Impaired periamygdaloid-cortex prodynorphin is characteristic of opiate addiction and depression

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Negative affect is critical for conferring vulnerability to opiate addiction as reflected by the high comorbidity of opiate abuse with major depressive disorder (MDD). Rodent models implicate amygdala prodynorphin (*Pdyn*) as a mediator of negative affect; however, evidence of *PDYN* involvement in human negative affect is limited. Here, we found reduced *PDYN* mRNA expression in the postmortem human amygdala nucleus of the periamygdaloid cortex (PAC) in both heroin abusers and MDD subjects. Similar to humans, rats that chronically self-administered heroin had reduced *Pdyn* mRNA expression in the PAC at a time point associated with a negative affective state. Using the in vivo functional imaging technology DREAMM (DREADD-assisted metabolic mapping, where DREADD indicates designer receptors exclusively activated by designer drugs), we found that selective inhibition of *Pdyn*-expressing neurons in the rat PAC increased metabolic activity in the extended amygdala, which is a key substrate of the extrahypothalamic brain stress system. In parallel, PAC-specific *Pdyn* inhibition provoked negative affect–related physiological and behavioral changes. Altogether, our translational study supports a functional role for impaired *Pdyn* in the PAC in opiate abuse through activation of the stress and negative affect neurocircuitry implicated in addiction vulnerability.

Introduction

Chronic negative emotional states are a compelling motivation for relapse to most drugs of abuse, especially opiates (1). The cluster of symptoms that make up these negative emotional states include loss of motivation for natural reward, dysphoria, anhedonia, and anxiety (1, 2). Persistent negative affect is also a cardinal feature of major depressive disorder (MDD), which is the most common comorbid psychiatric diagnosis with opiate addiction (3). The high comorbidity rate of these disorders has been hypothesized to be due to overlapping neurobiological abnormalities in neural circuits regulating emotion (4, 5).

In accordance with this hypothesis, human imaging studies of drug addiction and MDD patients converge on structural and functional disturbances in limbic neurocircuitry such as the amygdala (6–10). The amygdaloid complex comprises several heterogeneous nuclei that mediate emotional responses to fear, dysphoria, and activation of the brain's stress system (11–13). Accordingly, neurotransmitters within the amygdala may underlie the neuropathology of MDD and addiction disorders and are target candidates for understanding the neurobiological basis of negative affect to opiate addiction.

Across species, the amygdala displays a dense expression of the endogenous opioid neuropeptide precursor prodynorphin (*PDYN*) (14), highly implicated in negative mood states (13, 15, 16). Preclinical animal studies support that *PDYN* disturbances are characteristic of negative affect states in drug self-administration, stress exposure, and depression-like behaviors (17–20). Specifically within the amygdala, *PDYN* expression is altered by acute and chronic administration of heroin (18–20). Although such animal studies point to amygdala *PDYN* as a relevant factor in negative affect– and opiate disorder–related phenotypes, very few studies have investigated *PDYN* mRNA expression in the human amygdala related to neuropsychiatric disorders. Therefore, our goal was to investigate whether amygdala *PDYN* expression was abnormal in opiate abuse subjects and was a shared neuropathological feature of MDD. The current results highlighted a common *PDYN* impairment within a virtually unexplored sub-region of the amygdala, the periamygdaloid cortex (PAC) nucleus. This unusual finding prompted us to utilize a rat behavioral model to interrogate the in vivo functional connectivity of PAC-*PDYN* neurons using a novel molecular imaging technique.

Results

Heroin abusers and MDD subjects share a reduction of PDYN in the PAC. We assessed molecular alterations of amygdala *PDYN* gene expression in human opiate abusers. *PDYN* mRNA expression was evaluated using in situ hybridization histochemistry (ISHH) on amygdala from 2 postmortem heroin abuse cohorts: (a) an ethnically mixed cohort from the US (ref. 21) and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI70395DS1); and (b) a white Hungarian population (ref. 21 and Supplemental Table 2). Congruent with previous

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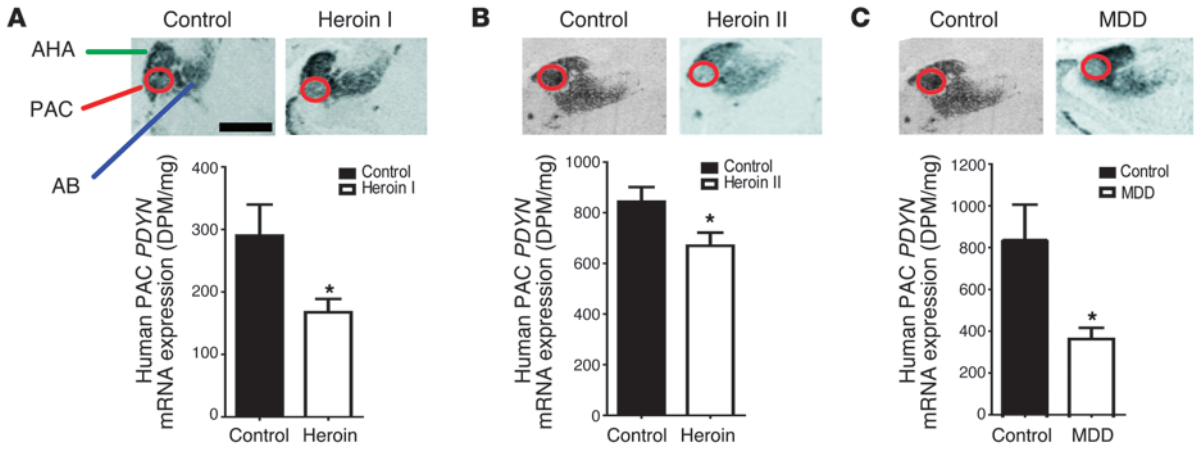

Figure 1

PDYN is reduced in the PAC of postmortem chronic heroin subjects and MDD subjects. (**A**–**C**) Representative film autoradiograms of *PDYN* mRNA expression on coronal cryosections of the amygdala and corresponding bar graphs of *PDYN* mRNA expression levels in the PAC. Values are expressed in DPM/mg (mean ± SEM) and the PAC is encircled in red. Scale bar: 5 mm (**A**) Cohort I of heroin abusers and matched controls. (**B**) Cohort II of heroin abusers and matched controls. (**C**) MDD subjects and matched controls. **P* < 0.05.

studies (21, 22), *PDYN* in the human amygdala had the greatest expression within the nuclei of the PAC, amygdalohippocampal area (AHA), and accessory basal nucleus (AB) (Figure 1).

A selective reduction of *PDYN* mRNA expression was observed in the PAC of the cohort I heroin population (Figure 1A; $n_{Control}$ = 7, n_{Heroin} = 8; P = 0.045). No other amygdala nuclei assessed showed any difference (Supplemental Figure 1). Similarly a second population of heroin abusers in cohort II displayed a specific reduction of *PDYN* mRNA expression in the PAC (Figure 1B; $n_{Control}$ = 18, n_{Heroin} = 28; P = 0.037; Supplemental Figure 2). Further, no significant correlation was found between heroin metabolites and *PDYN*-PAC expression in either heroin cohort, suggesting that the *PDYN* changes were likely due to chronic heroin abuse.

Next, we examined whether amygdala *PDYN* disturbances were evident in an MDD population of the same ethnic makeup as heroin cohort II (Supplemental Table 3). Similar to those in both heroin cohorts, MDD subjects had a significant downregulation of PAC-*PDYN* mRNA expression (Figure 1C; $n_{Control}$ = 10, n_{MDD} = 14; P = 0.030). Distinct from the heroin populations, MDD subjects also exhibited decreased *PDYN* expression in the other amygdala nuclei of the AB and AHA (Supplemental Figure 3).

Reduced Pdyn mRNA in the PAC and concomitant sensitization of neural stress marker induced in rats with chronic heroin self-administration. The shared alterations of *PDYN* mRNA levels in heroin abusers and MDD subjects brought attention to the PAC. However, information about this subnucleus was limited, thus, warranting studies in animal models to help address the limitations of human postmortem studies such as incomplete knowledge of drug use history and time of death following drug use. To this end, we used the well-validated model of heroin self-administration in rats (23). First, we found that there was detectable *Pdyn* mRNA expression in the analogous structure of the PAC in rats (Figure 2A). Next we used the operant paradigm of heroin self-administration in which animals robustly self-administered heroin (30 μg/kg/infusion on a designated "active" lever), but not saline (Figure 2B; n = 6–7 per group ANOVA, P < 0.001). After 10 days of chronic heroin self-administration, animals were euthanized 24 hours after last drug use, a time point characterized by negative affective state and stress activation associated with heroin withdrawal and drug seeking (24). Figure 2C shows that *Pdyn* mRNA expression was reduced in the PAC at 24 hours after the last heroin session (P = 0.031, n = 5–6).

In order to verify that animals were in a heightened stress state 24 hours after last heroin intake, we examined the expression of the brain stress marker corticotropin-releasing factor (*Crf*) within the central amygdala (CeA), an extrahypothalamic brain stress site. Increased mRNA expression of CeA *Crf*, has been correlated with the aversive motivational properties of opiate withdrawal (25) and was indeed apparent at the 24-hour withdrawal period (P = 0.045; Figure 2D; n = 5–7).

DREAMM in vivo behavioral imaging reveals a direct link of inhibition of PAC-Pdyn neurons with hyperactivation of the extended amygdala. Due to the unknown functional connectivity of PAC-*Pdyn* neurons in relation to the complex heterogeneous organization of the amygdala, we used a novel strategy to identify in vivo functional brain circuits associated with modulation of PAC-*Pdyn* neuronal activity (Supplemental Methods). This technique, entitled DREAMM, integrates viral-mediated designer receptors exclusively activated by designer drugs (DREADD) (26) and metabolic mapping using a direct marker of brain metabolism, [^{18}F]Fluorodeoxyglucose (FDG) and μPET imaging. (27).

Rats were unilaterally infused in the right PAC with a neuronal *Pdyn* promoter-specific DREADD viral construct that encodes expression of a G_i-coupled receptor (hM4D$_i$) solely activated by the otherwise inert pharmacological substance, clozapine-n-oxide (CNO) (Supplemental Figure 4) (26, 28). CNO binding to the hM4D$_i$-*Pdyn* DREADD receptor activates G_i-mediated signaling, leading to a transient inhibition of *Pdyn* neuronal firing (26, 28). FDG uptake occurred in behaving animals, and rats were scanned in 2 states: following vehicle or CNO. As compared with the vehicle state, CNO administration and subsequent reduction of PAC-*Pdyn* neuronal activity led to a strong increase (P < 0.05) of FDG uptake in the ipsilateral (right) macrostructure known as the extended amygdala (ExA)

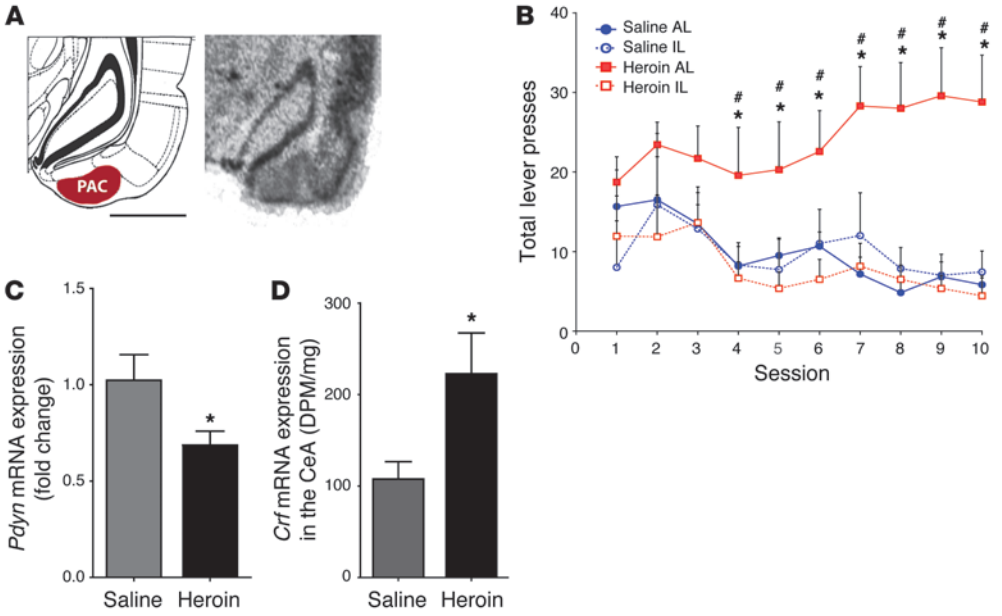

Figure 2

Reduced *Pdyn* mRNA in the PAC and concomitant sensitization of a neural stress marker induced in rats with chronic heroin self-administration. (**A**) Rodent PAC schematic and in situ hybridization image of *Pdyn* in the PAC. Scale bar: 2 mm. (**B**) Lever-pressing behavior (active versus inactive lever presses) of rats receiving heroin or saline euthanized 24 hours after last self-administration session. Data shown as mean ± SEM. $^{*}P < 0.05$ between active lever (AL) and inactive lever (IL) presses of animals receiving heroin. $^{\#}P < 0.05$ in active lever presses between heroin and saline animals. (**C**) Reduction of PAC-*Pdyn* mRNA expression in rats 24 hours after final heroin self-administration session versus saline animals. Data represent mean ± SEM, fold change difference. $^{*}P < 0.05$. (**D**) Increased *Crf* mRNA expression in the CeA 24 hours following heroin self-administration. Values are expressed in DPM/mg (mean ± SEM). $^{*}P < 0.05$.

(Figure 3A; Supplemental Figure 5). The ExA is a functional circuit associated with negative affect and stress that consists of the medial amygdala (MeA), CeA, bed nucleus stria terminalis (BNST), and nucleus accumbens (NAc) shell (Figure 3B; Supplemental Figure 5) (29).

To validate the specificity of this global ExA activation, we quantified individual responses based on voxel intensity in the discrete substructures of the ExA, including the BNST, CeA, MeA, and NAc shell (Figure 4A). Administration of CNO versus vehicle significantly increased voxel intensity within the majority of the right ExA components with a trend in the CeA, ipsilateral to the site of injection (BNST, $P = 0.002$; right NAc shell, $P = 0.031$; MeA, $P = 0.008$; CeA, $P = 0.1$; Figure 4B). The percentage increases (3%–10%) in voxel intensity of FDG uptake are within the range associated with physiological and clinical changes in humans (30–32) and with lesions of the susbtantia nigra in rodents (33).

Inhibitory modulation of PAC-Pdyn neurons induces depression-related behavioral and physiological phenotypes. The ExA as a circuit and its individual components are strongly implicated in aversive state and serves as an interface between stress and addiction (1). As such, we next evaluated behavioral and physiological parameters of stress in PAC-hM4D$_i$-*Pdyn* animals. First, we found that CNO administration in PAC-hM4D$_i$-*Pdyn* rats led to significantly elevated corticosterone levels as compared with vehicle administration in the same animal ($P = 0.014$; Figure 5A) and this effect was not attributable to the order of CNO administration, as the animals were counterbalanced ($P = 0.93$). No effect of CNO was observed in control PAC-GFP-*Pdyn* animals (Supplemental Figure 6). Hedonic state was examined using the sucrose preference task in animals expressing either PAC-hM4D$_i$-*Pdyn* or PAC-GFP-*Pdyn* (control). CNO administration led to a decrease in sucrose consumption in PAC-hM4D$_i$-*Pdyn* animals as compared with control GFP-*Pdyn* ($P = 0.032$; Figure 5B). Last, depressive-like phenotype was assessed using the forced swim test, a commonly used depression model of behavioral despair (34). Rats expressing either PAC-hM4D$_i$-*Pdyn* or PAC-GFP-*Pdyn* were exposed to a 15-minute swim pretest 24 hours before the test. On test day, animals were evaluated for 5 minutes and time spent immobile was scored. PAC-hM4D$_i$-*Pdyn* animals displayed significantly higher immobility as compared with control rats, which was indicative of increased depression-like behavior ($n = 7$/group, $P = 0.003$; Figure 5C). This impairment was not due to a gross motor deficit, as there was no significant difference between hM4D$_i$-*Pdyn* and PAC-GFP-*Pdyn* animals in overall locomotor behavior assessed in an open field (Supplemental Figure 7).

Discussion

Using a translational approach, we have shown that impaired *PDYN* mRNA expression in the human PAC is a shared characteristic of the diagnostically distinct but symptomatically related disorders of opiate addiction and MDD. The consistent disturbance of PAC-*PDYN* expression is intriguing but raises questions as to the potential functional relevance of this impairment, since the PAC, unlike other amygdala nuclei, has not been extensively studied. As such, we expanded insights about the PAC-*Pdyn* neurons by examining their in vivo functional connectivity. Our use of the molecular in vivo imaging strategy DREAMM in rats highlighted the selective recruitment of the ExA in relation to impaired PAC-

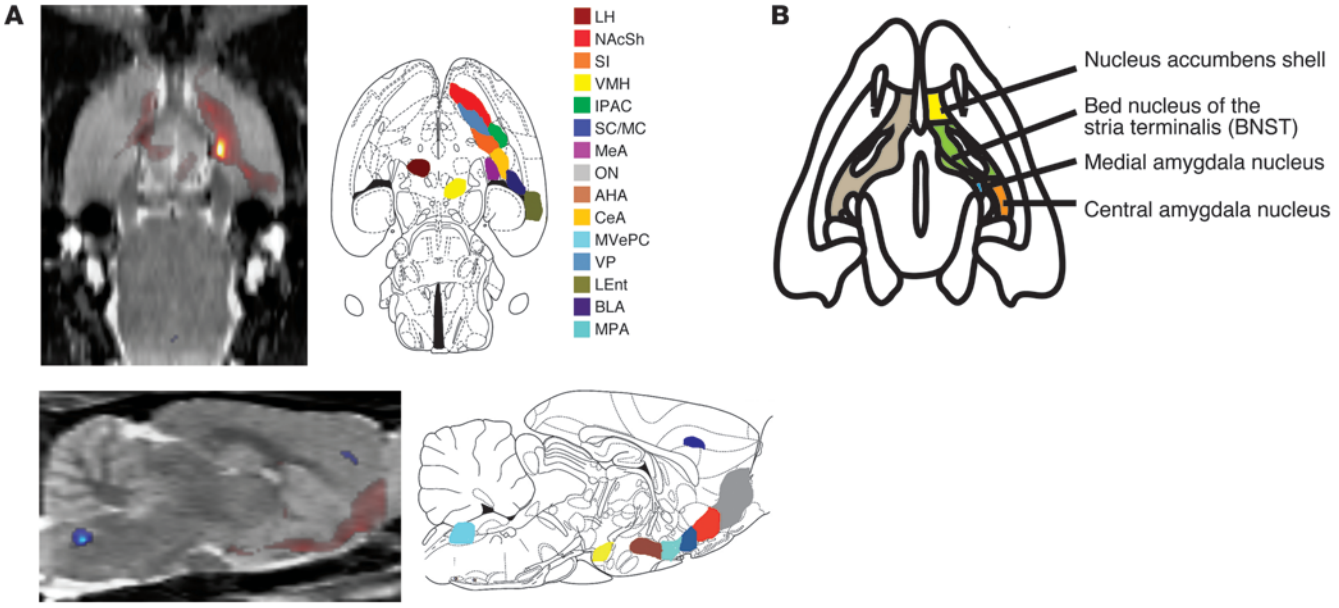

Figure 3
DREAMM imaging reveals that neuronal inhibition of *Pdyn* neurons in the PAC increases metabolic activity in the ExA. (**A**) Axial and right sagittal views showing that activation of G_i-mediated signaling in *Pdyn* neurons of the PAC with CNO leads to a profound induction of neuronal activity primarily in the right ExA as compared with vehicle administration in the same animal (red, relative increase; blue, relative decrease). LH, lateral hypothalamus; NAcSh, NAc shell; SI, substantia inominata; VMH, ventral medial hypothalamus; IPAC, interstitial nucleus of posterior limb of anterior commissure; SC/MC, sensory cortex/motor cortex; ON, olfactory nuclei; CeA, central amygdala; MVePC,: medival vestibular nucleus, parvicellular part; VP, ventral pallidum; LEnt, lateral entorhinal cortex; BLA, basolateral amygdala; MPA, medial preoptic area. (**B**) Schematic diagram of the ExA circuit and its substructures.

Pdyn neuronal activity, therefore linking the Pdyn in the PAC to a functional neural network that regulates negative affect responses relevant to opiate addiction vulnerability.

Our finding of an association between the *Pdyn* system and negative affect is in line with a large body of evidence. While much of the published data have been obtained from experimental animal studies (15, 16, 35), recent clinical investigations have also demonstrated that variants of the *PDYN* gene relate to transcriptional alterations of *PDYN* mRNA expression and are associated with depressive traits and negative craving in alcoholics (36, 37). The current study adds to the field by directly examining the human brain to provide neuropathophysiological insights that have not been previously reported. The shared reduction of *PDYN* mRNA expression in the amygdala PAC of human heroin and MDD subjects supports the hypothesis that common disturbances in brain areas linked to emotional regulation underlie the high comorbidity rates of drug addiction with MDD. Although human postmortem studies have many challenges, the *PDYN* mRNA impairments observed were replicated in multiple cohorts of opiate abusers and disturbances detected in the MDD subjects also matched those previously reported (38). In addition, unlike the PAC, where PDYN expression was similarly reduced in addiction and MDD subjects, *PDYN* mRNA is differentially affected in the striatum of presumably depressed suicide subjects (39) and heroin abusers (40).

The prevailing hypothesis in the field posits that increased dynorphin mediates negative affect. While the reduction of *PDYN* mRNA expression observed in the current study might seem contrary, there may be compensatory upregulation of peptide levels. Indeed, it has been demonstrated in the striatum that reduced *PDYN* mRNA expression can occur concomitant to elevated dynorphin levels in some heroin abusers (40). Future insights about the release of *PDYN*-derived peptides from PAC-*PDYN* neurons will be very informative. It is also important to emphasize that the relationship between PDYN and different aspects of negative affect is complex and still requires further investigation. For example, although stress-induced aversion is established to be mediated by elevated dynorphin (41, 42), a number of investigations have documented that animals lacking the *Pdyn* gene have enhanced anxiety behavior (43, 44). Moreover, our study underscores the heterogeneity of PDYN expression even within discrete brain regions, suggesting different PDYN circuits could contribute to distinct behavioral components relevant to negative affect.

A notable finding in the present study was that MDD subjects, in addition to the PAC, also had reduced *PDYN* mRNA expression in the AB and AHA nuclei. These disturbances were not, however, evident in the lateral nucleus (Supplemental Figure 3), which also expresses PDYN, suggesting that the alterations in MDD individuals are not global but instead discretely localized. Further studies are needed to delineate the specific contribution of PDYN AB and AHA neurons to distinct components of MDD neuropathophysiology.

Direct molecular studies of the human brain are of marked importance, but some limitations of human postmortem investigations are the lack of complete knowledge about the subjects' drug abuse and other confounding factors, including nicotine consumption, medical history, and time of last drug use prior to death, which are relevant for interpretation of the data. However, interestingly, a reduction of PAC-*Pdyn* mRNA expression was also found in rats that self-administered heroin at a time point associated with acute withdrawal related

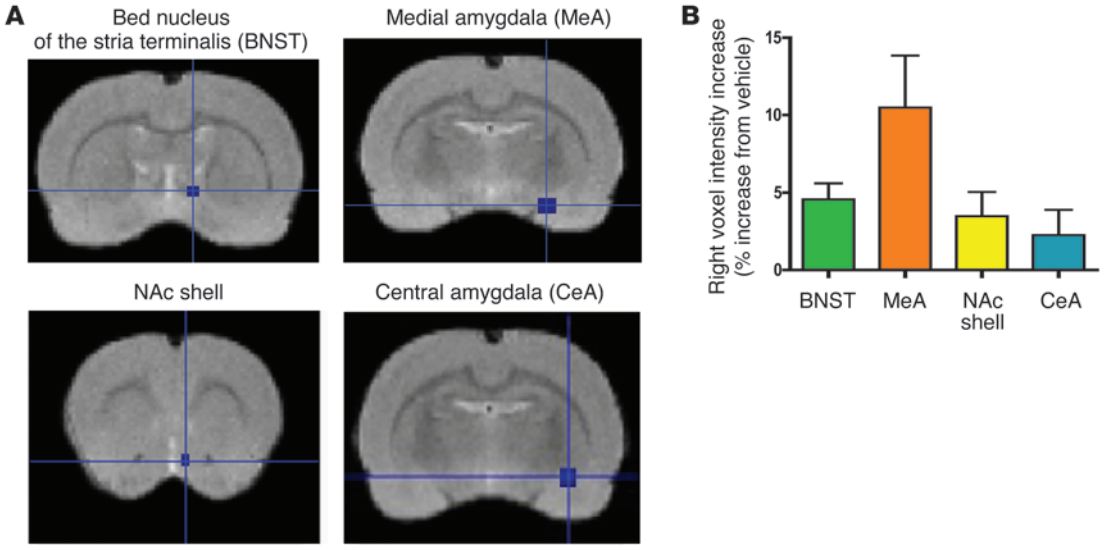

Figure 4
Voxel analysis of ExA substructures confirms increase of metabolic activity following PAC-*Pdyn* inhibition. (**A**) Representative coronal images of ExA substructures analyzed for voxel analysis (**B**) Quantitative bar graphs of 3D voxel intensities in the right ExA substructures. Evaluation of each substructure demonstrated that CNO administration enhanced voxel intensity selectively in the right substructures as compared with vehicle administration. BNST, $P = 0.002$; NAc shell, $P = 0.031$; MeA, $P = 0.008$; CeA, $P = 0.1$, paired *t* test.

to hyperactivation of brain stress systems (upregulation of *Crf* expression in the CeA). Indeed, extracellular and mRNA *Crf* changes in the CeA are consistently associated with acute withdrawal from opiates (25, 35, 45). Our human data also underscore the importance of the PAC-*PDYN* alterations related to chronic neuroadaptations, given that acute toxicology of heroin metabolites did not correlate with amygdala *PDYN* expression. Altogether, these observations are in line with PAC-*PDYN* impairments underlying negative affect that may contribute to the motivational drive for drug-seeking and relapse behavior and that have been associated with a decrease in the function of neuronal reward circuits that contributes to addiction, which Koob et al. have characterized as the "dark side" of addiction (46).

Our use of the innovative molecular and in vivo imaging strategy of DREAMM in rats also clearly highlighted the striking selective association of PAC-*Pdyn* neurons with the ExA that serves as an interface between drug abuse and negative affect (13, 47). Moreover, the fact that impaired firing of *Pdyn* neurons directly enhanced corticosterone levels and also induced behavioral anhedonia and despair provides strong evidence for a direct causal relationship between PAC-*Pdyn* neuronal function and negative affect. Recent imaging studies in humans and primates have expanded the concept of the ExA in higher order species and support a role for its dysfunction in responses to antidepressants (48, 49). As such, the activation of the ExA due to inhibition of *Pdyn* neurons in the PAC of behaving animals suggests that chronic PAC-*Pdyn* dysregulation may be relevant to relapse that is driven by enhanced negative affect/stress/anxiety that maintains the insidious cycle of drug abuse. Further studies are needed to determine whether PAC-*Pdyn* neurons are a feasible target for addiction-related behaviors, particularly during the withdrawal phase.

It is important to note that species differences exist in the expression pattern of *Pdyn* in the amygdala of humans and rodents. Whereas humans have pronounced *Pdyn* expression in cortical amygdala nuclei such as the PAC and in the AB (22), rodents have the strongest expression in the CeA, basolateral, and medial nuclei (50). Despite the species difference, the PAC circuitry variations examined in several primate and nonprimate models have been shown to be complementary (51, 52). As such, the similar changes in PAC-*Pdyn* observed in the rodent heroin self-administration model and in human heroin abusers would be predicted to affect similar types of neural circuitry.

Overall, the common disturbance of *PDYN* in the PAC in opiate addiction and MDD offers new pathophysiological insights into the amygdala relevant to stress and negative affect endophenotypes common in opiate addiction. Moreover, the in vivo imaging technique of DREAMM to manipulate neurochemically distinct cells in discrete brain areas with high molecular specificity in awake freely moving animals will be a valuable neurobiological tool in helping to enhance knowledge about discrete neuronal circuits relevant to neuropsychiatric disorders.

Methods

Human postmortem subjects

Cohort I heroin abuse population. The postmortem human brain specimens used for this population were previously described (21). Briefly, specimens from heroin users and nonabuse control subjects were collected within 24 hours after death. The brains were immediately frozen using dry-ice–cooled isopentane, subsequently cryosectioned, and thaw-mounted onto poly-L-lysine–treated slides. Cause and manner of death were determined after medico-legal examination by the medical examiner. Demographics of this cohort are described in Supplemental Table 1. Inclusion criteria were death associated with heroin intoxication, as verified by toxicology, physical signs of heroin use such as needle track marks, and a history of heroin abuse. Exclusion criteria for all subjects were postmortem interval (PMI) of greater than 24 hours, age less than 50 years, HIV-positive status, and history of alcoholism. Positive alcohol was evident in some of the controls and heroin subjects; however, ethanol concentrations in both groups were not significantly different.

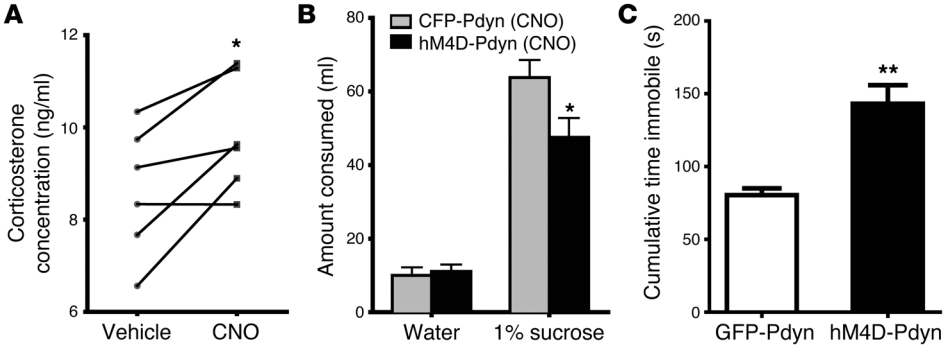

Figure 5

Inhibitory modulation of PAC-*Pdyn* neurons induces depression-related behavioral and physiological phenotypes. (**A**) There is a significant increase in corticosterone levels in PAC-*Pdyn*-hM4Di rats administered CNO as compared with vehicle administration in the same animal. Values represent ng/ml, $^{*}P < 0.05$. (**B**) PAC-*Pdyn*-hM4Di animals consumed significantly less sucrose than PAC-*Pdyn*-GFP animals ($P = 0.032$, ANOVA) although they did not differ in water consumption. Data are shown in ml, $^{*}P < 0.05$ (**C**) PAC-*Pdyn*-hM4Di rats show increased immobility compared with *Pdyn*-GFP animals in the forced swim test. Data represent cumulative time in seconds, $^{**}P < 0.01$.

Cohort II heroin abuse and MDD populations. Postmortem human brain specimens from white Hungarian subjects were collected for 3 study populations: (a) heroin abusers, (b) those who committed suicide by hanging with a diagnosis of MDD, and (c) healthy control subjects. All specimens were without head trauma and retrieved at autopsy within 24 hours after death. The brains were collected and stored similarly to those from cohort I. The demographics and general characteristics for cohort II are described in Supplemental Tables 2 and 3. All cases were assessed for common drugs of abuse (including alcohol and marijuana) and also for therapeutic agents. Cigarette toxicology was not assessed, but cigarette use was common for subjects in all groups.

Subjects in the MDD group had a verified psychiatric history and diagnosis of depression and had no documented history or positive toxicology for illicit drug abuse. The same control group was used for both heroin and MDD cohort II subjects (but studied in separate experiments), since the brains of these individual cadavers were collected during the same time, were ethnogeographically similar, and adhered to the following criteria: negative toxicology of illicit drugs and antidepressants; no history of illicit drug abuse or mood and anxiety disorders. Positive alcohol was evident in very few controls in which ethanol concentrations were similar to those of the limited alcohol-positive subjects identified in the heroin group (none of the MDD subjects were alcohol positive). Exclusion criteria for all subjects were as follows: PMI greater than 24 hours, age greater than 50 years, HIV-positive status, and history of alcoholism.

ISHH

Briefly, as previously described, riboprobes complementary to the human *PDYN* gene were synthesized from a 1.2-kb human cDNA from the main exon of the *PDYN* gene (38). The *Crf* riboprobe used for the rat tissue was a 760-bp cDNA fragment of the gene (1196 bases; GenBank 81648). ISHH was performed on 20-μM coronal cryosections of human or rodent amygdala specimens as previously described (38). The high quality of the postmortem human brain samples suitable for mRNA expression studies is evident in our ability to routinely detect mRNAs of various genes in these samples (21, 40, 53, 54).

Image analysis

Optical density values were measured using Scion Image (NIH) from digitalized images or FujiFilm Multigauge V3.0 from phosphofilm exposed slides. Measurements were converted to disintegrations per minute (DPM)/mg by reference to coexposed C14 standards. Measurements were taken within discrete amygdala subnuclei at similar levels according to previously published data on the human brain (38). Background noise was also measured and subtracted from the measurements taken. DPM/mg values from duplicate slides were averaged.

Animal studies

Adult (PND 55-69) male Long-Evans rats were obtained from Charles River Laboratories and housed on a reversed 12-hour dark/12-hour light cycle with food and water available ad libitum except as noted below.

Heroin self-administration

Rats were anesthetized with isoflurane (2.5%–4.0% in O_2) and catheters (Brian Fromant) implanted into the right jugular vein. Following 1 week recovery, the heroin self-administration paradigm was initiated as previously described (55). Briefly, self-administration experiments were conducted during the dark phase of the light/dark cycle in standard operant chambers housed in sound-attenuating boxes (MED Associates Inc.) with 2 retractable levers. Depression of the drug-paired lever (defined as the active lever) resulted in an intravenous heroin (NIDA Drug Supply) injection, whereas depression of the inactive lever had no programmed consequence. Rats were allowed to self-administer heroin (30 μg/kg/injection) under a fixed-ratio-1 (FR1) reinforcement schedule in daily 3-hour sessions for 10 days. Brains were collected at 24 hours following the final drug self-administration session.

Quantitative PCR gene expression

Fresh-frozen bilateral 12-gauge punches were taken from the rat posterior medial lateral cortical (PM/LCo) nuclei equivalent of the PAC; total RNA was isolated using the 5 Prime PerfectPure RNA Tissue Kit and reverse transcribed to cDNA (qScript Kit; VWR). Quantitative real-time PCR (qPCR) analysis was performed using TaqMan-based *Pdyn* primers and probes (*Pdyn* Rn00571351_m1; Applied Biosystems). Reactions were run in triplicate, and eukaryotic 18S rRNA (Applied Biosystems) multiplexed as an endogenous control. qPCR and subsequent analysis were performed with a Roche Light Cycler 480 sequence detection system. Quantification of *Pdyn* gene expression was normalized to eukaryotic 18S rRNA and analyzed using the ΔΔCT method (56).

Viral-mediated DREADD expression

All rats were stereotaxically injected with 2 μl (0.2 μl/min) of purified herpes simplex virus (HSV) vectors expressing a triple hemagglutinin (HA) epitope-tagged hM4Di gene (1567 Kb) under the control of the

Pdyn promoter (PAC-hM4D$_i$-*Pdyn*) or a control vector that expresses GFP, also under the control of the *Pdyn* promoter (PAC-GFP-*Pdyn*) (28). The vectors were injected unilaterally (right hemisphere) into the PAC (PM\LCo) using the following coordinates (from bregma): AP, –4.3; ML, 3.7; DV, 9.6 mm.

DREAMM

The DREAMM technique was conducted as described in Supplemental Methods. Rats received an i.p. injection of either vehicle-saline or the DREADD-activating ligand CNO (7–10 days post vector injection) (1 mg/kg). Immediately after vehicle or CNO injection, rats were injected i.p. with FDG (~0.6 mCi). Order of CNO administration was counterbalanced in animals due to the within subjects design of the experiment. Thirty minutes later, rats were anesthetized with 1.5% isoflurane, placed in a prone position on the bed of an R4 microPET rodent scanner (Siemens Medical Solutions), and scanned for 20 minutes using a static acquisition protocol. Analysis of the scans, reconstruction of the images, and statistical parametric mapping (SPM) were conducted as described in Supplemental Methods. Briefly, anatomical regions were based on Paxinos coordinates, and sites of voxel analysis were validated in conjunction with a rat brain imaging atlas intrinsic to the analytical software.

Corticosterone assessment

Whole blood was obtained from a tail-vein catheter under isoflurane anesthesia in hM4D$_i$-*Pdyn* rats (given either 1 mg/kg of CNO or vehicle i.p. thirty minutes prior to anesthesia). After centrifugation, the remaining supernatant (plasma) was stored at –80°C. Levels of corticosterone were measured from the blood plasma using the IDS Corticosterone HS EIA kit (Immunodiagnostics Systems) according to the manufacturer's instructions.

Sucrose preference test

To measure anhedonic traits, the sucrose preference test was carried out in hM4D$_i$-*Pdyn* or GFP-*Pdyn* rats. Rats were given the choice of drinking from 2 bottles, one of which contained a 1% sucrose solution and the other of standard water. Baseline sucrose preference of each animal was determined over a 24-hour period prior to the test. Subsequently, animals were food and water restricted for 15 hours prior to the test session. One hour prior to the test session, all animals (hM4D$_i$-*Pdyn* or GFP-*Pdyn*) received 1 mg/kg i.p. of CNO. Choice preference was observed over 24 hours.

Forced swim test

The procedure used was based on that initially described by Porsolt and modified as described (34). Behavioral studies were carried out from 11:00 to 17:00. Rats were placed individually in a Plexiglass cylinder 46 cm in height with a 21-cm internal diameter, which was filled with water (23°C–24°C) to a depth sufficient to keep adult rats from supporting themselves by placing their paws or tails on the base of the cylinder. Water was changed between swim sessions. There were 2 swim sessions: a pretest 15-minute session and, 24 hours after, a 5-minute swim test. The cumulative time spent in immobile posturing (minimal effort to keep head above water) during the 5-minute test was recorded.

Open field/locomotor activity

Prior to testing, rats were acclimated to the test room for 1 hour. General locomotor activity was examined in a MED Associates activity arena (16 in. × 16 in.) outfitted with a grid of 32 infrared beams. All movements were automatically tracked by beam breaks in 1-hour sessions run in dim lighting for each subject. Between animals, all cages were thoroughly wiped clean with ethanol followed by vinegar.

Statistics

For ISHH, parametric univariate analysis for normal distribution was used to determine group differences in DPM/mg levels. Independent variables (age, PMI, sex, toxicology [prescription and illicit], and brain pH] were included in the general linear model if results from univariate analysis (ANOVA) showed a P value of less than 0.05. For unequal variances (as determined by the 2-sided F test), Welch's test was used to determine P value. We used 2-tailed, unpaired Student's t tests (for comparison of 2 groups), 1-way ANOVAs followed by Tukey's honest significant difference test or 2-tailed Student's t tests when appropriate (for 3 groups), and 1-way repeated-measures ANOVAs followed by 1-way ANOVAs (to examine significant repeated-measure effects). All values represent mean ± SEM. $P > 0.05$ and statistical calculations were performed using JMP software (SAS) or GraphPad Prism 5.0.

Study approval

Postmortem human brains for cohort I were obtained as part of the routine autopsy process under protocols approved by Wayne State University's Human Investigation Committee. Postmortem human brains in cohort II were obtained through the Department of Forensic Medicine at Semmelweis University and from the National Institute of Forensic Medicine (Karolinska Institutet). All of the specimens from cohort II were collected under guidelines approved by the Semmelweis University Research Ethical Committee and the Karolinska Institute Research Ethical Committee. All animal experiments were approved by the institutional animal care and use committees at the Icahn School of Medicine at Mount Sinai and Brookhaven National Laboratory and were conducted in accordance with NIH guidelines.

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