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

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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 (Wand et al, 2011). In this study we examined whether naloxone-induced cortisol and 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 adrenocorticotropin (ACTH) levels and area under the cortisol and ACTH curve did not differ by group. There were negative relationships between cortisol AUC to naloxone and [¹¹C] MeNTL 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.

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Keywords

mu opioid receptors; naloxone; PET imaging; HPA axis; cortisol

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 and 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 release of opioid peptides and release of dopamine.

Investigators have administered opioid receptor antagonists to interrogate the HPA axis in part to elucidate the pathophysiologic 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 MOR (Oswald and Wand, 2004; Roche et al, 2010; Adinoff et al, 2005; Wand et al, 1998; Wand et al, 1999; Mangold et al, 2000; Wand et al 2001; Wand et al, 2002; Uhart et al, 2006; Mangold and Wand, 2006).

Employing the MOR selective PET ligand, [¹¹C]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 DOR system is also important in stress regulation and is involved in maintaining alcohol consumption (Mendez and 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 CRF neurons, but also in other stress-related regions including the anterior cingulate cortex, infra-limbic cortex, central and medial amygdala and the bed nucleus of the stria terminalis (Fallon and Leslie, 1986; Guthrie and Basbaum, 1984; Harlan et al, 1987; Hurd, 1996; Khachaturian et al, 1983; Petrusz et al, 1985; Watson et al, 1982). The relationship between naloxone-induced cortisol and DOR availability has never been studied.

Individuals at increased risk for alcohol dependence (eg., 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 opioids receptor activity (Wand, 2008). Moreover, opioid receptor antagonists have been an important therapeutic modality for the treatment of alcohol dependence (Kranzler and Edenberg, 2010). Employing PET imaging with [^{11}C]CFN and [^{11}C]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 (Krishnan-Sarin et al, 1985; Froehlich et al, 1991; Franck et al, 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 5 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 JHU 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 NIAAA-defined hazardous levels as determined by completion of a 90-day Time Line Follow Back (Sobel and Sobel, 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, 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 and Allen, 1982) was administered to characterize alcohol use and associated problems. Prior to the 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 Clinical Research Unit (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 3 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 FNNDT score of 3 or higher, received a transdermal nicotine patch (21 mg nicotine) at hospital admission, each morning while on the CRU, and three 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) 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 (33,34) 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 sec, 5 × 60 sec, 5 × 120 sec, 9 × 480 sec) were acquired during a 90-min period for each study. Arterial blood samples were collected every 5 seconds initially, and then increasing intervals throughout out the [¹¹C] MeNTL scans (e.g., 1-min, 2-min, 5-min, 10-min and 15-min) and counted for the radioactivity. Blood samples taken at 0, 5, 15, 30, 60 and 90 min were analyzed with HPLC for radioactive metabolites (Hilton et al, 2000). PET images were reconstructed employing the back projection algorithm using the software provided by the manufacturer (Kinahan et al, 1989).

Volumes of Interest (VOI) Analyses

Fifteen VOIs were selected to include brain regions that had moderate to high [¹¹C] MeNTL BP_{ND} and included regions that are involved in responding to stressful stimuli and/or are altered in alcohol dependence (table 4). 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 [¹¹C] MeNTL. We analyzed data using plasma reference graphical analysis (PRGA) (Logan et al., 1990) to obtain distribution volume (V_T); [¹¹C]MeNTL BP_{ND} was obtained as target-reference V_T ratio less 1 using cerebellum as the reference region. Plasma Logan plots for [¹¹C] MeNTL approached asymptotes in all regions examined by 20 min in all subjects. Therefore t* was set at 20 min (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 (Wand et al, 2011; Mangold et al, 2000). 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 min before placebo administration. Baseline blood samples were obtained -30 min, -15 min 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 ug/kg) dissolved in 0.9% saline were administered every 30 min thereafter. Blood samples were obtained 15 and 30 min after placebo and each naloxone dose, and then every 30 min through 240 min. Plasma cortisol (Diagnostic Products Corporation, Inc.; Los Angeles, CA). and ACTH concentrations (DiaSorin immunoradiometric assay) 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-square 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 min 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 $\mu\text{g/dl} \cdot \text{hour}$ for cortisol and $\text{pg/mL} \cdot \text{hour}$ 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 and 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 M 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, 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 (Figure 1). Magnitude of peak cortisol responses did not differ by group nor did area under the cortisol curve (AUC) (Table 2). In contrast, time to peak response did differ by group. The majority of the healthy control subjects mounted a peak cortisol response between 75–90 min corresponding to the 50 ug/kg dose whereas the majority of the alcohol dependent subjects mounted a peak cortisol response between 105–120 min corresponding to the 100ug dose (Table 2; $p=.05$).

We measured [^{11}C] MeNTL BP_{ND} in fifteen 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 8 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 7 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 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 ($p=.0531$). In contrast, there was no significant correlations between naloxone-induced cortisol responses and [^{11}C] MeNTL BP_{ND} in alcohol dependent subjects in any of the 15 brain region (Table 4).

As an example of these relationships, the partial residual plots show the association between [^{11}C] MeNTL BP_{ND} in the ventral striatum, fusiform gyrus and cingulate cortex with cortisol responses to naloxone in both groups (Figure 2A–F). Figure 3 shows brain images of mean [^{11}C] 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 [^{11}C] 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 5 incremental doses of naloxone. Naloxone has been shown to dose-dependently block mu, delta-1 and delta-2 opioid receptors (Hirose et al, 2005; Lewandowitsch and Irvine, 2003). 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 [^{11}C] 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 [^{11}C] 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 correlated with recent drinking (average drinks per drinking day) in alcohol dependent subjects. In the present study we showed [^{11}C] MeNTL BP_{ND} measured in 7 additional brain regions that had not been previously reported. Again we found no group differences in [^{11}C] MeNTL BP_{ND} except for a trend in the temporal cortex. With these measurements in hand we went on to correlate [^{11}C] MeNTL BP_{ND} and cortisol AUC. In the healthy control subjects, there were significant negative relationships between cortisol response to naloxone and [^{11}C] MeNTL BP_{ND} in several mesolimbic structures. Subjects who mounted a higher cortisol response to naloxone had lower [^{11}C] MeNTL BP_{ND} compared to subjects with a higher cortisol response. Lower [^{11}C] MeNTL BP_{ND} indicates either increased enkephalin peptide occupancy or decreased DOR number/affinity compared to a state of higher [^{11}C] MeNTL BP_{ND}; the scanning technique cannot distinguish among these possibilities. In contrast, in alcohol dependent subjects, cortisol responses did not correlate with [^{11}C] MeNTL BP_{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 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 (Boosook and Hyman 1995; Szekely, 1990). 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}C] MeNTL BP_{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}C] MeNTL BP_{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 (Narita et al, 2006; Hebb et al, 2004). 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 and 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 [¹¹C] 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 [¹¹C]Carfentanil (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 and Wand, 2011). Although a less likely explanation for the relationship between [¹¹C] 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 [¹¹C] 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 (Young and Lightman, 1992; Ceccatelli and Orazzo, 1993; Watts, 1991). Cortisol-induced increases in synaptic concentrations of enkephalinergic peptides could reduce [¹¹C] 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 [¹¹C] 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 [¹¹C] MeNTL BP_{ND} in this study and [¹¹C] 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; Barson, et al 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 [^{11}C] 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 [^{11}C] 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 et al. 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 et al, 1992). This supports that [^{11}C]MeNTI and [^{11}C]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.

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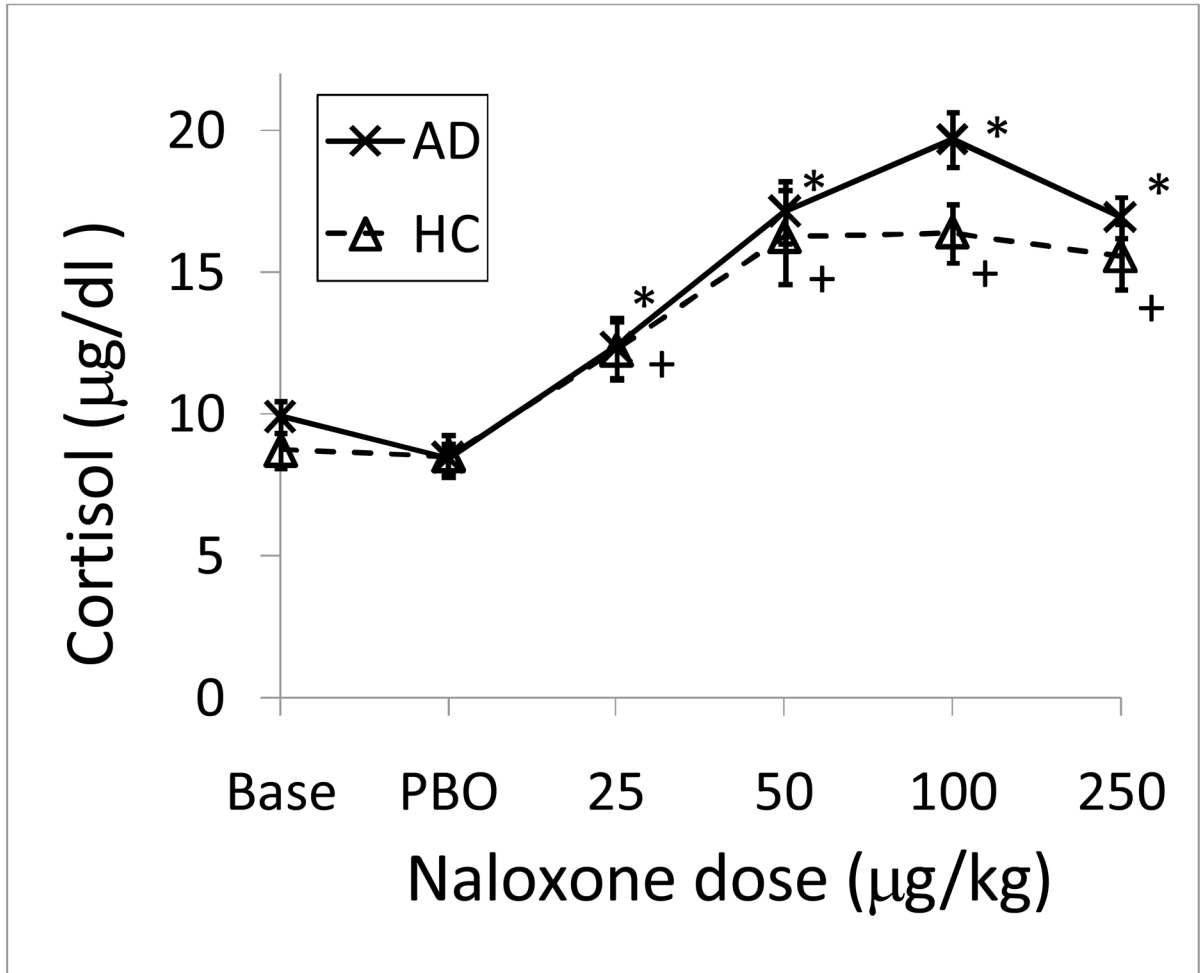


Figure 1.

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 $p < .05$, adjusting for sex and smoking status. + denotes time points in healthy control subjects that were significantly different from mean of placebo time points with $p < .05$, adjusting for sex and smoking status.

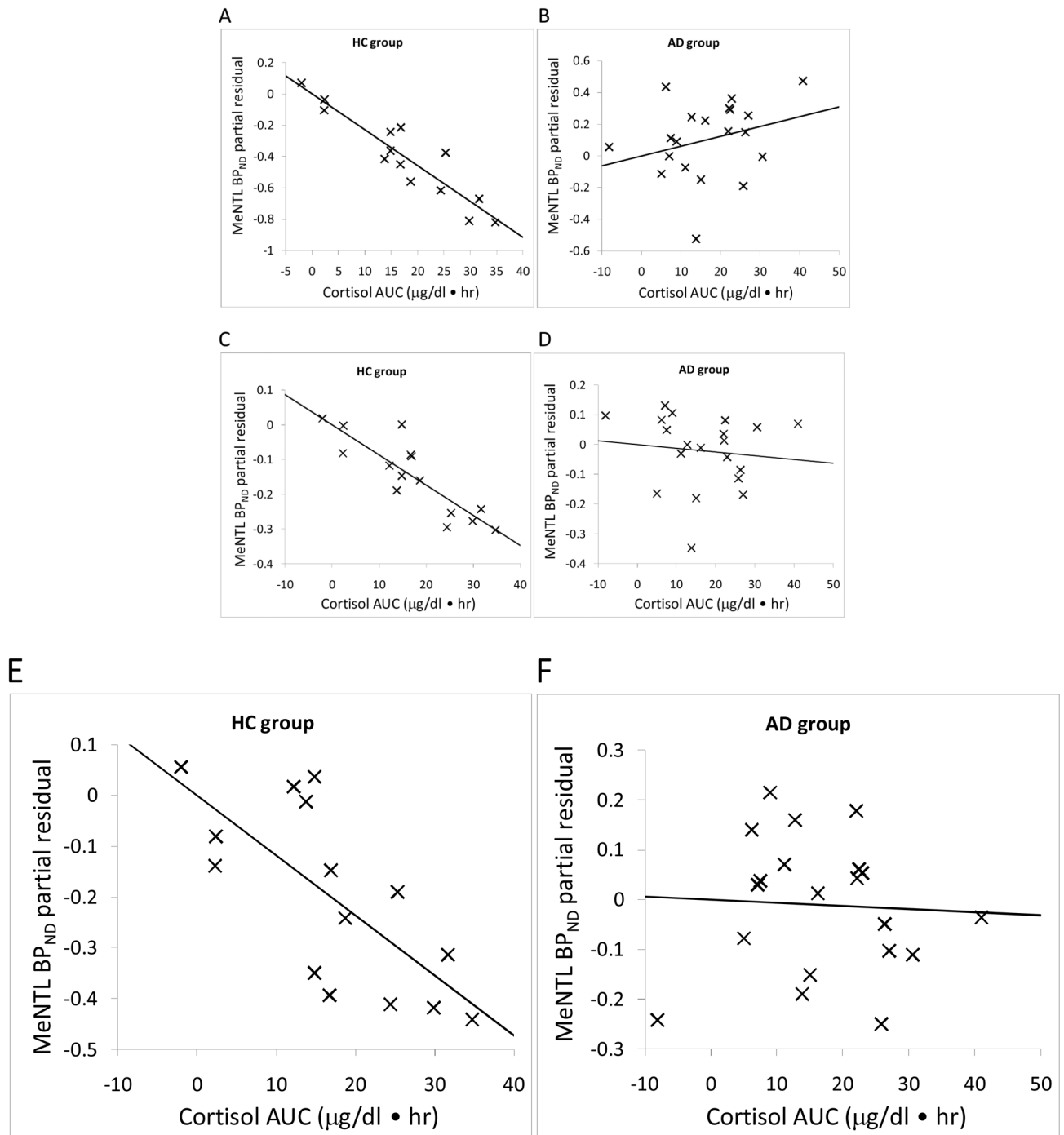


Figure 2. Partial residual plot of $[^{11}\text{C}]\text{MeNTL BP}_{\text{ND}}$ and cortisol response AUC (0–240 min) adjusted for sex and smoking. Statistics are displayed in Table 4. A and B, ventral striatum, C and D, fusiform; E and F, cingulate

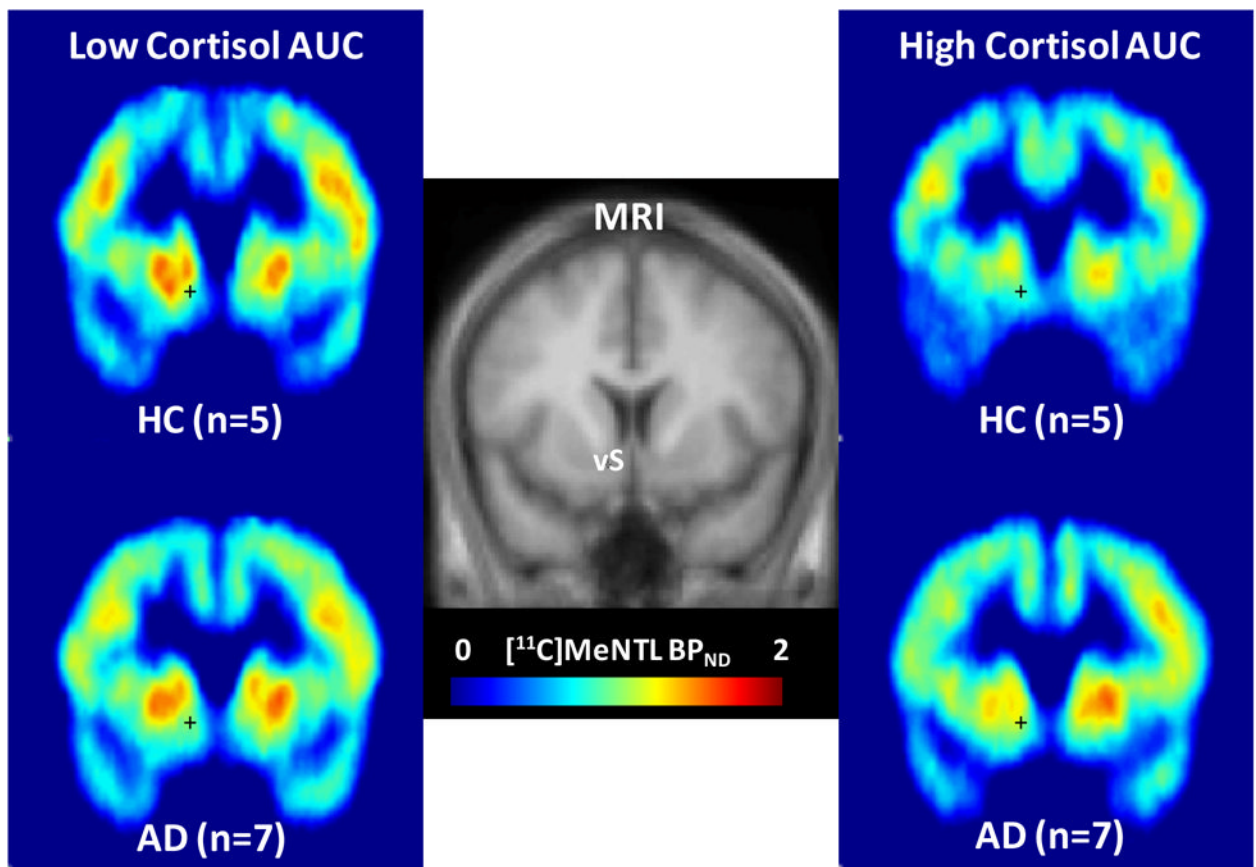


Figure 3.

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).

Table 1

Demographics for alcohol dependent (AD) and healthy control (HC) subjects. Data shown are group means (SD) or number (n) of subjects as indicated.

	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	<.001
Alcohol-related measures: mean (SD)			
Age (Yrs) met criteria for Alcohol	N/A	28 (6.1)	
Years of Dependent Alcohol Drinking	N/A	16.4 (9.1)	
Alcohol Dependence Scale (ADS)	0.1 (0.4)	20.3 (7.3)	<.001
Number of Drinks per drinking day	1.5 (0.9)	12.9 (7.1)	<.001
Number of Drinking Days per Week	0.8 (1.3)	5.5 (1.2)	<.001
Psychological Assessments on			<.001
Beck Depression Inventory (BDI-II)	1.3 (1.4)	11.8 (8.9)	<.001
Beck Anxiety Inventory (BAI)	1.3 (2.5)	9.9 (8.2)	<.001
Brief Symptom Inventory (BSI)	0.1 (0.1)	0.5 (0.6)	0.003

Table 2

Comparisons of mean cortisol variables between healthy control (HC) alcohol dependent (AD), 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.h)	17.2 (3.4)	16.7 (2.8)	0.923
Time at peak (min)	87.5 (10.2)	118.1 (8.5)	0.050

Table 3

Mean [^{11}C]MeNTLBP_{ND} healthy control (HC) and alcohol dependent (AD) subjects. Data shown are group means and SEM adjusted for sex and smoking for each VOI.

VOI	HC (N = 15)	AD (N = 20)	Adjusted P	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 ^{**}	0.727 (0.059)	0.761 (0.049)	0.696	0.696

* Adjusted for multiple comparison using the adaptive step-up Bonferroni method for multiple comparisons (Hochberg and Benjamini, 1990).

** dorsal lateral prefrontal cortex

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Table 4

Correlation of cortisol AUC (0 minute to 240 minute) response to naloxone and [¹¹C]MeNTL BP_{ND} adjusted by sex and smoking. The adaptive step-up Bonferroni method was used to adjust P values for multiple comparisons.

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 *	-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***	-0.123	0.6892	0.6892	-0.059	0.974	0.815

* , one outlier removed;

** DL PFC, dorsal lateral prefrontal cortex