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Cocaine reduces cytochrome oxidase activity in the prefrontal cortex and modifies its functional connectivity with brainstem nuclei

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Abstract

Cocaine-induced psychomotor stimulation may be mediated by metabolic hypofrontality and modification of brain functional connectivity. Functional connectivity refers to the pattern of relationships among brain regions, and one way to evaluate this pattern is using interactivity correlations of the metabolic marker cytochrome oxidase among different regions. This is the first study of how repeated cocaine modifies: (1) mean cytochrome oxidase activity in neural areas using quantitative enzyme histochemistry, and (2) functional connectivity among brain regions using inter-correlations of cytochrome oxidase activity. Rats were injected with 15 mg/kg i.p. cocaine or saline for 5 days, which lead to cocaine-enhanced total locomotion. Mean cytochrome oxidase activity was significantly decreased in cocaine-treated animals in the superficial dorsal and lateral frontal cortical association areas Fr2 and Fr3 when compared to saline-treated animals. Functional connectivity showed that the cytochrome oxidase activity of the noradrenergic locus coeruleus and the infralimbic cortex were positively inter-correlated in cocaine but not in control rats. Positive cytochrome oxidase activity inter-correlations were also observed between the dopaminergic substantia nigra compacta and Fr2 and Fr3 areas and the lateral orbital cortex in cocaine-treated animals. In contrast, cytochrome oxidase activity in the interpeduncular nucleus was negatively correlated with that of Fr2, anterior insular cortex, and lateral orbital cortex in saline but not in cocaine groups. After repeated cocaine specific prefrontal areas became hypometabolic and their functional connectivity changed in networks involving noradrenergic and dopaminergic brainstem nuclei. We suggest that this pattern of hypofrontality and altered functional connectivity may contribute to cocaine-induced psychomotor stimulation.

Keywords

Functional connectivity; Cytochrome oxidase; Cocaine; Prefrontal networks; Hypofrontality

1. Introduction

Cocaine is a powerful psychomotor stimulant and its abuse and subsequent addiction are persistent public health problems. Human studies have shown a hypofrontality produced by chronic use of cocaine (Volkow et al., 1988; London et al., 1990; Matochik et al., 2003;

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Bolla et al., 2004). In animal models, repeated exposure to cocaine results in a progressive and enduring enhancement in locomotion (Post, 1980; Wise and Bozarth, 1987; Stewart and Badiani, 1993) and changes in various brain circuits, especially lower metabolic activity in frontal cortical areas and ventral striatum (Robinson and Berridge, 1993; Porrino et al., 2007). This study was conducted to investigate whether cocaine-enhanced locomotion may involve systems-level alterations in the interactivity or functional connectivity of specific prefrontal areas. While anatomical connectivity refers to patterns of structural relationships among brain regions, functional connectivity refers to patterns of relationships in metabolic activity among brain regions (McIntosh and Gonzalez-Lima, 1994a,b; Nair et al., 1999). If the enhancement of locomotion by repeated cocaine exposure is an emergent property of affected prefrontal areas interacting with subcortical regions, understanding it requires a network analysis of the patterns of interaction between brain regions.

Network functional connectivity uses covariance analyses that cannot determine directionality but can describe the patterns of interaction between brain regions, as has been evaluated by inter-regional correlation changes in cytochrome oxidase activity (Sakata et al., 2000; Padilla et al., 2011). This particular functional connectivity method using inter-correlations of cytochrome oxidase activity describes stable metabolic relationships among areas, and it also describes how the regions are modified across sustained behavioral paradigms (Puga et al., 2007; Conejo et al., 2010; Fidalgo et al., 2012) or drug treatments (Padilla et al., 2011; Riha et al., 2011). Characterizing which specific neural systems modify their metabolic capacity and functional connectivity as a result of repeated cocaine exposure may advance our understanding of cocaine-enhanced locomotion.

Cytochrome oxidase (also called cytochrome *c* oxidase, ferrocytochrome *c*: O_2 oxidoreductase, EC 1.9.3.1, cytochrome aa3, or the respiratory enzyme) is a ubiquitous mitochondrial membrane integral protein responsible for the last step of the electron transport chain that catalyzes the transfer of electrons to oxygen, which serves to generate ATP via oxidative phosphorylation (Wong-Riley, 1989; Gonzalez-Lima and Garrosa, 1991). Neurons depend mostly on oxidative metabolism as an energy source. For this reason, the enzymatic activity of cytochrome oxidase is used as a metabolic marker for neuronal activity (Wong-Riley, 1989) and cytochrome oxidase enzyme histochemistry serves to map sustained changes in brain energy metabolism (Wong-Riley, 1989; Gonzalez-Lima and Garrosa, 1991; Hevner et al., 1993; Sakata et al., 2005). In particular, we have not seen acute effects on cytochrome oxidase histochemistry one hour after a single drug injection, but the longer-term oxidative capacity for energy metabolism (protein-synthesis-dependent enzyme induction over hours or days) of brain regions can be investigated using quantitative cytochrome oxidase histochemistry (Gonzalez-Lima and Cada, 1994; Padilla et al., 2011; Riha et al., 2011). However, to date, there has not been any cytochrome oxidase study in animals exposed to cocaine. Therefore, we were interested in using cytochrome oxidase to investigate altered relationships between neural areas after 5 days of cocaine exposure, rather than monitoring acute effects of cocaine exposure.

Quantitative enzyme histochemistry of cytochrome oxidase (Gonzalez-Lima and Cada, 1994; Gonzalez-Lima, 1998) has been used successfully in over a hundred previous studies to map alterations in brain oxidative metabolism in numerous learning tasks and drug treatments (Poremba et al., 1997, 1998; Villarreal et al., 2002; Hu et al., 2006; Gonzalez-Pardo et al., 2008; O'Reilly et al., 2009; Conejo et al., 2010; Padilla et al., 2011; Rojas et al., 2012). Analysis of inter-regional correlations of cytochrome oxidase activity (Sakata et al., 2000; Padilla et al., 2011) between cortical and subcortical regions after cocaine administration, especially between the prefrontal cortex and monoaminergic nuclei, may also identify underlying initial brain effects of repeated cocaine.

2. Results

2.1. Cocaine enhanced locomotion from days 1–5

The behavioral protocol showed that rats treated with cocaine (15 mg/kg i.p.) for five days had an increase in total locomotion relative to saline-injected rats, two way ANOVA $F_{(9,110)}$=3.20 (p<0.001). Additionally on day 5, subjects injected with cocaine had a significantly (p<0.05) increased total locomotor activity (5621±533 pcc) when compared to day 1 (2966±552 pcc), two-way ANOVA $F_{(9,110)}$=3.20 (p<0.05). There was no significant change in total locomotor activity between day 1 (655±88) and day 5 (542±82) in saline-treated rats (Fig. 1).

2.2. Prefrontal regions became hypometabolic after repeated cocaine

Mean regional cytochrome oxidase effects of cocaine were focused on the prefrontal cortex. Cytochrome oxidase activity was significantly (p<0.05) decreased in cocaine-treated animals in the superficial layers of dorsal (Fr2) and lateral (Fr3) frontal cortex regions (DFS mean=217±8 and LFS mean=242±9) when compared to saline-treated animals (DFS mean=244±8 and LFS mean=265±6) (Fig. 2). Means and standard errors for all regions measured are reported in Table 1, which showed that the hypometabolic effect of repeated cocaine (15 mg/kg i.p. for 5 days) was specific to prefrontal cortical areas.

2.3. Prefrontal regions increased their functional connectivity with noradrenergic and dopaminergic subcortical nuclei after repeated cocaine

Specific prefrontal-subcortical nuclei inter-regional cytochrome oxidase correlations were significantly different between cocaine- and vehicle-treated animals (absolute value of Z_{abs}>1.96, p<0.05), indicating that cocaine had significant effects on the functional connectivity of these regions, as illustrated in Fig. 3. Inter-regional correlations of cytochrome oxidase activity showed significant cocaine effects focused on prefrontal regions and noradrenergic and dopaminergic nuclei listed in Table 2. No significant cocaine-induced inter-correlations differences were found among other regions. For simplicity, correlations with no significant effects were not listed in Table 2. Three types of significant effects were found:

First, the noradrenergic locus coeruleus (LC) and deep layers of the infralimbic medial frontal cortex (ILD) were positively correlated in animals treated with cocaine (r=0.802) but not in saline-treated animals (r= −0.076, significant group difference p=0.013) (Fig. 4).

Second, significant positive correlations were observed in cocaine subjects between the dopaminergic substantia nigra compacta (SNc) and the superficial layer of the prelimbic medial frontal cortex (MFS, r=0.809), the deep layer of the lateral (Fr3) frontal cortex (LFD, r=0.770) and the superficial layer of the lateral orbital cortex (LOS, r=0.717). These positive correlations were absent in saline-treated animals, revealing significant group differences (MFS, r=0.109 p=0.031, LFD, r= −0.118 p=0.015, and LOS r=−0.157 p=0.025). A positive correlation between the substantia nigra reticulata (SNr) and the medial septum (MS) was present in saline (r=0.602) but not in cocaine (r=−0.296 p=0.033) treated animals.

Third, in cholinergic pathways, interpeduncular nucleus (IP) cytochrome oxidase activity was negatively correlated with the activities of the deep layer of the dorsal (Fr2) frontal cortex (DFD, r=−0.851), the superficial and deep layers of the anterior insular cortex (AIS, r=−0.672 and AID, r=−0.954), the superficial and deep layers of the lateral orbital cortex (LOS, r=−0.697 and LOD, r=0.847) in saline treated animals but not in cocaine treated animals (DFD, r=0.000 p=0.008, AIS, r=0.164 p=0.032, AID, r=−0.173 p=0.0001, LOS, r=0.238 p=0.018, and LOD, r=0.300 p=0.001).

3. Discussion

Here we demonstrated two sets of metabolic changes in cytochrome oxidase activity in the brains of rats after a 5-day cocaine exposure paradigm. The rich datasets obtained from this mapping revealed not only mean cytochrome oxidase activity decreases in prefrontal regions (hypofrontality) but also modified functional connectivity between specific prefrontal regions and subcortical noradrenergic and dopaminergic nuclei. This cytochrome oxidase analysis demonstrated novel effects in subcortical brainstem nuclei that are too small to be reliably measured with noninvasive neuroimaging studies in humans. We speculate that these metabolic alterations produced by repeated cocaine impair prefrontal networks for inhibitory psychomotor control and might contribute to the observed enhanced locomotion.

3.1. Difference between mean activity and functional connectivity

Means provide measures of the average metabolic activity of individual regions (*univariate measure*), whereas functional connectivity provides measures of the interactivity among regions (*covariance measure*) (McIntosh and Gonzalez-Lima, 1994a, 1994b). These two statistical approaches have been embraced by functional neuroimaging studies because they are complementary, as the nervous system can be viewed as a complex network of interacting individual regions (Gonzalez-Lima and McIntosh, 1994). The analysis of regional means of cytochrome oxidase activity focuses on how the baseline metabolic activity of specific brain regions change after repeated cocaine, whereas the analysis of functional connectivity focuses on how baseline interactions among brain regions change after repeated cocaine. Two regions can have similar means across groups, but different functional connectivity across groups (Nair et al., 1999; Sakata et al., 2000). Fig. 4 helps to explain how two brain areas may have similar means across groups but different functional connectivity. For example, the mean cytochrome oxidase activity in the infralimbic deep (ILD) cortical area and the locus coeruleus (LC) nucleus are not different between cocaine and saline groups, but the functional connectivity between cocaine and saline groups is substantially different (LC-ILD inter-regional correlations=0.80 cocaine vs. −0.08 saline). Therefore, in addition to frontal hypometabolism, it is plausible that cocaine-enhanced locomotor behavior may be mediated by different patterns in the interactivity or functional connectivity among particular frontal-subcortical brain regions.

3.2. Cytochrome oxidase vs. other metabolic markers

Elegant studies with the 2-deoxyglucose (2-DG) method in rhesus monkeys have mapped regional decreases in metabolic activity of frontal cortical areas 32 (prelimbic), 25 (infralimbic) and 24 (anterior cingulate) and the ventral striatum after 5 days of cocaine (Porrino et al., 2007). Similar 2-DG studies in rats have shown glucose utilization decreases only in the nucleus accumbens after 5 days of cocaine self-administration, while there were decreases in infralimbic and prelimbic cortical regions after 30 days of cocaine (Macey et al., 2004).

Other studies using Fos immunohistochemistry and c-fos mRNA have been used to measure neuronal activity after repeated cocaine administration. These investigations differ in terms of number of injections given, doses and time of measurement after the last injection, and most importantly the fact that c-fos is a transient activity marker as opposed to cytochrome oxidase histochemistry that is a maker for chronic, sustained metabolic demand over days. Due to the transient effects inherent to immediate early gene expression, different results in c-Fos activity were found depending on whether repeated cocaine administration was given in the home cage or in the locomotor activity chambers. Home cage injections diminished Fos expression and c-Fos mRNA in the nAcc and caudate–putamen (Hope et al., 1992; Steiner and Gerfen, 1993). In contrast, repeated cocaine administration inside the activity

chambers increased Fos expression in the nAcc without changes in the caudate–putamen (Crombag et al., 2002). Experiments using a similar cocaine administration paradigm to ours found a significant decrease in c-fos density in the medial prefrontal cortex and two subdivisions of the nAcc in animals challenged after a 2-days withdrawal period (Todtenkopf et al., 2002). These findings suggest that chronic cocaine administration induces a decrease in pre-frontal activity.

Interestingly, regions related to self-administration reward, such as the nucleus accumbens, showed no changes in mean cytochrome oxidase activity or in functional connectivity in our experimenter-administered cocaine paradigm (15 mg/kg i.p. for 5 days). It is possible that involvement of nucleus accumbens may require a different behavioral protocol such as self-administration to reveal self-stimulation effects of cocaine. But lack of effects on the midbrain ventral tegmental area (VTA) are consistent with the 2-DG mapping studies of cocaine-treated rats by Koch et al. (1997) and Porrino and Kornetsky (1988), although here we provide further alternative explanations (Porrino and Kornetsky, 1988; Koch et al., 1997). For example, after cocaine exposure the VTA changes from tonic to phasic neuronal firing (Porrino and Kornetsky, 1988; Koch et al., 1997; Schultz, 1998). This transition does not necessarily result in a mean change in neuronal metabolic profile. Thus, it is possible that total neuronal activity in the VTA is not changed, only the pattern of firing. Furthermore, we have recently shown that a decrease in VTA dopaminergic neuronal cell size occurs after cocaine sensitization (Arencibia-Albite et al., 2012). This diminution may compensate for the increase in metabolic activity that one may expect with the long-term potentiation known to occur after chronic cocaine treatment (Ungless et al., 2001). Therefore, some changes in neuronal activity patterns in areas like the VTA and the nucleus accumbens may not be detected with cytochrome oxidase histochemistry or with the cocaine protocol we used. These regions may show alterations only if a different methodology for mapping neuronal activity or a different behavioral protocol such as self-administration are used.

The cytochrome oxidase technique, like 2-DG and fluorodeoxyglucose (FDG), offers a functional marker for neuronal energy metabolism (Gonzalez-Lima 1998). Cytochrome oxidase is the final step in the electron transport chain; therefore, its catalytic activity is critical for glucose oxidation and the creation of ATP (Wong-Riley, 1989; Wong-Riley et al., 1998). But cytochrome oxidase mapping was used to assess the cumulative effects of repeated drug manipulations over days (Padilla et al., 2011). While 2-DG/FDG and immediate early genes as c-fos evaluate on-going changes in neuronal activity during the period of tracer uptake or gene expression (usually 45–90 min), cytochrome oxidase measures longer-term alterations in enzyme levels that are induced by days of sustained metabolic changes on the implicated brain region (Gonzalez-Lima, 1998). These markers provide measures of neuronal metabolism, but some (2-DG, FDG, c-fos) measure more the on-going neuronal activation while the other (cytochrome oxidase) gives information on longer-term changes on neuronal oxidative metabolic capacity. The oxidative capacity for energy metabolism, as measured by quantitative cytochrome oxidase histochemistry, reflects the energy demand history of different brain regions after repeated cocaine exposure because cytochrome oxidase levels change by enzymatic induction (a process that requires protein synthesis) in a cumulative way after repeated daily changes in energy demand (Gonzalez-Lima and Cada, 1994). Thus, we found that cytochrome oxidase served to determine cumulative effects on prefrontal neural oxidative metabolic capacity and functional connectivity after a 5-day cocaine treatment, although it should be acknowledged that a more extended cocaine treatment or a different protocol using a withdrawal period could produce different changes.

3.3. Prefrontal cortical involvement

Mean cytochrome oxidase was decreased in cocaine treated animals when compared to saline injected rats in the superficial layers of dorsal (Fr2) and lateral (Fr3) frontal cortex regions (Table 1). Hypometabolic effects circumscribed to the superficial cortical layers of Fr2 and Fr3 imply an alteration of intercortical communication of association fibers between the superficial layers, as opposed to ascending or descending projection fibers that innervate deeper cortical layers. These Fr2 and Fr3 areas of Zilles and Wree shown in the atlas of Paxinos and Watson (1986), correspond to the most anterior secondary and tertiary association areas of the rat frontal cortex that are regarded as prefrontal cortical areas (Paxinos and Watson, 1986; Paxinos, 1995; Shumake and Gonzalez-Lima, 2003). They are not part of the rat primary motor cortex (Fr1) but may be prefrontal cortical areas involved in descending inhibitory control of psychomotor behavior. Therefore, cocaine-induced hypometabolism of Fr2 and Fr3 areas may lead to diminished inhibitory control that may manifest as enhanced locomotion.

Other prefrontal cortical areas in the rat showed cocaine-modified functional connectivity, including the prelimbic medial frontal cortex (area 32), the infralimbic medial frontal cortex (area 25), and the medial and lateral orbital frontal cortex (Fig. 2). These prefrontal regions receive projections from most of the mesocorticolimbic system such as the VTA, substantia nigra, amygdala, lateral hypothalamus, hippocampus, and other areas of the cortex (Groenewegen et al., 1997; Dalley et al., 2004b). They also project back to most of these regions (Groenewegen et al., 1997; Dalley et al., 2004a). But these mesocorticolimbic regions did not show significant changes in mean cytochrome oxidase or functional connectivity differences with the prefrontal areas after our cocaine paradigm. This may be related to the fact that cocaine was not self-administered by the rats and thus may not engage the mesocorticolimbic self-stimulation system.

As discussed below, there were only specific noradrenergic and dopaminergic brainstem nuclei that showed cytochrome oxidase inter-regional correlation differences with prefrontal areas when saline-treated animals are compared to cocaine-treated rats (Fig. 3). Since cocaine not only increases synaptic levels of noradrenaline and dopamine, but also that of serotonin and glutamate, simply increasing transmitter levels is unlikely to explain the specific cocaine-induced modification of functional connectivity observed in this study. We speculate that 5 days of cocaine leads to neuro-metabolic adaptations that are more specific for prefrontal cortical areas and their functional connectivity with noradre-nergic and dopaminergic brainstem nuclei. Future studies would be needed to further evaluate the mechanisms of these changes.

3.4. Noradrenergic pathways

Our studies found that the noradrenergic locus coeruleus (LC) and deep layers of the infralimbic cortex were positively correlated in cocaine-treated animals but not in control rats (Table 2 and Fig. 4). But inter-regional correlations do not provide information on the direction of the influence, so they need to be interpreted based on other known anatomical and functional data (Gonzalez-Lima and McIntosh, 1994). The noradrenergic system contributes to control stress responses, arousal, mood and alters learning and memory (Huether, 1996; Sved et al., 2001) and plays a key role in mediating reward (Poschel and Ninteman, 1963; Stein, 1964, 1975; Wise, 1978). The LC is the principal site for norepinephrine (NE) synthesis in the brain and it projects to extensive areas that may help modulate the observed cocaine-enhanced locomotion (projections reviewed by Foote et al. (1983), Grzanna and Fritschy (1991), Holstege and Bongers (1991), Jones (1991), Westlund et al. (1991) and Berridge and Waterhouse (2003)). It is also possible that a modified pattern of LC functional connectivity might underlie cocaine-induced behaviors not investigated in

this study, such as self-stimulation, and future studies manipulating behavior or brain mechanisms would be important to test this possibility. Regions of the mesolimbic dopamine (DA) system, like the VTA, nucleus accumbens and amygdala receive noradrenergic inputs (Ungerstedt, 1971; Alheid and Heimer, 1988; Liprando et al., 2004; Mejias-Aponte et al., 2009). Also, noradrenergic neurons in the LC receive afferents from the infralimbic prefrontal cortex (Heidbreder and Groenewegen, 2003).

The links between the LC and the infralimbic cortex with the VTA are so strong that activation of alpha-1 receptors in the LC or in the infralimbic cortex by themselves increase VTA DA neuronal activity (Lategan et al., 1990; Blanc et al., 1994). In addition, our lab has shown that glutamate release onto VTA DA neurons is modulated by pre-synaptic alpha-1 receptors (Velasquez-Martinez et al., 2012); and systemic inhibition of alpha-1 and activation of alpha-2 receptors block the development and expression of cocaine sensitization (Jimenez-Rivera et al., 2006). Together with the observed change in functional connectivity between the LC and the infralimbic cortex, these data suggest the hypothesis that NE pathways to the infralimbic cortex play a critical role in the development of cocaine-induced neuroadaptations. Based on this hypothesis, the observed significant pair-wise positive correlation in cytochrome oxidase activity may be interpreted as repeated cocaine modifying the noradrenergic LC influence on the infralimbic cortex. This hypothesis deserves testing in future studies.

3.5. Dopaminergic pathways

There were significant positive correlations in cocaine-treated subjects between the substantia nigra compacta (SNc) and the superficial layer of the prelimbic medial frontal cortex (MFS), the deep layer of the Fr3 lateral frontal cortex (LFD) and the superficial layer of the lateral orbital cortex. These correlations were not present in saline-treated rats (Fig. 3). A positive correlation between the SN reticulata and the medial septum was present in saline and not cocaine treated animals. The SN is a predominantly dopaminergic area most commonly known for its involvement in Parkinson's disease and the extra-pyramidal motor system linked to involuntary movement control. The presence of a positive correlation between the SN and specific prefrontal regions suggests a role of DA in the control of these areas after repeated cocaine exposure. Augmentation of DA release with repeated drug exposure is the basis of theories that suggest drugs of abuse impair adaptive circuitry to become hyper-responsive to drug stimuli (Berridge and Robinson, 1998; Goldstein and Volkow, 2002; Everitt and Robbins, 2005; Kalivas, 2008). In addition, there is a direct interaction from prefrontal cortex to SN (Watabe-Uchida et al., 2012).

The orbital frontal cortex projects to central parts of caudate–putamen and to the lateral part of nucleus accumbens shell (Schilman et al., 2008). This area also receives afferents from regions like the VTA, ventral pallidum and the medial temporal lobe (Krettek and Price, 1977; Groenewegen, 1988; Ray and Price, 1992) and it is thought to be important in drug vulnerability (Schoenbaum and Shaham, 2008). Hyper-activity of the orbitofrontal cortex in humans results in impulsive behavior (Baxter et al., 1987, 1989; Zametkin et al., 1990; Andreason et al., 1994) and this area compensates this hyperactivity by an inhibitory mechanism (Winstanley, 2007). Monkeys and humans show orbital cortex dysfunction after cocaine exposure (Franklin et al., 2002; Jentsch et al., 2002; Olausson et al., 2007). Increases in metabolic rates in the orbitofrontal cortex and basal ganglia in humans correlate negatively with the duration of abstinence (Volkow et al., 1991). Further studies suggest that orbital cortex abnormalities are a consequence of drug exposure and not a predisposing factor for drug addiction (Perry et al., 2011). The observed changes in cytochrome oxidase correlations between specific regions of the prefrontal cortex and the SNc may be relevant to a role of DA pathways in the loss of inhibitory control and increased locomotion seen after cocaine intake.

3.6. Cholinergic pathways

Cytochrome oxidase activity in the interpeduncular nucleus (IP) was negatively correlated with the activities of the deep layer of the Fr2 dorsal frontal cortex, the superficial and deep layers of the anterior insular cortex, the superficial and deep layers of the lateral orbital cortex in saline but not in cocaine-treated animals (Fig. 3). Although IP neurons are mainly GABAergic, its major inputs are cholinergic projections from the medial habenula. Indeed, the IP receives more acetylcholine input than any other region in the mammalian brain (Herkenham and Nauta, 1979; Villani et al., 1983; Artymyshyn and Murray, 1985; Contestabile et al., 1987; Eckenrode et al., 1987; Fasolo et al., 1992). Studies have shown that interpeduncular pathways (i.e. habenulaip) and the mesolimbic pathways are mutually inhibitory, (Sutherland and Nakajima, 1981; Nishikawa et al., 1986). In addition, it has been reported that cocaine injections decrease the extracellular levels of acetylcholine in the IP (Hussain et al., 2008). Nicotininc AChR subunits in the IP have been also been shown to play a crucial role in somatic withdrawal symptoms in drugs of abuse like nicotine (Salas et al., 2009). Acetylcholine is an integral component of the mesolimbic system (Hoebel et al., 2007). Cholinergic projections may be altered in learning and memory processes like those changed in drug abuse (Vorel et al., 2001; See et al., 2003). For example, nicotinic receptor inactivation decreases sensitivity to cocaine whereas nicotine exposure enhances cocaine seeking (Zachariou et al., 2001; Bechtholt and Mark, 2002). On the other hand, changes in the neurochemistry of the IP and its projections induce changes in motor behavior (Shannon and Peters, 1990; Salas et al., 2004; Taraschenko et al., 2007). For example, muscarinic antagonists enhance cocaine and amphetamine-induced locomotor effects (Shannon and Peters, 1990; Bymaster et al., 1993; Ichikawa et al., 2002), whereas mice lacking muscarinic M5 receptors self-administer less cocaine and show reduced conditioned place preference (Fink-Jensen et al., 2003). These studies suggest that cholinergic receptors mediate some component of cocaine-induced changes. Recent evidence has shown that blockade of nicotinic cholinergic receptors in the VTA prevents the cocaine-induced DA increase (Mark et al., 2011). There is also evidence that supports that disruption of IP signaling can lead to increased drug intake (Picciotto, 1998; Cui et al., 2003; Tapper et al., 2004; Fowler et al., 2011). The loss of connectivity of the IP observed in our experiments suggest that cocaine exposure produces an abnormality in acetylcholine signaling that may affect the connections between the IP and frontal cortical structures that could further enable cocaine sensitization.

3.7. Conclusion

First, the hypofrontality results presented here are in agreement with previous glucose metabolic mapping studies in cocaine-exposed animals (Porrino and Kornetsky, 1988; Koch et al., 1997). They also agree with previous human studies that suggest a hypofrontality produced by chronic use of drugs of abuse such as cocaine (Volkow et al., 1988; London et al., 1990; Matochik et al., 2003; Bolla et al., 2004). Second, our findings also indicate, for the first time, that repeated cocaine modifies the functional connectivity between specific prefrontal regions and subcortical noradrenergic, dopaminergic and cholinergic pathways. Taken together these patterns of functional connectivity suggest the general hypothesis that prefrontal networks change from cholinergic influences to networks driven by noradrenergic and dopaminergic nuclei after repeated cocaine administration. Finally, we speculate that when these prefrontal networks for inhibitory control are modified by repeated cocaine, enhanced locomotion may arise, which suggests that treatments that increase the metabolic capacity of these prefrontal networks may antagonize the psychomotor effects of cocaine (Chen et al., 2013).

4. Experimental procedures

4.1. Subjects

All procedures were performed according to the US Public Health Service publication "Guide for the Care and Use of Laboratory Animals" and were approved by the Animal Care and Use Committee at the University of Puerto Rico Medical Sciences Campus. Twenty-four Sprague-Dawley male rats (Taconic Farms) weighing between 250–300 g were housed two per cage and maintained at constant temperature and humidity with a 12-h light/dark cycle. All behavioral experiments were carried out during the light period. Water and food were provided *ad libitum*.

4.2. Cocaine protocol and behavior

Animals were randomly divided into two groups (saline n=12 and cocaine n=12). Prior to any drug treatment, all animals (n=24) were habituated to locomotor chambers (AccuScan Instruments Inc., Columbus, OH) for two daily 1 h sessions. Each cage frame houses sensor panels and a row of 16 infrared beams. When a rodent intersects the beams, the software detects a photocell count. On experimental day 1, animals were placed for 15 min in the photocell box. After 15-min habituation, animals were treated with either 15 mg/kg i.p. of cocaine (Sigma, St. Louis, MO, USA) or isovolumetric saline injections. This particular dose repeated for five injections was chosen because it is a well-established protocol to induce progressive cocaine-enhanced locomotion that is commonly used in our laboratory (Jimenez-Rivera et al., 2006; Arencibia-Albite et al., 2012; Santos-Vera et al., 2013) and others (Pierce et al., 1996; Robinson and Berridge, 2001; Yoon et al., 2007). Therefore, using this protocol makes this study more relevant to compare with similar cocaine rat studies in the literature. Immediately after injections, total locomotor activity was assessed for 1 h. The total number of beam breaks (photocell counts) determined total locomotion. This procedure was repeated for 5 consecutive days. On day 5, after locomotor activity was assessed for 1 h, rats' brains were collected for tissue processing.

4.3. Tissue processing and cytochrome oxidase staining

Brains were quickly removed and frozen in isopentane. Using a cryostat microtome (Leica CM3000, Germany) at −20 °C, brains were sectioned at 40 μm, mounted on slides and kept frozen at −40 °C until they were processed using quantitative cytochrome oxidase enzyme histochemistry (Gonzalez-Lima, 1998). Series of coronal sections from each brain were used to perform cytochrome oxidase histochemistry following the validated quantitative protocol described by Gonzalez-Lima and collaborators (Gonzalez-Lima and Cada, 1994; Gonzalez-Lima and Jones, 1994) that has been used in hundreds of other cytochrome oxidase studies. The staining reaction signal is produced when diaminobenzidine is oxidized to a visible indamine polymer. The reaction product is further intensified by the addition of cobalt to the preincubation solution. Since continuous reoxidation of cytochrome *c* by cytochrome oxidase is needed for the accumulation of the visible product, this reaction under linear conditions serves to visualize cytochrome oxidase reactivity. Enzymatic activity units are calculated using calibration standards made of brain paste which showed a linear relationship (r=0.99) between cytochrome oxidase activity units measured spectrophotometrically and cytochrome oxidase reactivity measured with densitometry. Briefly, frozen slides were fixed for 5 min using a 10% sucrose phosphate buffer (0.1 M pH 7.6) containing 0.5% glutaraldehyde to adhere the sections to the slides. Next, slides were rinsed in three changes of a 10% sucrose phosphate buffer (0.1 M) for 5 min each to remove red blood cells and warm tissue to room temperature. Then the slides were pre-incubated in 0.05 M Tris buffer (pH 7.6), with 275-mg/l cobalt chloride, 10% sucrose, and 0.5% dimethylsulfoxide, for 10 min to enhance staining contrast (metal intensification). Slides were then rinsed for 5 min in phosphate buffer and incubated in 700 ml of oxygen-saturated

0.1 M phosphate buffer (350 mg diaminobenzidine tetrahydrochloride, 52.5 mg cytochrome c, 35 g sucrose, 14 mg catalase, and 1.75 ml dimethylsulfoxide) at 37 °C for 1 h. The tissue was fixed in buffered formalin to stop the last reaction (for 30 min at room temperature with 10% sucrose and 4% formalin). Finally, the slides were dehydrated in a series of ethanol baths (increasing from 30% to 100% ethanol), cleared by immersion in xylene, and cover slipped with Permount (Fisher Scientific, Pittsburgh, PA, USA).

Each batch of slides was accompanied by a set of brain paste standards to quantify enzymatic activity and to control for staining variability across batches. For these standards, the brains from 12 additional Sprague-Dawley male rats were removed after decapitation, stored at 4 °C (in sodium phosphate buffer, pH 7.6), and then homogenized at 4 °C (Gonzalez-Lima, 1998). Cytochrome oxidase activity of the brain paste was spectrophotometrically assessed as described by Gonzalez-Lima and Cada (1998), and activity units were defined at pH 7 and 37 °C, where 1 unit oxidizes 1 μmol of reduced cytochrome *c* per min (μmol/min/g tissue wet weight). Remaining paste was frozen in the same manner as the experimental brains and stored at −40 °C. Immediately prior to each cytochrome oxidase staining procedure, cryostat sections of different thickness (10, 20, 40, 60 and 80 μm) were obtained from the rat brain paste and mounted on a slide. These sets of sections of known cytochrome oxidase activity were used as calibration standards in each cytochrome oxidase staining bath.

4.4. Cytochrome oxidase activity mapping

Using a stereotaxic atlas of the rat brain (Paxinos and Watson, 1986) as well as a cytochrome oxidase atlas of the rat brain (Gonzalez-Lima, 1998), cytochrome oxidase-stained sections were carefully selected for both the appropriate levels of brain regions of interest and the integrity of the sections. The regions of interest examined are illustrated by Bregma level in Fig. 2. An image-processing system consisting of a high-gain video camera, Targa-M8 image capture board, Everex computer, Sony color monitor, DC-powered illuminator, and JAVA software (Jandel Scientific, San Rafael, CA, USA) was used to sample optical density (OD) from each ROI. This system was calibrated before each measurement session using an OD tablet (Kodak, Rochester, NY, USA). The film had a known set of absolute OD units in seven standards ranging from 0 to 0.92 OD units. Background subtraction of the clear part of the slide without sections was used to correct possible optical artifacts from the camera. The histochemical reaction product from cytochrome oxidase staining was measured in OD units. In each measured region, four readings of each section were taken on each of three adjacent sections to yield 12 readings per region per brain. For each region measured, size of the square-shaped sampling window was adjusted so that it was as large as possible while still allowing two, non-overlapping readings to be taken bilaterally (four total). The size of the window was held identical across subjects, as was the number of readings for each ROI. The OD values of these readings were then converted to cytochrome oxidase activity units (μmol/min/g tissue w/w) using a regression curve (r^2>0.90) that was obtained from the mean OD values and enzymatic activity of the tissue standards stained in the same batch and imaged in the same measurement session (Gonzalez-Lima, 1998).

4.5. Statistical analysis of locomotion, mean cytochrome oxidase activity and functional connectivity

Total locomotor activity, expressed as photocell counts, between groups were analyzed using Two-way ANOVA for repeated measures followed by Bonferroni post-test in order to establish behavioral locomotor effects (numbers are presented as mean ± standard error). A significant day 1 to day 5 difference in mean photocell counts at p<0.05 was considered a successful enhanced locomotor effect. Group differences in mean cytochrome oxidase

activity measured in each brain region were evaluated by one-way ANOVA. Functional connectivity was assessed by computing separate pair-wise Pearson correlation matrices of cytochrome oxidase activity across all regions of interest for each group (within-group analysis) (Puga et al., 2007). In this data-driven approach, all the brain regions are evaluated and the brain effects determine which are the relevant inter-regional correlations, as opposed to using a more restricted theory-driven or arbitrary selection of regions for analysis (McIntosh and Gonzalez-Lima, 1994a, 1994b). A "jackknife" procedure was used to ensure the reliability of significant correlations and to protect against the effects of outliers to avoid inflated correlation estimates which sometimes result from small sample sizes. In this procedure, an individual is removed from the data set and correlations are computed on the data set with $n-1$ subjects. The individual is then replaced into the data set, and another individual is removed. Correlations and significance tests are recomputed on the data set with $n-1$ subjects. This procedure is repeated until all individuals have been removed once. Based on the calculation of all possible pairwise correlations resulting from removing one subject at a time, this procedure takes into consideration only those correlations that remain significant ($p<0.01$) across all possible combinations. These correlations were then tested for significant differences between groups (between-group analysis). The Fisher Z transformation was used to convert each correlation to a Z score to test differences in inter-regional correlations between groups (Jones and Gonzalez-Lima, 2001; Bruchey and Gonzalez-Lima, 2006; Puga et al., 2007). Significant group differences of pair-wise inter-regional correlations between cocaine- and vehicle-treated animals were calculated as absolute value of $Z_{abs}>1.96$ ($p<0.05$). As with all functional connectivity methods, inter-regional correlations do not provide information on the direction of the influence, so they need to be interpreted based on known anatomical pathways. For example, if region A that influences region B via an anatomical path showed a significant pair-wise positive correlation in cytochrome oxidase activity after treatment, it may be inferred that a change in activity in region A was functionally related to a corresponding alteration in region B (Puga et al., 2007). The term functional connection was used to refer to jackknife-tested reliably significant cytochrome oxidase activity correlations between two brain regions (Padilla et al., 2011).

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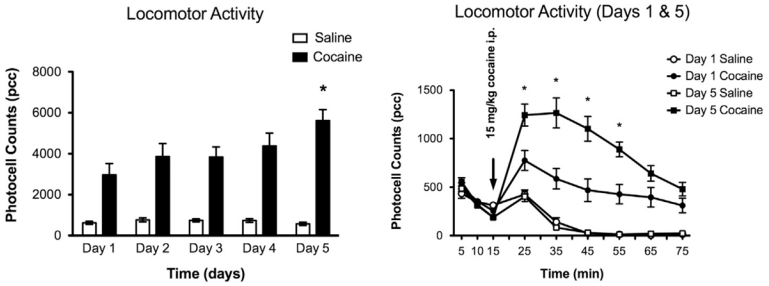

Fig. 1. Locomotor activity

(A) Graph shows mean total locomotor activity (photocell counts/60 min, S.E.M. error bars) after 15 mg/kg cocaine (*n*=12) or saline (*n*=12) daily injections (i.p.) for days 1–5. (B) Graph shows comparison of day 1 and day 5 time course of total locomotor activity. Asterisks (*) denote a significant mean group difference as compared to day 1 ($p<0.05$).

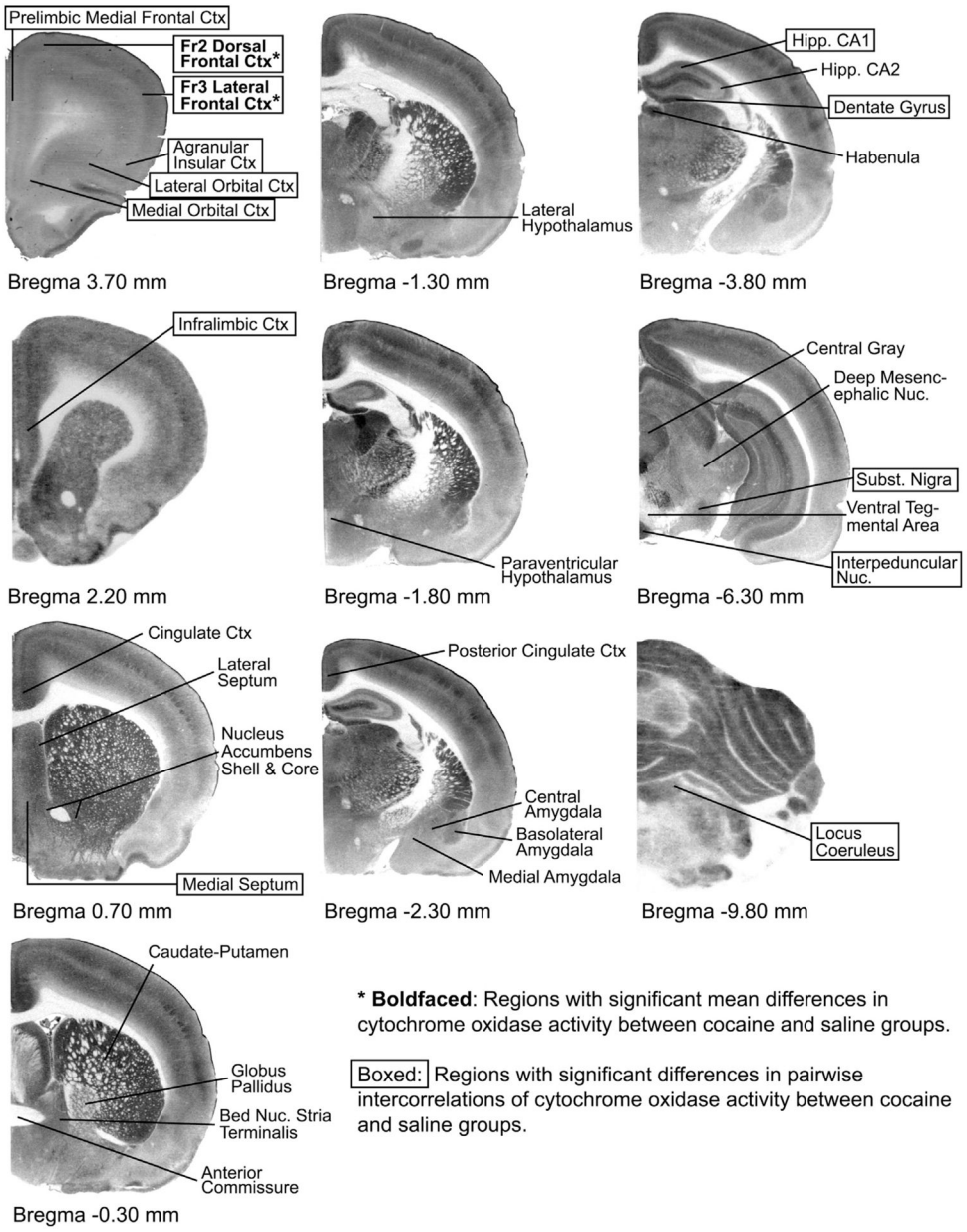

Fig. 2. Cytochrome-oxidase stained sections indicating regions of interest by Bregma level

The purpose of this figure is to show representative control sections at the Bregma levels where the regions of interest were investigated in each brain and to illustrate schematically which were the affected regions. The quantitative densitometric cytochrome oxidase differences and functional connectivity changes cannot be seen with the naked eye by comparing sections, and are thus presented in Tables 1 and 2. To map these changes schematically in one figure, cocaine effects were illustrated so that regions appearing with boldfaced asterisks showed significant mean differences ($p<0.05$) in cytochrome oxidase (μmol/min/g tissue wet weight) between saline ($n=12$) and cocaine ($n=12$) groups (Table 1); and boxed regions showed significant differences ($p<0.05$) in pair-wise inter-regional correlations between saline and cocaine groups (Table 2).

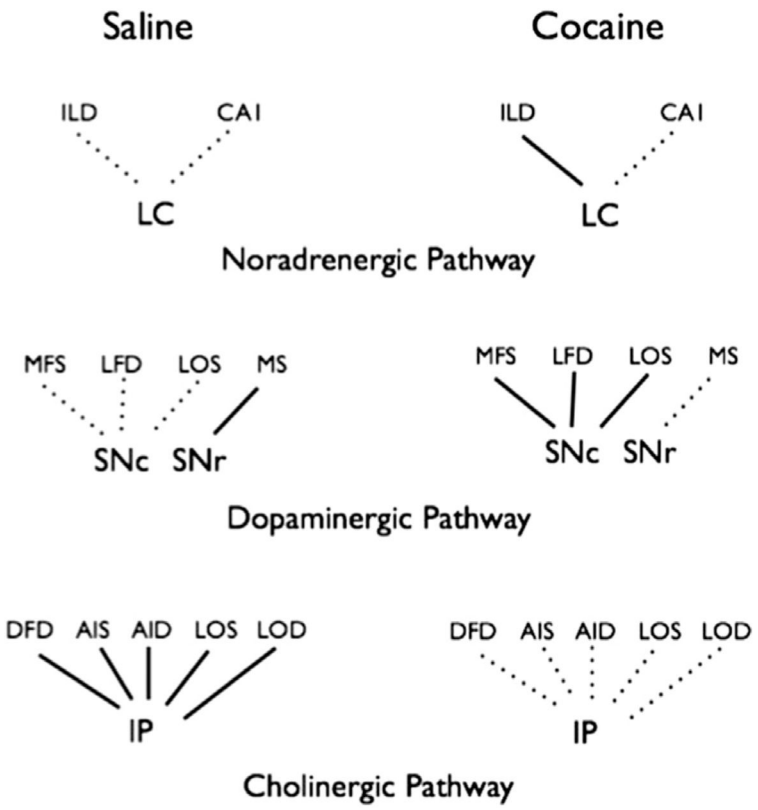

Fig. 3.

Schematic diagram illustrating the significant inter-regional correlations of cytochrome oxidase activity calculated in both saline (n=12) and cocaine (n=12) groups. Abbreviations are the same as in Table 1. Solid lines represent pair-wise Pearson's correlations significantly different from zero (p<0.05) and dotted lines represent no significant correlation. The value and sign of the correlations with significant group differences are listed in Table 2.

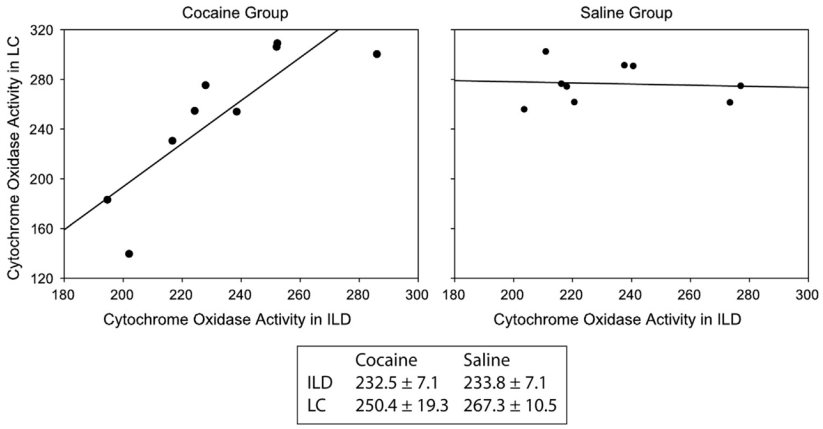

Fig. 4.

Inter-regional correlations patterns among nucleus locus coeruleus (LC) and deep infralimbic frontal cortex (ILD). Plots show significantly (p=0.01) different inter-regional correlations of cytochrome oxidase activity between the LC and the ILD in cocaine and saline treated animals. The line shows the best fit linear regression (r=0.80 in cocaine and r= −0.08 in saline treated animals). Box shows the mean cytochrome oxidase activity units (μmol/min/g tissue wet weight), which are not significantly different for both regions in saline (n=12) and cocaine treated rats (n=12).

Table 1

Means and standard errors of cytochrome oxidase activity units (μmol/min/g tissue w/w) for all regions measured.

Region	Bregma	Cocaine	Saline
Fr2 Dorsal Frontal Cortex Superficial Layer (DFS)	3.7	216.8±8.3*	243.5±8.4*
Fr3 Lateral Frontal Cortex Superficial Layer (LFS)		241.5±8.7*	264.8±6.3*
Prelimbic Medial Frontal Cortex Superficial (MFS)		227.3±4.6	225.7±4.7
Fr2 Dorsal Frontal Cortex Deep Layer (DFD)		218.6±7.0	236.4±7.6
Prelimbic Medial Frontal Cortex Deep (MFD)		221.8±4.3	228.0±4.7
Fr3 Lateral Frontal Cortex Deep Layer (LFD)		242.4±7.8	259.0±5.9
Anterior Insular Cortex Superficial Layer (AIS)		210.1±8.1	219.6±6.9
Anterior Insular Cortex Deep Layer (AID)		231.9±7.8	248.0±6.4
Lateral Orbital Cortex Superficial Layer (LOS)		238.3±6.9	239.6±5.2
Lateral Orbital Cortex Deep Layer (LOD)		239.4±6.9	253.3±8.0
Medial Orbital Cortex Superficial Layer (MOS)		226.7±7.2	230.2±6.0
Medial Orbital Cortex Deep Layer (MOD)		235.6±6.3	244.4±8.9
Infralimbic Cortex Superficial Layer (ILS)	2.2	229.8±6.5	226.4±6.9
Infralimbic Cortex Deep Layer (ILD)		232.5 ±7.1	233.8±7.1
Anterior Cingulate Cortex Superficial Layer (CGS)	0.7	266.9±7.7	256.1 ±8.3
Anterior Cingulate Cortex Deep Layer (CGD)		257.5±8.7	247.4±7.9
Lateral Septum Nucleus (LS)		245.8±6.1	242.4±3.1
Medial Septum Nucleus (MS)		198.4±5.2	194.3 ±2.1
Accumbens Nucleus Shell (ACS)		220.7±6.1	219.7±5.5
Accumbens Nucleus Core (ACC)		245.0±7.9	246.7±6.4
Caudate Putamen (CPU)	−0.3	234.8±9.4	231.5±6.7
Globus Pallidus (GP)		158.5±5.2	150.7±3.2
Bed Nucleus Stria Terminalis (BST)		213.2±5.7	214.4±2.4
Anterior Commissure (AC)		55.6±3.6	53.4±3.9
Lateral Hypothalamic Area (LH)	−1.3	186.4±6.3	178.8±4.6
Paraventricular Hypothalamic Nucleus (PVH)	−1.8	194.2±11.1	204.4±12.2
Basolateral Amygdala (BLA)	−2.3	221.3±7.2	209.2±11.5
Central Amygdala (CAM)		228.5±6.8	234.3±8.4
Medial Amygdala (MAM)		198.9±7.1	212.1±4.2
Posterior Cingulate/Retrosplenial Cortex (PCN)		284.6±7.5	282.4±8.7
Field CA1 of Hippocampus (CA1)	−3.8	208.5±8.6	212.8±9.3
Field CA2 of Hippocampus (CA2)		226.0±11.3	225.0±12.1
Dentate Gyrus (DG)		337.4±15.7	327.5±15.0
Medial Habenula (MHB)		221.3±12.6	215.5±14.4
Lateral Habenula (LHB)		293.8±13.4	288.6±17.9
Central Gray (CG)	−6.3	256.8±6.3	248.9±5.2
Deep Mesencephalic Nucleus (DPME)		168.0±5.8	183.7±13.0
Interpeduncular Nucleus (IP)		378.4±13.3	353.5±20.6

Region	Bregma	Cocaine	Saline
Ventral Tegmental Area (VTA)		102.8±9.8	95.9±12.3
Substantia Nigra Reticulata (SNR)		234.7±7.4	234.3±11.3
Substantia Nigra Compacta (SNC)		202.0±5.2	197.7±7.8
Locus Coeruleus (LC)	−9.8	250.4±19.3	267.3±10.5

* Cytochrome oxidase activity was significantly ($p<0.05$) decreased in cocaine-treated animals ($n=12$) as compared to saline ($n=12$) in the superficial layers of dorsal (Fr2) and lateral (Fr3) frontal cortex regions.

Table 2

Value and sign of pair-wise correlation coefficients (*r*) for the saline and cocaine groups and *p* values of Zabs for the group comparison of each regional pair. Abbreviations are the same as in Table 1.

	Saline (*r*)	Cocaine (*r*)	*p* value
LC/ILD	−0.08	0.8	0.01
LC/CA1	−0.56	0.31	0.05
SNC/MFS	0.11	0.81	0.03
SNC/LFD	−0.12	0.77	0.02
SNC/LOS	−0.16	0.72	0.03
SNR/MS	0.6	−0.3	0.03
IP/DFD	−0.85	0	0.01
IP/AIS	−0.67	0.16	0.03
IP/AID	−0.95	−0.17	0.001
IP/LOS	−0.7	0.24	0.02
IP/LOD	−0.85	0.3	0.001
LFS/DG	0	0.74	0.05
MOS/AIS	0.4	0.89	0.04