	2(8%)	1 (12.5%)	2 (10%)
Other	2 (16.7%)	0	0	1 (5%)
Gender (Male)	7 (58.3%)	17 (68%)	6 (75%)	9 (45%)
Age *	37.6 (7.06)	37.6 (5.85)	34.8 (9.85)	29.1 (9.85)
Average Years of Education *	13.1 (1.78)	12.6 (1.23)	14.5 (1.97)	14.9 (1.77)
Average Years of Alcohol Use	12.4 (8.32)	13.7 (8.13)	8.9 (8.41)	7.5 (8.46)
Average Days of Alcohol Use/Month*	14.8 (9.04)	15.9 (11.97)	3.2 (3.35)	5.8 (8.33)
Total Amount (drinks) of Alcohol Use /Month*	187.7 (210.18)	242.3 (237.5)	8.0 (6.93)	11.5 (10.35)
Average Years of Cocaine Use	9.54 (4.72)	8.00 (7.26)	-	-
Average Days of Cocaine Use/Month	21.00 (10.43)	14.24 (13.23)	-	-
Total Amount (g) of Cocaine Use/Month	44.80 (38.22)	33.88 (23.14)	-	-
Average Days of Marijuana Use/Month	9.5 (12.94)	0.22 (0.52)	-	-
Total Amount (g) of Marijuana Use/Month	23.52 (26.60)	0.6 (0.94)	-	-
Regular Cigarette Smokers: N(%)*	8 (66.67%)	23 (92%)	4 (50%)	5 (25%)
Lifetime Prevalence of PTSD	2 (16.7%)	2 (8%)	0	2 (10%)
Lifetime Other Anxiety Disorders	3 (25%)	3 (12%)	0	0
Lifetime Major Depression	1 (8.3%)	7 (28%)	2 (25%)	1 (5%)

Note:

* SD significantly different from HC group, $p<0.05$, but no differences between HH and HLLL groups; HH: *NPY* Diplotype predicting high NPY mRNA expression; HLLL: *NPY* diplotype predicting moderate and low NPY mRNA expression; PTSD: post-traumatic stress disorder