

The relationship between naloxone-induced cortisol and delta opioid receptor availability in mesolimbic structures is disrupted in alcohol-dependent subjects

Gary S. Wand^{1,2}, Elise M. Weerts², Hiroto Kuwabara³, Dean F. Wong^{2,3,4,5}, Xiaoqiang Xu¹ & Mary E. McCaul^{1,2}

Departments of Medicine¹, Psychiatry and Behavioral Sciences², Radiology³, Neuroscience⁴ and Environmental Health Sciences⁵, The Johns Hopkins University School of Medicine, Baltimore, MD, USA

ABSTRACT

Hypothalamic-pituitary-adrenal (HPA) axis responses following naloxone administration have been assumed to provide a measure of opioid receptor activity. Employing positron emission tomography (PET) using the mu opioid receptor (MOR) selective ligand [¹¹C] carfentanil (CFN), we demonstrated that cortisol responses to naloxone administration were negatively correlated with MOR availability. In this study, we examined whether naloxone-induced cortisol and adrenocorticotropic (ACTH) responses in 15 healthy control and 20 recently detoxified alcohol-dependent subjects correlated with delta opioid receptor (DOR) availability in 15 brain regions using the DOR-selective ligand [¹¹C] methyl-naltrindole (MeNTL) and PET imaging. The day after the scan, cortisol responses to cumulative doses of naloxone were determined. Peak cortisol and ACTH levels and area under the cortisol and ACTH curve did not differ by group. There were negative relationships between cortisol area under curve to naloxone and [¹¹C] MeNTL-binding potential (BP_{ND}) in the ventral striatum, anterior cingulate, fusiform cortices, temporal cortex, putamen and a trend in the hypothalamus of healthy control subjects. However, in alcohol-dependent subjects, cortisol responses did not correlate with [¹¹C]MeNTL BP_{ND} in any brain region. Plasma ACTH levels did not correlate with [¹¹C]MeNTL BP_{ND} in either group. The study demonstrates that naloxone provides information about individual differences in DOR availability in several mesolimbic structures. The data also show that the HPA axis is intimately connected with mesolimbic stress pathways through opioidergic neurotransmission in healthy subjects but this relationship is disrupted during early abstinence in alcohol-dependent subjects.

Keywords Cortisol, delta opioid receptors, HPA axis, mu opioid receptors, naloxone, PET imaging.

Correspondence to: Gary Wand, The Johns Hopkins University School of Medicine, 720 Rutland Ave, Ross building, rm 863, Baltimore, MD 21205, USA. E-mail: gwand@jhmi.edu

INTRODUCTION

Central nervous system (CNS) opioid pathways are important modulators in the adaptation to stress as well as to alcohol. Strong evidence indicates that endogenous opioids attenuate or terminate the emotional consequences of the stress response as part of the allostatic reaction (Drolet *et al.* 2001). There is also strong evidence that CNS opioid systems play a significant role in alcohol dependence (Oswald & Wand 2004). The mesocorticolimbic system mediates the rewarding effects of most drugs of abuse including alcohol, and both dopamine and opioid neurotransmission play a crucial role in this

reward pathway. Within this key region of the brain, the reinforcing effects of alcohol are modulated by the release of opioid peptides and release of dopamine.

Investigators have administered opioid receptor antagonists to interrogate the hypothalamic-pituitary-adrenal (HPA) axis in part to elucidate the pathophysiological role of opioid systems in regulating stress and contributing to substance abuse disorders. The non-selective opioid receptor antagonist, naloxone, activates the HPA axis by blocking opioid inhibitory tone directed at the hypothalamic and possibly mesolimbic regulators of ACTH secretion. ACTH, in turn, stimulates cortisol release. Studies using opioid receptor antagonists have

assumed to identify differences in opioid activity due to nicotine and alcohol dependence, family history of alcoholism, gender, neuroticism and genetic variations in the mu opioid receptor (MOR) (Wand *et al.* 1998, 2001, 2002; Wand, Mangold & Ali 1999; Mangold *et al.* 2000; Oswald & Wand 2004; Adinoff *et al.* 2005; Mangold & Wand 2006; Uhart *et al.* 2006; Roche *et al.* 2010).

Employing the MOR-selective positron emission tomography (PET) ligand, [¹¹C] carfentanil (CFN) to measure [¹¹C]CFN-binding potential (BP_{ND}), in healthy subjects, we recently demonstrated that cortisol responses to a naloxone challenge were negatively correlated with MOR availability in the mesolimbic region (Wand *et al.* 2011). This finding demonstrated that naloxone is not merely a non-specific pharmacologic activator of the HPA axis but it also provides information about individual differences in MOR availability in important brain regions involved in responding to stress. The data also suggested that in addition to the hypothalamus, the ventral striatum, caudate and putamen may contribute endorphinergic inhibitory tone directed to the hypothalamic regulators of ACTH, and that this inhibition could be relieved by blocking MORs.

Similar to MORs, the delta opioid receptor (DOR) system is also important in stress regulation and is involved in maintaining alcohol consumption (Méndez & Morales-Mulia 2008). The majority of stress-related brain nuclei receives enkephalinergic innervation, or contains enkephalin perikarya (Drolet *et al.* 2001). In addition to MORs, DORs also place inhibitory tone on the hypothalamic regulators of HPA axis (Drolet *et al.* 2001). Indeed, enkephalinergic neurons are present not only in the paraventricular (PVN) nucleus of the hypothalamus and median eminence contiguous to corticotropin releasing factor (CRF) neurons, but also in other stress-related regions including the anterior cingulate cortex, infralimbic cortex, central and medial amygdala and the bed nucleus of the stria terminalis (Watson *et al.* 1982; Khachaturian, Lewis & Watson 1983; Guthrie & Basbaum 1984; Petrusz, Merchenthaler & Maderdrut 1985; Fallon & Leslie 1986; Harlan *et al.* 1987; Hurd 1996). The relationship between naloxone-induced cortisol and DOR availability has never been studied.

Individuals at increased risk for alcohol dependence (e.g. non-alcohol-dependent offspring of alcohol-dependent parents) have altered cortisol responses to naloxone and naltrexone compared to individuals at low risk for alcohol dependence, suggesting differences in opioid receptor activity (Wand 2008). Moreover, opioid receptor antagonists have been an important therapeutic modality for the treatment of alcohol dependence (Kranzler & Edenberg 2010). Employing PET imaging with [¹¹C]CFN and [¹¹C] methyl-naltrindole (MeNTL), our group reported significantly greater MOR availability in

mesolimbic regions in alcohol-dependent subjects when compared to controls; there was a similar trend for higher DOR availability in many of these brain regions in alcohol-dependent subjects, although differences did not achieve statistical significance (Weerts *et al.* 2011). In laboratory animals, antagonists that are selective for the MOR or DOR decrease alcohol consumption in laboratory animals (Froehlich *et al.* 1991; Krishnan-Sarin *et al.* 1995; Franck, Lindholm & Raaschou 1998).

The current study examined the utility of using a naloxone challenge procedure to characterize the relationship between naloxone-induced cortisol and DOR availability in healthy control subjects and to also detect defects in these systems in alcohol-dependent subjects. Cortisol responses were assessed using a technique, which allows administration of five incremental doses of naloxone in a single session (Mangold *et al.* 2000). This cumulative naloxone dosing procedure is believed to activate the HPA axis by blocking MORs at lower doses and DORs at higher doses (Traynor *et al.* 1990). Based on our recent findings that naloxone-induced cortisol is negatively correlated with MOR availability, we hypothesized a negative correlation between naloxone-induced cortisol and DOR availability in stress-related brain regions in healthy control subjects. Since chronic alcohol exposure is known to disrupt opioid and stress systems, we hypothesized that the relationship between cortisol and DOR availability would be disrupted in alcohol-dependent subjects.

METHODS

Subjects

Current heavy, alcohol-dependent drinkers and healthy control subjects between 21 and 58 years of age were recruited by advertisement and provided informed consent, in the sober state, using the John Hopkins University Institutional Review Board approved procedures. A masters-level research assistant utilized a battery of standardized diagnostic and psychological instruments to interview potential subjects as described previously (Weerts *et al.* 2008). Inclusion criteria for alcohol-dependent subjects included meeting DSM-IV criteria for alcohol dependence based on the Semi-Structured Assessment of the Genetics of Alcoholism (Bucholz *et al.* 1994) and active drinking at National Institute of Alcohol Abuse and Alcoholism (NIAAA)-defined hazardous levels as determined by completion of a 90-day Time Line Follow Back (Sobell & Sobell 1992). Aged-matched healthy control subjects did not drink above the NIAAA-recommended guidelines (less than 8 drinks/week for women and less than 15 drinks/week for men) and did not meet current or lifetime DSM-IV criteria for either alcohol abuse or dependence. For both groups,

Table 1 Demographics for alcohol-dependent (AD) and healthy control (HC) subjects.

	HC (n = 15)	AD (n = 20)	P
Mean years of age (SD)	46.7 (9.7)	44.3 (7.4)	0.263
Gender (n)			0.344
Male	9	15	
Female	6	5	
Race (n)			
Caucasian	9	12	1.000
Black	6	8	
Smoking status (n)			
Non-smokers	13	4	<0.001
Alcohol-related measures: mean (SD)			
Age (years) met criteria for alcohol dependence	N/A	28 (6.1)	—
Years of dependent alcohol drinking	N/A	16.4 (9.1)	—
Alcohol dependence scale (ADS) score	0.1 (0.4)	20.3 (7.3)	<0.001
Number of drinks per drinking day	1.5 (0.9)	12.9 (7.1)	<0.001
Number of drinking days per week	0.8 (1.3)	5.5 (1.2)	<0.001
Psychological assessments on admission: mean (SD)			<0.001
Beck Depression Inventory (BDI-II)	1.3 (1.4)	11.8 (8.9)	<0.001
Beck Anxiety Inventory (BAI)	1.3 (2.5)	9.9 (8.2)	<0.001
Brief Symptom Inventory (BSI)	0.1 (0.1)	0.5 (0.6)	0.003

Data shown are group means (SD) or number (n) of subjects as indicated.

exclusionary criteria included: (1) current or lifetime DSM-IV diagnostic criteria for any other Axis I disorder, including other drug abuse/dependence (except nicotine); (2) positive urine drug toxicology at screening or hospital admission; (3) other ongoing health problems or (4) subject report of maternal drinking during pregnancy. In addition, alcohol-dependent subjects were excluded if they reported alcohol-related seizures or the need for medication during previous detoxifications. Basic demographic characteristics for alcohol-dependent and healthy control subjects are shown in Table 1. The alcohol dependence scale (ADS; Skinner & Allen 1982) was administered to characterize alcohol use and associated problems. Prior to the clinical research unit (CRU) admission, subjects underwent magnetic resonance imaging (MRI) to rule out persons with clinically significant brain abnormalities, and allow within-subject anatomical localization of brain regions (Meltzer *et al.* 1990).

Inpatient procedures following admission to CRU

Healthy control subjects were admitted to the clinical research unit and completed an inpatient protocol that included PET imaging and the naloxone challenge. Alcohol-dependent subjects completed the study under an inpatient protocol that included hospital admission and medically supervised alcohol withdrawal prior to PET imaging on day 5 of supervised abstinence. Alcohol-dependent subjects remained on the clinical research unit for subsequent naltrexone treatment and PET imaging to determine naltrexone blockade of MORs and DORs

(Weerts *et al.* 2008). Both groups underwent the naloxone challenge the day after the PET scans.

The CRU nursing staff completed the Clinical Institute Withdrawal Assessment-Alcohol Revised (CIWA-Ar; Sullivan *et al.* 1989) with alcohol-dependent participants three times each day for the first 5 days. No subject required withdrawal medication based on CIWA scores, vital signs and physician assessment. During the CRU stay and all study procedures, cigarette smoking was prohibited. Nicotine-dependent smokers determined by a Fagerstrom nicotine dependence test (FNDT) score of 3 or higher, received a transdermal nicotine patch (21 mg nicotine) at hospital admission, each morning while on the CRU, and 3 hours prior to the PET scan. This standardized approach was used to mitigate potential impact of nicotine withdrawal on scan outcome.

PET procedures

PET scans were acquired in 3D mode on a GE Advance PET scanner (GE Medical Systems, Milwaukee, WI, USA) as previously described (Weerts *et al.* 2011). In brief, subjects underwent two PET scans in a fixed order on the same day; the [¹¹C]MeNTL, a specific DOR antagonist (Madar *et al.* 1997) and [¹¹C]CFN, a specific mu opioid agonist (Titeler *et al.* 1989; Frost *et al.* 1990) scans were conducted at 8:30 and 10:45 AM, respectively. This study only presents results from the [¹¹C] MeNTL scan.

Twenty-five frames with variable time period (6 × 30 seconds, 5 × 60 seconds, 5 × 120 seconds, 9 × 480 seconds) were acquired during a 90-minute period

for each study. Arterial blood samples were collected every 5 seconds initially, and then increasing intervals throughout out the [^{11}C] MeNTL scans (e.g. 1 minute, 2 minutes, 5 minutes, 10 minutes and 15 minutes) and counted for the radioactivity. Blood samples taken at 0, 5, 15, 30, 60 and 90 minutes were analyzed with high performance liquid chromatography for radioactive metabolites (Hilton *et al.* 2000). PET images were reconstructed employing the back projection algorithm using the software provided by the manufacturer (Kinahan & Rogers 1989).

Volumes of interest analyses

Fifteen volumes of interest (VOIs) were selected to include brain regions that had moderate to high [^{11}C] MeNTL BP_{ND} and included regions that are involved in responding to stressful stimuli and/or are altered in alcohol dependence. The VOIs were manually defined as previously described (Weerts *et al.* 2011).

Pharmacokinetic modeling

The primary outcome variable of interest for DOR was binding potential (BP_{ND}; Innis *et al.* 2007) of [^{11}C] MeNTL. We analyzed data using plasma reference graphical analysis (Logan *et al.* 1990) to obtain distribution volume (V_T); [^{11}C]MeNTL BP_{ND} was obtained as target-reference V_T ratio less 1 using cerebellum as the reference region. Plasma Logan plots for [^{11}C] MeNTL approached asymptotes in all regions examined by 20 minutes in all subjects. Therefore, t^* was set at 20 minutes (Weerts *et al.* 2011).

Naloxone cumulative dosing procedure

Generating a dose-response curve to naloxone in a single session was carried out based on our previously published procedures (Mangold *et al.* 2000; Wand *et al.* 2011). The session was conducted the afternoon following the PET scan. Following a calorie-controlled lunch, participants had an intravenous catheter inserted into a forearm vein 60 minutes before placebo administration. Baseline blood samples were obtained -30 minutes, -15 minutes and immediately prior to placebo administration. At time 0, placebo (0.9% saline) was administered as a bolus. Sequential doses of naloxone (25, 50, 100 and 250 $\mu\text{g}/\text{kg}$) dissolved in 0.9% saline were administered every 30 minutes thereafter. Blood samples were obtained 15 and 30 minutes after placebo and each naloxone dose, and then every 30 minutes through 240 minutes. Plasma cortisol (Diagnostic Products Corporation, Inc.; Los Angeles, CA, USA) and ACTH concentrations (Diasorin immunoradiometric assay, Stillwater, MN, USA) were assayed by radioimmunoassay. Intra-assay and

inter-assay coefficients of variance were less than 8% for cortisol and 6% and 9%, respectively, for plasma ACTH.

Statistical plan

Chi-squared tests, t -tests or the non-parametric equivalent were performed to compare demographics, smoking status, alcohol-related-measures and psychological assessment between the two groups.

The hormone level at baseline and following each naloxone dose was plotted for each group. The hormone level for each dose was calculated as the average of the two measures 15 and 30 minutes after the dose was given. To compare hormone levels following various naloxone doses to the placebo level, a linear mixed model with random intercept was constructed for each group. An unstructured covariance matrix was used to obtain robust standard errors.

We calculated the hormone area under the curve (AUC) measures to estimate the total amount of response. AUC was calculated as the sum of the trapezoids between every pair of consecutive time points subtracting baseline hormone level. Calculated AUC value had units of $\mu\text{g}/\text{dl} \cdot \text{hour}$ for cortisol and $\text{pg}/\text{ml} \cdot \text{hour}$ for ACTH. The hormone response AUC for the time period from time 0 through time 240 minutes was calculated as the main outcome. For each subject, the hormone peak response and time to peak was also calculated. Then linear models were constructed to compare AUC, peak, and time to peak between the two groups. Smoking status and sex were included as covariates. Similar linear models were constructed to compare [^{11}C] MeNTL level between AD and HC group on indicated regions. The obtained P -values across the regions were adjusted using the adaptive step-up Bonferroni method for multiple comparisons (Hochberg & Benjamin 1990).

To model the correlation between hormone response and [^{11}C] MeNTL BP_{ND}, multi-linear models were constructed with BP_{ND} as the dependent variables and cortisol or ACTH response AUC as the independent variable. Although the covariates of sex and smoking did not have significant effects on any dependent measures, both covariates have been shown to modulate opioid activity (Micevych *et al.* 1997; al'Absi *et al.* 2008; Berrendero *et al.* 2010). Therefore, we included sex and smoking status (smoker/non-smoker) as covariates. Partial residual plots with the component line were plotted for both groups in selected regions to show the relationship between hormone AUC and [^{11}C] MeNTL BP_{ND} given that the covariates were also in the model. Again, the adaptive step-up Bonferroni method was used for multiple comparison correction. All the analyses were carried out using SAS 9.2, (Carey, NC, USA) and a two-sided P -value of < 0.05 was considered statistically significant.

RESULTS

Baseline cortisol levels (mean of -30, -15 and 0 time points) did not differ by group. Compared to the effect of placebo, cumulative naloxone dose administration induced statistically significant cortisol responses in both healthy and alcohol-dependent subjects (Fig. 1). Magnitude of peak cortisol responses did not differ by group nor did area under the cortisol curve (AUC; Table 2). In

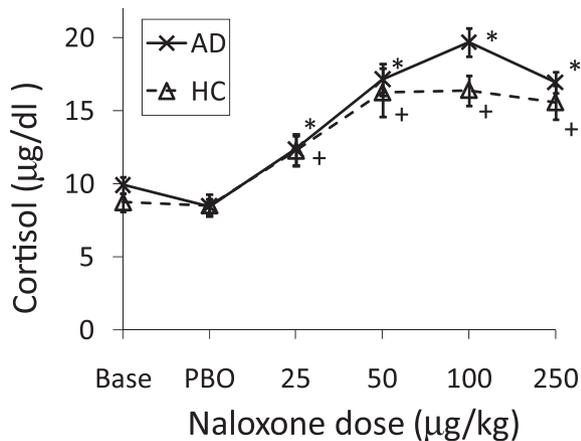


Figure 1 Cortisol responses to five graded doses of naloxone. Data points are mean (SEM). BASE=baseline; PBO=placebo. *denotes time points in alcohol-dependent subjects that were significantly different from mean of placebo time points with $P < 0.05$, adjusting for sex and smoking status. + denotes time points in healthy control subjects that were significantly different from mean of placebo time points with $P < 0.05$, adjusting for sex and smoking status.

contrast, time to peak response did differ by group. The majority of the healthy control subjects mounted a peak cortisol response between 75–90 minutes corresponding to the 50 µg/kg dose, whereas the majority of the alcohol-dependent subjects mounted a peak cortisol response between 105–120 minutes corresponding to the 100 µg dose (Table 2, $P = 0.05$).

We measured [^{11}C] MeNTL BP_{ND} in 15 brain regions in healthy control and alcohol-dependent subjects a day before the naloxone session was completed. We previously reported mean [^{11}C] MeNTL BP_{ND} on eight regions in healthy control and alcohol-dependent subjects: cingulate cortex, amygdala, insula, ventral striatum, putamen, caudate, globus pallidus and thalamus (Weerts *et al.* 2011). We found no statistical differences in [^{11}C] MeNTL BP_{ND} between healthy control and alcohol-dependent subjects, although there were trends for alcohol-dependent subjects to have higher [^{11}C] MeNTL BP_{ND} in the insula, putamen, caudate and globus pallidus. In Table 3, we compare [^{11}C] MeNTL BP_{ND} in seven additional brain regions. Again, there were no group differences in [^{11}C] MeNTL BP_{ND} except for a trend in the temporal cortex.

We then examined the correlation between [^{11}C] MeNTL BP_{ND} and naloxone-induced cortisol response in all 15 brain regions. There were no significant correlations between baseline cortisol concentration and [^{11}C] MeNTL BP_{ND} in healthy subjects or alcohol-dependent subjects. However, there were significant negative correlations between area under the cortisol response curve and [^{11}C] MeNTL BP_{ND} in the fusiform gyrus, ventral

Table 2 Comparison of mean cortisol variables between healthy control (HC) and alcohol-dependent (AD) subjects, adjusted for smoking status and sex.

Variable	Adjusted mean (SE)		P
	HC	AD	
Peak (ug/dL)	19.4 (1.4)	20.2 (1.2)	0.712
Response AUC (ug/dL·hour)	17.2 (3.4)	16.7 (2.8)	0.923
Time at peak (minutes)	87.5 (10.2)	118.1 (8.5)	0.050

Table 3 Mean [^{11}C]MeNTL BP_{ND} in healthy control (HC) and alcohol-dependent (AD) subjects.

VOI	HC (n = 15)	AD (n = 20)	Adjusted P ^a	Unadjusted P
Fusiform	0.548 (0.037)	0.601 (0.03)	0.696	0.330
Temporal	0.864 (0.05)	0.993 (0.042)	0.266	0.089
Hypothalamus	0.082 (0.062)	-0.002 (0.051)	0.696	0.357
Frontal cortex	0.801 (0.044)	0.89 (0.037)	0.526	0.175
Hippocampus	0.455 (0.047)	0.528 (0.039)	0.696	0.292
Parietal	0.849 (0.053)	0.925 (0.044)	0.696	0.333
DLPFC ^b	0.727 (0.059)	0.761 (0.049)	0.696	0.696

^aAdjusted for multiple comparison using the adaptive step-up Bonferroni method for multiple comparisons (Hochberg & Benjamin 1990). ^bDorsal lateral prefrontal cortex. Data shown are group means and SEM adjusted for sex and smoking for each VOI.

Table 4 Correlation of cortisol AUC (0 minute to 240 minutes) response to naloxone and [¹¹C]MeNTL BP_{ND} adjusted by sex and smoking.

Region	HC			AD		
	Partial correlation coefficient	Adjusted P	Unadjusted P	Partial correlation coefficient	Adjusted P	Unadjusted P
Fusiform	-0.826	0.0015	0.0005	-0.115	0.974	0.651
Ventral striatum ^a	-0.839	0.0019	0.0006	0.288	0.974	0.246
Cingulate	-0.661	0.0418	0.0139	-0.05	0.974	0.843
Temporal	-0.633	0.0604	0.0201	0.017	0.974	0.948
Putamen	-0.619	0.0727	0.0242	0.098	0.974	0.699
Hypothalamus	-0.547	0.1593	0.0531	0.027	0.974	0.916
Thalamus	-0.527	0.1936	0.0645	0.246	0.974	0.326
Caudate	-0.495	0.2574	0.0858	-0.188	0.974	0.455
Insula	-0.486	0.2764	0.0921	0.045	0.974	0.86
Frontal cortex	-0.455	0.3537	0.1179	0.008	0.974	0.974
Hippocampus	-0.374	0.6241	0.208	0.247	0.974	0.324
Globus pallidus	-0.246	0.6892	0.4174	-0.119	0.974	0.638
Parietal	-0.198	0.6892	0.5161	0.009	0.974	0.971
Amygdala	-0.185	0.6892	0.5442	-0.243	0.974	0.33
DLPFC ^b	-0.123	0.6892	0.6892	-0.059	0.974	0.815

The adaptive step-up Bonferroni method was used to adjust *P*-values for multiple comparisons. ^aOne outlier removed. ^bDLPFC = dorsal lateral prefrontal cortex. Bold values reached statistical significance.

striatum and cingulate cortex in healthy subjects (Table 4). The data, without adjustment for multiple comparisons, also includes significant correlations with the temporal cortex and putamen as well as a trend in the hypothalamus (*P* = 0.0531). In contrast, there were no significant correlations between naloxone-induced cortisol responses and [¹¹C] MeNTL BP_{ND} in alcohol-dependent subjects in any of the 15 brain regions (Table 4).

As an example of these relationships, the partial residual plots show the association between [¹¹C] MeNTL BP_{ND} in the ventral striatum, fusiform gyrus and cingulate cortex with cortisol responses to naloxone in both groups (Fig. 2a–f). Figure 3 shows brain images of mean [¹¹C] MeNTL BP_{ND} in the ventral striatum for the control and alcohol-dependent subjects in the lowest cortisol response tertile compared to subjects in the highest cortisol response tertile.

The magnitude of peak ACTH responses did not differ by group nor did area under the ACTH curve or time to peak (data not shown). There were no significant correlations between ACTH AUC and [¹¹C] MeNTL BP_{ND} in any VOI in either group (data not shown).

DISCUSSION

In the current study, we compared two CNS measures in healthy control and alcohol-dependent subjects. Cortisol response to opioid receptor blockade was interrogated by administering five incremental doses of naloxone.

Naloxone has been shown to dose dependently block mu, delta-1 and delta-2 opioid receptors (Lewanowitsch & Irvine 2003; Hirose *et al.* 2005). The alcohol-dependent subjects mounted a cortisol response that was minimally different compared to the healthy control subjects. The peak and the AUC cortisol response did not differ between groups; however, time to peak response differed significantly. The physiological significance of this group difference in time to peak is unclear. It could reflect differential sensitivity to naloxone dosing between alcohol-dependent and control subjects or may indicate a more global injury of the HPA axis in alcohol-dependent subjects. If the latter is true, it implies that the alcohol-dependent group may have an impaired response to both pharmacologic and physiologic activation. Mounting a delayed response to stress could jeopardize health of the organism.

Second, we examined whether the naloxone-induced cortisol response correlated with DOR availability. To do this, we first measured [¹¹C] MeNTL BP_{ND} in 15 brain regions in the healthy control and alcohol-dependent subjects. We previously published these data in 8 of the 15 regions (Weerts *et al.* 2011) finding no significant group differences in [¹¹C] MeNTL BP_{ND}. However, there were trends in many brain regions for alcohol-dependent subjects to have higher MeNTL than control subjects, although differences did not achieve statistical significance due to limited sample sizes (Weerts *et al.* 2011). Interestingly, DOR availability in the caudate was

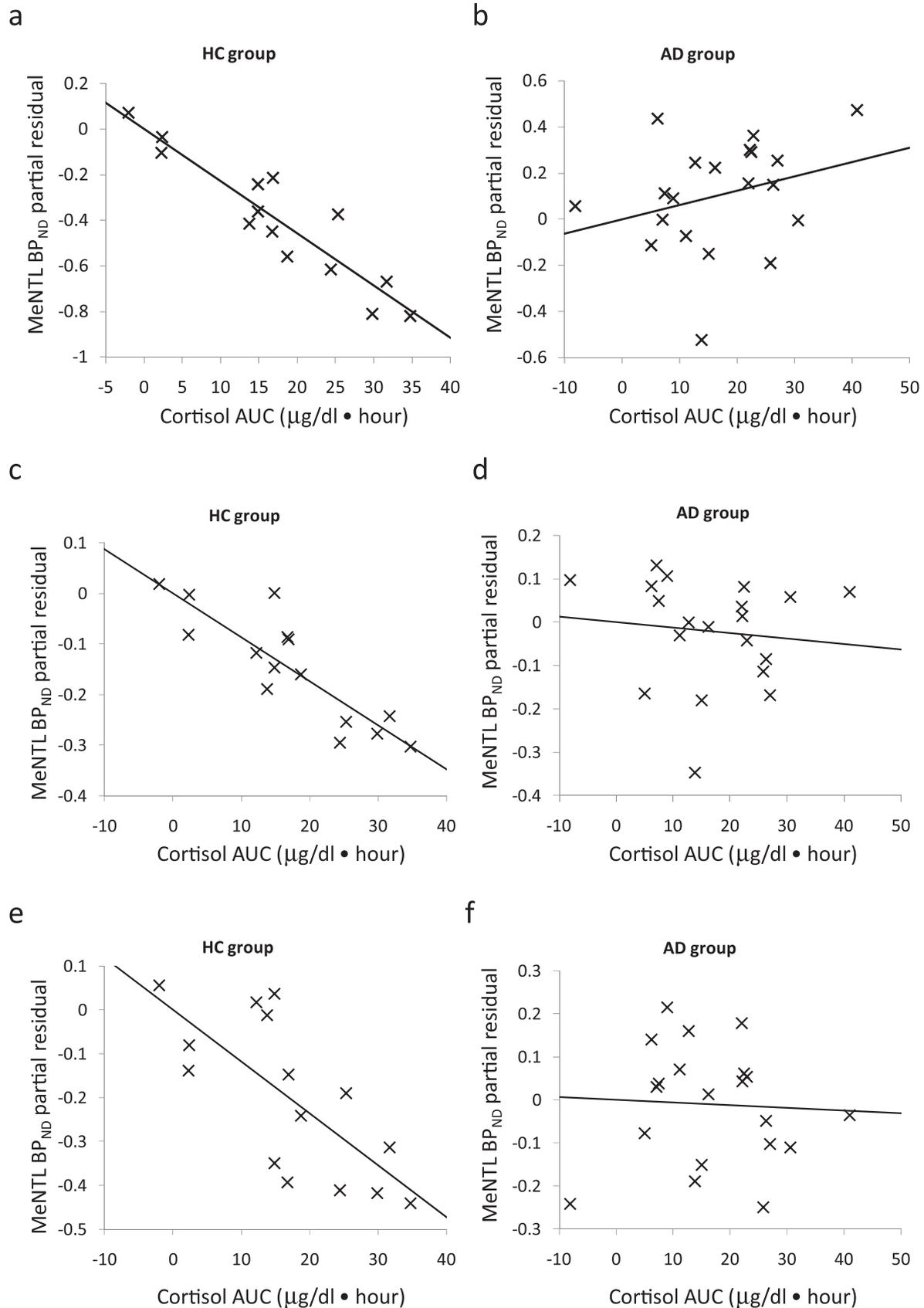


Figure 2 Partial residual plot of [¹¹C]MeNTL BP_{ND} and cortisol response AUC (0–240 minutes) adjusted for sex and smoking. Statistics are displayed in Table 4. a and b, ventral striatum, c and d, fusiform, e and f, cingulate.

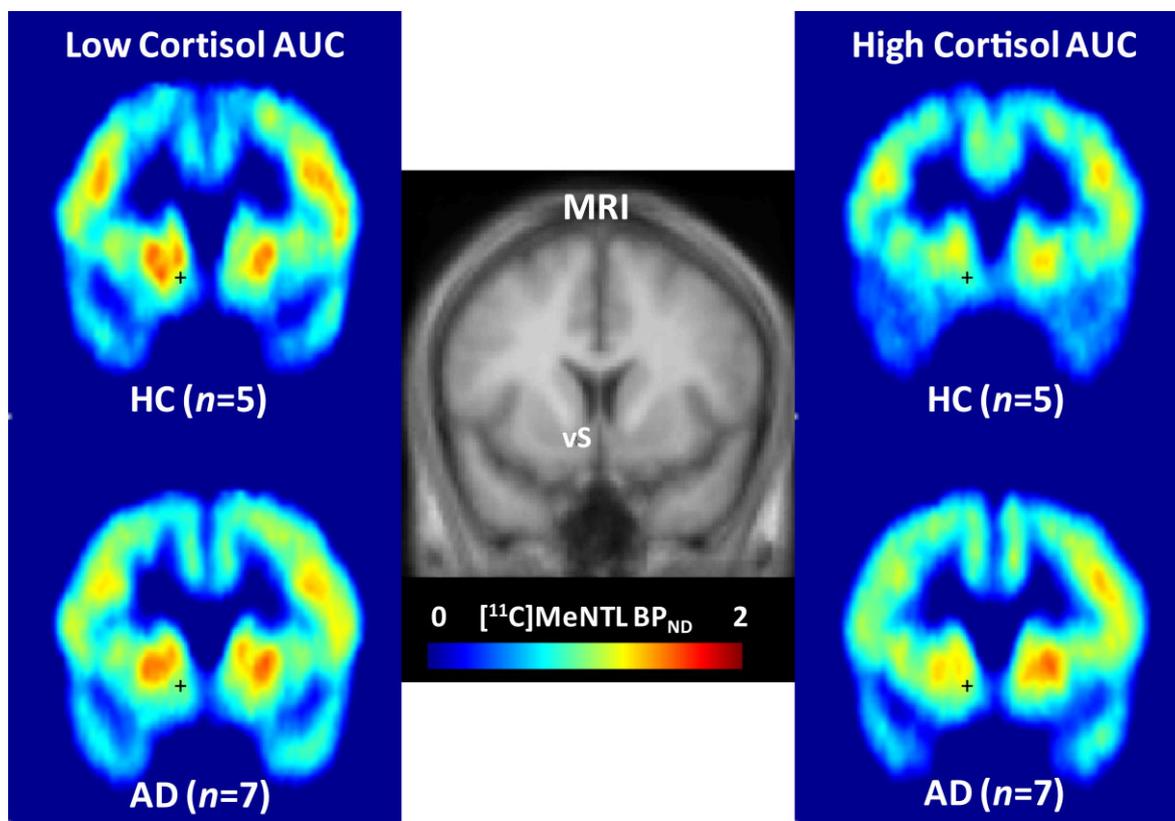


Figure 3 Coronal view images of $[^{11}\text{C}]\text{MeNTL BP}_{\text{ND}}$, averaged across the subjects in the lowest tertile of cortisol responses (left) compared to the subjects in the highest tertile of cortisol responses (right). A standard MRI to which BP_{ND} images were spatially normalized is displayed in the middle panel to indicate image locations around the anterior-posterior center of ventral striatum (vS). Colored legend depicts $[^{11}\text{C}]\text{MeNTL BP}_{\text{ND}}$ from 0 (light blue) to 2.0 (red).

correlated with recent drinking (average drinks per drinking day) in alcohol-dependent subjects. In the present study, we showed $[^{11}\text{C}]\text{MeNTL BP}_{\text{ND}}$ measured in seven additional brain regions that had not been previously reported. Again, we found no group differences in $[^{11}\text{C}]\text{MeNTL BP}_{\text{ND}}$ except for a trend in the temporal cortex. With these measurements in hand, we went on to correlate $[^{11}\text{C}]\text{MeNTL BP}_{\text{ND}}$ and cortisol AUC. In the healthy control subjects, there were significant negative relationships between cortisol response to naloxone and $[^{11}\text{C}]\text{MeNTL BP}_{\text{ND}}$ in several mesolimbic structures. Subjects who mounted a higher cortisol response to naloxone had lower $[^{11}\text{C}]\text{MeNTL BP}_{\text{ND}}$ compared to subjects with a higher cortisol response. Lower $[^{11}\text{C}]\text{MeNTL BP}_{\text{ND}}$ indicates either increased enkephalin peptide occupancy or decreased DOR number/affinity compared to a state of higher $[^{11}\text{C}]\text{MeNTL BP}_{\text{ND}}$; the scanning technique cannot distinguish among these possibilities. In contrast, in alcohol-dependent subjects, cortisol responses did not correlate with $[^{11}\text{C}]\text{MeNTL BP}_{\text{ND}}$ in any brain region. Apparently, factors associated with alcohol dependence and/or withdrawal disrupted the relationship identified in the healthy control participants.

The primary regulators of ACTH secretion, and thus cortisol, are CRF and arginine vasopressin (AVP) neurons located in the parvocellular region of the paraventricular nucleus. Enkephalins and other opioid peptides modify the synthesis and secretion of hypothalamic releasing factors like CRF (Szekely 1990; Borsook & Hyman 1995). Furthermore, endorphinergic and enkephalinergic innervation places inhibitory tone on these neurons; opioid receptor antagonists can release this inhibition resulting in stimulation of ACTH and cortisol (Schluger *et al.* 1998). In the healthy control group, we identified negative correlations between $[^{11}\text{C}]\text{MeNTL BP}_{\text{ND}}$ and cortisol responses to naloxone in the fusiform gyrus, ventral striatum and the cingulate cortex; these relationships remained significant after statistically adjusting for analyses in the 15 brain regions. The three regions have high $[^{11}\text{C}]\text{MeNTL BP}_{\text{ND}}$ and are enriched in enkephalin perikarya (Drolet *et al.* 2001; Narita *et al.* 2006). DORs in the ventral striatum and cingulate cortex have been shown to modulate anxiety-like behaviors in rodents (Hebb *et al.* 2004; Narita *et al.* 2006). When we did not statistically adjust for the analyses across the 15 brain regions, additional correlations with several other brain

regions including the hypothalamus were significant. The weaker correlation for the hypothalamus may relate to this region being difficult to study with PET.

There are several possible mechanisms to explain the correlation between naloxone-induced activation of the HPA axis and [^{11}C] MeNTL BP_{ND} in the identified brain regions in healthy controls. The mechanisms are not mutually exclusive. Although causality cannot be proven by these data, the findings suggest that the three regions are under enkephalinergic inhibitory tone and release of that inhibition by naloxone results in communications with the PVN to activate the HPA axis. In this scenario, cumulative naloxone dosing activated the HPA axis proportionately to DOR availability. This assumes that these brain regions communicate to PVN neurons through the enkephalinergic system. In fact preclinical studies have shown that mesolimbic brain regions that participate in responding to stressful stimuli also communicate with PVN neurons to stimulate CRF and AVP release or are involved in glucocorticoid negative feedback (Jankord & Herman 2008). It is thought that the release of enkephalin peptides from several mesolimbic structures attenuates the impact of the stress response on emotional and affective states helping the organism cope with the stressful event (Drolet *et al.* 2001). Supporting this are studies showing higher anxiety levels in mice with a targeted disruption of the DOR gene (Filliol *et al.* 2000) or the proenkephalin gene (Konig *et al.* 1996). Also, naloxone-induced activation of the HPA response is stronger in chronically stressed animals compared to unstressed control animals showing the impact of chronic stress on opioid systems (Janssens *et al.* 1995). It is interesting that [^{11}C] MeNTL BP_{ND} measured in the fusiform gyrus correlated with cortisol responses. Similar to the amygdala, recent studies have shown that the fusiform gyrus is also involved in the perception of threatening facial stimuli (Radua *et al.* 2010).

It is noteworthy that our previous paper demonstrated negative correlations between MOR availability and cortisol responses to naloxone in the ventral striatum, putamen, caudate and hypothalamus of healthy control subjects using the mu selective ligand [^{11}C]CFN (Wand *et al.* 2011). Taken together, these observations suggest that certain mesolimbic regions that activate the hypothalamic regulators of ACTH are under enkephalinergic inhibitory tone (fusiform and cingulate), other regions are predominantly under endorphinergic inhibitory tone (caudate and putamen) and still other regions are under both forms of opioidergic inhibition (ventral striatum and hypothalamus).

There is ample evidence that genotype, early life traumas and current life stressors create individual differences in chronic cortisol exposure (cortisol burden; Stephens & Wand 2011). Although a less likely

explanation for the relationship between [^{11}C] MeNTL BP_{ND} and cortisol response to naloxone, it is possible that individual differences in cortisol burden modulate DOR availability. In this model, individuals with a phenotype of high chronic cortisol production have lower DOR availability compared to subjects with a low chronic cortisol exposure phenotype. The effects of chronic cortisol exposure on DOR expression would then account for the association of cortisol and [^{11}C] MeNTL BP_{ND}. Indeed, it has been shown that stress causes adaptive increases in pro-enkephalin gene expression in the paraventricular nucleus and other brain regions conducted using several different stress procedures including intraperitoneal injection of hypertonic saline (Watts 1991; Young & Lightman 1992; Ceccatelli & Orazzo 1993). Cortisol-induced increases in synaptic concentrations of enkephalinergic peptides could reduce [^{11}C] MeNTL BP_{ND} through competitive inhibition. This is precisely the relationship we see in our study. If we had observed a relationship between baseline cortisol levels and [^{11}C] MeNTL BP_{ND}, this would have provided stronger support for this model. Whatever the mechanisms responsible for these interesting correlations, the data show that the naloxone challenge procedure as conducted in this study provide measurement related to DOR availability in healthy control subjects.

The findings in the ventral striatum are particularly interesting. In this region, the correlations between cortisol and [^{11}C] MeNTL BP_{ND} in this study and [^{11}C] CFN BP_{ND} in our previous work (Wand *et al.* 2011) are robust and particularly important because endogenous opioids modulate dopaminergic transmission in the nucleus accumbens, modulating reinforcement and reward (Wise 2008). This brain region is central in understanding the neurobiology of alcohol and other drug dependencies (Barson *et al.* 2009, 2010). Moreover, naloxone has been shown to dose dependently block mu, delta 1 and delta 2 opioid receptors attenuating the effects of DAMGO, DPDPE and [D-Ser(2)]Leu-enkephalin-Thr on dopamine release in the nucleus accumbens (Hirose *et al.* 2005).

A third finding of this paper is that the alcohol-dependent subjects did not show correlations between cortisol responses to naloxone and [^{11}C] MeNTL BP_{ND} in any brain region. This suggests disruption of signaling between enkephalinergic pathways and the paraventricular nucleus. This analysis reveals an abnormality in the relationship between DOR availability and the HPA stress response in alcohol-dependent subjects that neither technique alone can identify. These data support a role of the DOR in alcohol dependence, particularly when coupled to our previous study showing that the FDA-approved dose of naltrexone (50 mg) produced only partial inhibition (21%) of DORs in alcohol-dependent subjects when the same dose of naltrexone produces near

complete inhibition (95%) of MORs (Weerts *et al.* 2008). Moreover, there is evidence from preclinical research showing efficacy of DOR antagonists for relapse prevention (Marinelli *et al.* 2009; Nielsen *et al.* 2011). It remains uncertain whether the failure to see a correlation between cortisol and [¹¹C] MeNTL BP_{ND} in alcohol-dependent subjects reflects a trait or state phenomenon. Through a variety of possible mechanisms (e.g. chronic alcohol toxicity, acute alcohol withdrawal, genetic regulation), this relationship has become disrupted. The clinical implications of this disruption merit further investigation, particularly in terms of the possible contribution to alcohol relapse in early recovery.

There are several limitations to the study. First, the sample size is small as this was an exploratory study. Second, we studied the relationship between DOR availability and naloxone challenge only using a cumulative dosing procedure developed in our laboratory (Mangold *et al.* 2000). It remains to be seen whether this would be observed following a single dose of naloxone or using other HPA provocateurs. A third limitation is the potential confound of smoking, which plagues most studies with alcohol-dependent subjects. Over 80% of alcohol-dependent subjects report regular tobacco use and smoke at high rates (Dawson 2000) when compared to social drinkers. It is somewhat reassuring that the covariate of smoking did not have a significant effect on any dependent measures. In addition, all smokers had nicotine patches placed to control for smoking, and smoking was a covariate in the statistical model. Fourth, we did not identify any association between ACTH AUC and [¹¹C] CFN BP_{ND} in any VOI. It is possible that cortisol negative feedback attenuates the ACTH response to naloxone, and therefore, a full ACTH response cannot be realized. It is also possible that the short half-life of ACTH in plasma prohibited capturing the true area under the ACTH curve given our blood sampling frequency. Finally, because the same measure of naloxone response is correlated with both mu and delta receptor availability in the brain, this raises the issue of ligand specificity. *In vitro* affinity, Ki values of MeNTL were reported to be 0.02, 14 and 65 nM for δ, μ and κ opioid receptors, respectively (Portoghese, Sultana & Takemori 1990), while Ki values of CFN were 0.024, 3.28 and 43.1 nM for μ, δ and κ opioid receptors, respectively (Cometta-Morini, Maguire & Loew 1992). This supports that [¹¹C]MeNTI and [¹¹C]CFN are specific radioligands of δ and μ opioid receptors (Madar *et al.* 1997). Accordingly, the two radioligand displayed distinctively different regional neuroreceptor distribution patterns in the human brain (Madar *et al.* 1997; Weerts *et al.* 2011).

In summary, the study demonstrates that naloxone provides information about individual differences in DOR availability in several mesolimbic structures. The data

also show that the HPA axis is intimately connected with mesolimbic stress pathways through opioidergic neurotransmission in healthy subjects but this relationship is disrupted during early abstinence in alcohol-dependent subjects. The mechanism and clinical significance behind this disruption needs further study.

Acknowledgements

The National Institute of Alcohol Abuse and Alcoholism (NIAAA) provided financial support for research related to the subject matter of this manuscript from the grants R37AA12303 (PI: G.S. Wand) and R01AA11855 (PI: M.E. McCaul). Dr. Wand is the recipient of a gift fund from the Kenneth Lattman Foundation. He is an investigator in a post-marketing study for Eli Lilly & Company, entitled The Global Hypopituitary Control and Complications Study (HypoCCS). He is an investigator in a post-marketing study for Ipsen entitled, Somatuline Depot (lanreotide) Injection for Acromegaly (SODA). Dr. Wong is a consultant for Amgen. Between 2009 and present, Dr. Wong has received funding from the following companies: Acadia, Amgen, Avid, Biotie, Bristol Myers Squibb, GE, Intracellular, J&J, Lilly, Luhdeck, Merk, Orexigen, Otuska, Roche, Sanofi-Aventis and Sepracor. Drs. Kuwabara, Xu and Weerts have no financial disclosures.

Authors Contribution

All authors contributed to the design and execution of the project as well as partaking in the writing and editing the manuscript.

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