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Alterations in the level of OFQ/N-IR in rat brain regions by cocaine

Kabirullah Lutfy¹, Hoa Lam², and Shridhar Narayanan^{2,*}

¹Department of Pharmaceutical Sciences, College of Pharmacy, Western University of Health Sciences, 309 East 2nd Street, Pomona, CA 91766

²Department of Psychiatry and Biobehavioral Sciences, NPI, UCLA School of Medicine, 760 Westwood Plaza, Los Angeles, CA 90024

Abstract

We have previously shown that administration of orphanin FQ/nociceptin (OFQ/N), the endogenous ligand of the opioid receptor-like (ORL-1) receptor, into the lateral ventricles or VTA blocked cocaine sensitization. In the present study, we determined the effect of acute and chronic cocaine treatment on the level of endogenous OFQ/N in rat brain regions. Male Sprague Dawley rats were tested for motor activity in response to saline or cocaine (20 mg/kg) injection once daily for three consecutive days. To determine the effect of single or repeated cocaine administration on the level of OFQ/N, rats were sacrificed one hour following saline or cocaine injection either on day 1 or 3, respectively. Additional groups of rats were treated similarly with saline or cocaine on days 1–3 and sacrificed or tested for locomotor sensitization on day 8. Consistent with previous studies, repeated cocaine administration induced locomotor sensitization to a challenge dose of cocaine (7.5 mg/kg) given on day 8. Measurements of tissue content of OFQ/N-IR using radioimmunoassay indicated that the rat hypothalamus and striatum respectively contained the highest and lowest level of the peptide among the brain regions tested. Acute cocaine decreased the level of OFQ-IR in the rat midbrain and to a lesser extent in the striatum. On the other hand, the level of OFQ/N was higher in rats treated with cocaine on days 1–3 and sacrificed on day 8. These findings suggest that endogenous OFQ/N may be involved in the actions of cocaine and possibly in cocaine-induced motor stimulation and locomotor sensitization.

Keywords

Cocaine; Orphanin FQ/Nociceptin (OFQ/N); Opioid receptor-like (ORL-1) receptor; Radioimmunoassay (RIA); Sprague Dawley rats; Behavioral sensitization

Introduction

Drug addiction is a chronic relapsing brain disorder that involves neuroadaptive alterations in numerous neuronal circuits leading to compulsive drug seeking and drug taking behaviors despite catastrophic consequences associated with continued drug use/abuse. Research in laboratory animals has also revealed neuroadaptions following administration of cocaine and

Corresponding author: Kabirullah Lutfy, Ph.D., klutfy@westernu.edu, Phone: (909) 469-5481, Fax: (909) 469-5600.

*Current address: Glenmark Pharmaceuticals Ltd, India

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other drugs of abuse in different brain circuits. Behavioral changes which accompany these neuroadaptive changes mimic some aspects of addictive behaviors in humans.

In rodents, repeated intermittent cocaine administration has been shown to induce a progressive and enduring increase in motor activity, a phenomenon referred to as locomotor sensitization (Kalivas and Weber 1988; Post and Rose 1976; Robinson and Becker 1986; Stripling and Ellinwood, Jr. 1977). This phenomenon is thought to play an important role in the development and maintenance of drug dependency through an increase in drug "wanting" upon repeated administration such that the urge to take the drug becomes irresistible, i.e., drug craving. Thus, behavioral sensitization is considered as an animal model of some aspects of addiction, particularly craving (Robinson and Berridge 1993; Robinson and Berridge 2000).

The phenomenon of behavioral sensitization is believed to be due to numerous changes that occur along the mesolimbic dopaminergic neurons following repeated drug administration (Anderson and Pierce 2005; Everitt and Wolf 2002; Vanderschuren and Kalivas 2000; White and Kalivas 1998; Woolverton and Johnson 1992). In particular, changes in the dynamics of dopaminergic neurotransmission, and dopamine receptor number and signaling have been reported (Kalivas and Duffy 1990; Pierce and Kalivas 1995; Pierce *et al.* 1995; Zahniser *et al.* 1988; Anderson and Pierce 2005). Furthermore, alterations in the function of the guanine regulatory binding proteins have been implicated in the phenomenon of sensitization (Cunningham and Kelley 1993; Hummel and Unterwald 2003; Nestler *et al.* 1990). Hyperactivity of the glutamatergic system is another hallmark of behavioral sensitization (for reviews, see Carlezon, Jr. and Nestler 2002; Everitt and Wolf 2002; Vanderschuren and Kalivas 2000). Recent evidence has also implicated protein kinase A (for review, see Anderson and Pierce 2005) as well as extracellular signal-regulated kinase (for review, see Girault *et al.* 2007) in the phenomenon of locomotor sensitization.

The endogenous opioid system has long been known to modulate the function of the mesolimbic dopaminergic neurons. Thus, while mu and delta receptor agonists increase, kappa receptor agonists decrease the function of the mesolimbic dopaminergic neurons (Di Chiara and Imperato 1988; Herz 1997). The endogenous opioid system may also be involved in the phenomenon of locomotor sensitization. For example, drugs that block the mu and delta opioid receptors (Hummel *et al.* 2004; Hummel *et al.* 2006; Kim *et al.* 1997; Schroeder *et al.* 2007) or activate the kappa opioid receptor (for review, see Shippenberg and Rea 1997) have been shown to attenuate the development of psychostimulant-induced locomotor sensitization. Repeated intermittent cocaine treatment has also been shown to modify the level of endogenous opioid peptides (Hurd and Herkenham 1992; Hurd *et al.* 1992; Hurd 1996; Hurd *et al.* 1999; Sivam 1989) and receptors (Hammer, Jr. 1989; Izenwasser *et al.* 1996; Unterwald *et al.* 1994).

In 1994, several laboratories cloned a receptor that showed approximately 65% homology to the classical (mu, delta and kappa) opioid receptors (Bunzow *et al.* 1994; Chen *et al.* 1994; Fukuda *et al.* 1994; Hammer, Jr. 1989; Mollereau *et al.* 1994). This receptor was termed as the opioid receptor-like (ORL-1) receptor. A year later, two independent laboratories isolated orphanin FQ/nociceptin (OFQ/N) as the endogenous ligand of the ORL-1 receptor (Meunier *et al.* 1995; Reinscheid *et al.* 1995). OFQ/N, a 17 amino acid peptide, is structurally similar to the endogenous opioid peptides, in particular to dynorphin A (1–17) (Julius 1995; Meunier *et al.* 1995). However, OFQ/N does not display appreciable affinity for the classical opioid receptors and the endogenous opioid peptides do not bind to the ORL-1 receptor. Thus, the endogenous OFQ/N/ORL-1 receptor system has its unique pharmacology.

The OFQ/N/ORL-1 receptor system regulates the function of the mesolimbic dopaminergic neurons and attenuates the rewarding and addictive effects of abused drugs. Thus,

intracerebroventricular OFQ/N administration has been reported to attenuate elevations in accumbal dopamine induced by morphine (Di Chiara *et al.* 1999) or cocaine (Lutfy *et al.* 2001). Furthermore, OFQ/N has been shown to block the development of behavioral sensitization (Lutfy *et al.* 2002), raising the possibility that the endogenous OFQ/N/ORL-1 receptor system may be involved in the phenomenon of locomotor sensitization. Thus, the present study was designed to determine whether repeated cocaine treatment that induces locomotor sensitization would alter the level of endogenous OFQ/N in various brain regions in rats. We also determined the effect of acute cocaine on the level of OFQ/N-immunoreactivity (OFQ/N-IR) in rat brain regions.

Materials and Methods

Subjects

Male Sprague Dawley rats, weighing 200–250 g, were obtained from Harlan Laboratories (San Diego, California, USA) and used in all experiments. Animals were maintained under a 12 h light/12 h dark cycle (light on at 7:00 AM) with free access to water and food in a humidity- and temperature-controlled room. All experiments were conducted according to the NIH guideline and approved by the Institutional Animal Care and Use Committee at Western University of Health Sciences (Pomona, California, USA).

Experimental Procedure

Measurement of OFQ/N-immunoreactivity (OFQ/N-IR) in rat brain regions—Rats ($n = 6$) were deeply anesthetized, their brains removed and placed in ice-cold buffer. Different brain regions (brain stem (mainly pons), cerebellum, midbrain (mainly VTA), thalamus, hypothalamus, hippocampus, striatum, and prefrontal and parietal cortices) were isolated and sonicated in 40 volumes of ice-cold acid acetone. The tissue was spun, the supernatant collected and lyophilized. A 1:10 dilution of each sample was prepared in artificial cerebrospinal fluid and assayed in triplicates for the measurement of OFQ-IR using a commercially available radioimmunoassay kit (Phoenix Pharmaceuticals, Belmont, CA). The sensitivity of the assay was 0.1 fmol and there was no cross-reactivity with opioid peptides, dynorphins A (1–17), enkephalins, beta-endorphin, endomorphin-1 and endomorphin-2. Total and non-specific bindings were 50% and 3%, respectively.

Measurement of OFQ/N-IR in rat brain regions following single cocaine administration—Rats were habituated to motor activity chambers for one hour, then injected with saline ($n = 6$) or cocaine (20 mg/kg, i.p.; $n = 6$) and motor activity was recorded for one hour (4×15 -min epochs) using a Videomex-V system (Columbus Instruments, Inc; Columbus, Ohio, USA). Rats were then immediately sacrificed and the level of OFQ/N-IR was measured in different brain regions, as described above.

Measurement of OFQ/N-IR in rat brain regions following repeated cocaine administration—Rats were habituated to the motor activity chambers for one hour, then injected with saline ($n = 6$) or cocaine (20 mg/kg, i.p.; $n = 6$) and motor activity was recorded for one hour (4×15 -min epochs). The same treatment was given once daily for three consecutive days. On day 3, rats were sacrificed one hour after the last cocaine or saline injection and their brains processed for the measurement of OFQ/N-IR, as described above.

Measurement of OFQ-IR in brain regions of rats sensitized to cocaine—Rats were habituated to the motor activity chambers, treated with saline ($n = 6$) or cocaine (20 mg/kg; $n = 6$) once daily for three consecutive days, as described above. Rats were then left untreated until day 8. On this day, rats were either sacrificed and their brains processed for the measurement of OFQ/N-IR, as described above, or tested for locomotor sensitization. To

confirm that the current cocaine treatment paradigm indeed induces locomotor sensitization, rats were habituated to the motor activity chambers, injected with saline or cocaine (7.5 mg/kg) and motor activity was recorded for one hour (4 × 15-min epochs).

Data Analysis

Data are presented as means ($\pm$ SEM). Behavioral data (the first 15-min epoch following cocaine administration) were analyzed using one-way analysis of variance (ANOVA) followed by the *post-hoc* Dunnett's test. Neurochemical data were analyzed by one-way ANOVA or unpaired student's t test. A $p < 0.05$ was considered statistically significant.

Results

Hypothalamus contained the highest level of OFQ/N-IR in rat brain

Our pilot studies showed that the level of OFQ/N-IR in some brain regions did not fall on the linear portion of the standard curve. To mitigate this problem, samples were diluted 10 times and 50 μ L of the samples yielded values falling on the linear portion of the standard curve. Additionally, the level of OFQ/N-IR in the diluted samples was above the level of detection (0.1 fmol) for each brain region (Table 1). A one-way ANOVA revealed a significant effect of brain region ($F_{8,41} = 27.32$; $p < 0.001$). The *post-hoc* test showed that the rat hypothalamus, as compared to the other brain regions tested in the current study, contained the highest amount of OFQ/N-IR ($p < 0.05$). Midbrain, thalamus, brain stem, cortical regions and hippocampus contained high to moderate levels of OFQ/N-IR. The lowest level of OFQ/N-IR was detected in the cerebellum and striatum (Table 1).

Acute cocaine treatment decreased the level of OFQ/N-IR in midbrain region

The level of OFQ/N-IR in brain regions of rats sacrificed one hour following saline or cocaine (20 mg/kg) treatment is shown in figure 1. Once again, analysis of the data using one-way ANOVA in the control group (saline-treated rats) revealed a significant effect of brain region ($F_{7,36} = 23.93$; $p < 0.001$). The *post-hoc* analysis revealed the highest level of OFQ/N-IR in the hypothalamus ($p < 0.05$ as compared to other brain regions) followed by midbrain, thalamus and brain stem (Fig. 1A). The lowest amount of OFQ/N-IR was observed in the striatum (Fig. 1B). Acute cocaine, as compared to saline, treatment induced a modest but significant reduction in the level of OFQ/N-IR in rat midbrain ($p < 0.05$). Also, cocaine produced a 20–40% reduction in OFQ/N-IR in the striatum and thalamic regions, but those changes were not statistically significant ($p > 0.05$). However, the level of OFQ/N-IR was comparable in the hypothalamus, brain stem, cortical regions and hippocampus of rats treated with cocaine or saline ($p > 0.05$).

Repeated cocaine administration (once daily for three consecutive days) failed to reduce the level of OFQ/N-IR in the midbrain

We have previously shown that rats treated with cocaine (20 mg/kg) once daily for three consecutive days express locomotor sensitization (Lutfy *et al.* 2002a). Therefore, we used a similar paradigm to determine whether the level of OFQ/N-IR would be altered in rat brain regions during the development of sensitization. Figure 2 depicts the level of OFQ/N-IR in rats treated with saline or cocaine once daily for three consecutive days and sacrificed one hour following the last saline or cocaine injection. Once again, the highest and lowest level of OFQ/N-IR was found in the hypothalamus (Fig. 2A) and striatum (Fig. 2B), respectively. Although a pattern toward reduction was observed in the hypothalamus, midbrain, and cortical regions in cocaine-treated rats, none of the changes were statistically significant in the cocaine-treated rats as compared to their saline-treated controls ($p > 0.05$). However, the reduction in OFQ-IR in the parietal cortex of cocaine treated rats approached a significant level as compared to their controls (Fig. 2B; $t_{1,10} = 1.99$; $p < 0.04$; one-tailed).

Repeated intermittent cocaine administration induced locomotor sensitization and increased the level of OFQ/N-IR in the hippocampus

Consistent with our previous results (Lutfy *et al.* 2002f), rats treated with cocaine on days 1–3 expressed locomotor sensitization following a challenge dose of cocaine (7.5 mg/kg) given on day 8 (Fig. 3). One-way ANOVA of the behavioral data (the first 15-min time point following saline or cocaine injection on day 8, when the action of cocaine was maximal) revealed a significant effect of treatment ($F_{3,20} = 6.72$; $p < 0.001$). The *post-hoc* test revealed that a challenge dose of cocaine, as compared to saline, injected on day 8, increased motor activity ($p < 0.05$; compare SAL-Coc versus SAL-Sal group). However, the magnitude of this response was significantly greater in rats treated with cocaine on days 1–3 as compared to their saline-treated control rats ($p < 0.05$; compare COC-Coc versus SAL-Coc group). This result demonstrates that sensitization developed to the motor stimulatory action of cocaine. To determine whether the level of OFQ/N-IR would be altered during the expression of sensitization, we determined the level of OFQ/N-IR in rats treated with saline or cocaine (20 mg/kg) once daily for three consecutive days and sacrificed on day 8 (Fig. 4). Once again, a similar pattern of OFQ/N-IR was detected in different brain regions, i.e., the highest and lowest level of OFQ/N-IR were detected in the hypothalamus (Fig. 4A) and striatum (Fig. 4B), respectively. The level of OFQ/N-IR was increased in the hippocampus of rats sensitized to cocaine as compared to their saline-treated controls ($p < 0.05$; Fig. 4B). However, the level of OFQ/N-IR was not different in other brain regions studied between the cocaine sensitized and control rats ($p > 0.05$; Fig. 4).

Discussion

Previous studies have shown that the neuropeptide OFQ/N is widely distributed throughout the central nervous system (CNS), particularly in brain regions involved in emotional and motivational behaviors (Neal, Jr. *et al.* 1999). Consistent with its central distribution, endogenous OFQ/N and its receptor have been implicated in learning and memory, stress response, motivational and emotional behaviors as well as in the rewarding and addictive properties of cocaine and other drugs of abuse (for review, see Calo *et al.* 2005; Meis 2003; Mogil and Pasternak 2001). In the current study, we report alterations in the level of OFQ/N-IR following single as well as repeated cocaine treatment in rat brains regions, showing that the endogenous OFQ/N/ORL-1 receptor system may be involved in the actions of cocaine.

Our neurochemical studies showed that the hypothalamus contained the highest level of OFQ/N-IR followed by midbrain, thalamus, brain stem, hippocampus and cortical regions. The lowest amount of OFQ/N-IR was detected in the cerebellum and striatum. The regional distribution of OFQ/N-IR in the present study is consistent with previous observations in rat (Lindholm *et al.* 2002), mouse (Ploj *et al.* 2000) or human (Witta *et al.* 2004) brain. The novelty of our study is that a single cocaine administration, as compared to saline, decreased the level of OFQ/N-IR in the midbrain and to a lesser extent in the striatum and cortical regions in rats, showing that cocaine could reduce tissue content of OFQ/N-IR, a response that we interpreted as an increase in the release or degradation of endogenous OFQ/N. Although the response was modest, it is of interest to note that the level of OFQ/N-IR was measured one hour following cocaine administration. It may be that more robust reduction could have been detected, if the levels had been measured at earlier time points.

Previous neurochemical (Murphy and Maidment 1999; Murphy *et al.* 2004), dual in situ hybridization (Norton *et al.* 2002), electrophysiological (Zheng *et al.* 2002) and behavioral (Lutfy *et al.* 2002; Narayanan *et al.* 2004) studies have demonstrated that VTA could be one of the brain regions where OFQ/N exerts its modulatory actions on mesolimbic dopaminergic neurons and alters the actions of cocaine and other drugs of abuse. For example, we have shown that intra-VTA administration of OFQ/N reduced cocaine-induced motor stimulation and also

blocked locomotor sensitization induced by repeated intermittent cocaine administration (Lutfy *et al.* 2002). The current neurochemical data demonstrating that the level of OFQ/N-IR was reduced by acute cocaine treatment raises the possibility that cocaine may cause the release of OFQ/N along the mesocorticolimbic dopaminergic pathway and might be a means through which the action of cocaine can be auto-regulated. Interestingly, we have recently demonstrated that the reinforcing effect of acute cocaine was enhanced in mice lacking the ORL-1 receptor or in wild-type mice treated with an ORL-1 receptor antagonist (Marquez *et al.* 2008).

Previous studies have shown changes in the level of endogenous opioid peptides following cocaine treatment (Hurd and Herkenham 1992; Hurd *et al.* 1992; Hurd 1996; Hurd *et al.* 1999; Sivam 1989). In the present study, we examined whether the level of endogenous OFQ/N, an anti-opioid peptide in the brain (Mogil and Pasternak 2001), would be altered in various brain regions of rats sensitized to cocaine. A recent report showed reduced levels of OFQ/N-IR in the substantia nigra and caudate putamen following continuous cocaine infusion. The level of OFQ/N was reduced in several other regions using immunohistochemistry but not radioimmunoassay (Romualdi *et al.* 2007). However, in the present study, the level of OFQ/N-IR was not reduced in midbrain or striatum of rats following repeated (once daily for three consecutive days) cocaine treatment (Fig. 2). Likewise, the level of OFQ/N-IR was not altered in midbrain or striatum of rats treated with cocaine once daily for three consecutive days and sacrificed on day 8 (Fig. 4). The previous study used a protocol of chronic cocaine infusion known to produce tolerance to the motor stimulatory action of cocaine (King *et al.* 1992; Reith *et al.* 1987). On the other hand, the present study determined the level of OFQ/N-IR under a repeated cocaine treatment paradigm that induces sensitization to the locomotor stimulatory effects of cocaine (Lutfy *et al.* 2002), which was confirmed in the current study (Fig. 3). Also, the dose of cocaine was higher (50 mg/kg/day) in the previous as compared to the current study. Lastly, in the previous study, rats were sacrificed while being infused with cocaine (Romualdi *et al.* 2007). Thus, the inconsistency in the results of the two studies could be due to the use of different experimental paradigms. However, we observed reduction in the level of OFQ/N-IR following acute cocaine administration in some brain regions (Fig. 1) that was consistent with the previous study (Romualdi *et al.* 2007).

Previous studies have shown that OFQ/N inhibits long-term potentiation (LTP) in the hippocampus (Yu *et al.* 1997) and therefore impairs spatial learning in rats (Sandin *et al.* 1997). Moreover, enhancement of LTP and spatial attention has been reported in mice lacking the ORL-1 receptor (Mamiya *et al.* 1998; Manabe *et al.* 1998), suggesting that the endogenous OFQ/N/ORL-1 receptor system is involved in synaptic plasticity. As cocaine sensitization involves conditioned learning and the hippocampus is involved in this process, our novel finding showing the level of OFQ/N-IR was increased in the hippocampus of cocaine sensitized rats (Fig. 4) could be important. As tissue content may represent the level of endogenous OFQ/N under the steady-state condition, the enhanced OFQ/N-IR in the hippocampus could be due to a decrease in the release or increase in the synthesis of endogenous OFQ/N. We interpreted this novel finding as an attempt of the internal milieu to reduce the strength of the conditioned response or to facilitate its extinction. However, the enhanced OFQ/N-IR in rats sensitized to cocaine may have been due to withdrawal from cocaine since these rats were sacrificed five days following the last cocaine treatment. Notably, we did not observe such changes in rats treated with cocaine for three days and sacrificed one hour following the last cocaine injection (Fig. 2). Thus, further studies are needed to shed more lights on the involvement of the endogenous OFQ/N in cocaine-induced locomotor sensitization. For example, dose-response and time-course data would provide important information about the dynamics of OFQ/N regulation following cocaine treatment in naïve and cocaine-sensitized rats. Further, while RIAs are more quantitative, anatomical resolution is lost with this method. Thus, inclusion of other approaches such as *in situ* immunohistochemical or immunohistochemical studies will provide useful information in this regard.

In summary, the level of OFQ/N-IR was reduced in the midbrain regions in response to acute but not repeated cocaine administration. On the other hand, the level of OFQ/N-IR was increased in the hippocampus of rats sensitized to cocaine as compared to their controls. Together, these results suggest that the endogenous OFQ/N/ORL-1 receptor system may play a functional role in the acute and chronic actions of cocaine.

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Fig. 1. Acute cocaine treatment reduced the level of OFQ/N-IR in the rat midbrain

Rats were habituated to the motor activity tests, injected with saline or cocaine (20 mg/kg, i.p.) and sacrificed one hour later. Brain regions were isolated and homogenized in acid acetone solution by means of sonication. Samples were lyophilized, resuspended in RIA buffer and 50 μ l of a 1:10 dilution of each sample were assayed in triplicates for the measurement of OFQ/N-IR using a commercially available RIA kit (Phoenix Pharmaceuticals; Belmont, CA). Data are mean ($\pm$ SEM) of 6 rats/group. *indicates a significant reduction as compared to saline group ($p < 0.05$).

Fig. 2. The level of OFQ/N-IR was not altered in the midbrain in rats repeatedly treated with cocaine Rats were habituated to the motor activity tests, injected with saline or cocaine (20 mg/kg, i.p.) and motor activity was recorded for one hour. The same treatment was given once daily for three days and rats were sacrificed one hour following the last saline or cocaine injection. Brain regions were isolated, homogenized and assayed as described under legend to figure 1. Data are mean ($\pm$ SEM) of 6 rats/group.

Fig. 3. Locomotor sensitization developed to the motor stimulatory action of cocaine in rats
Rats were treated with saline (SAL) or cocaine (COC; 20 mg/kg, i.p.) once daily for three consecutive days and tested for locomotor sensitization on day 8. On this day, rats were habituated to the testing chambers for one hour, injected with saline (Sal) or cocaine (Coc; 7.5 mg/kg, i.p.) and motor activity was recorded for one hour (4 × 15 min epochs). Data are mean ($\pm$ SEM) of distance traveled during the first 15-min epoch following saline or cocaine administration on day 8. * $p < 0.05$ as compared to the rats treated with saline on days 1–3 and injected again with saline on day 8 (SAL-Sal group); † $p < 0.05$ as compared to all other groups.

Fig. 4. The level of OFQ/N-IR was increased in the hippocampus of rats sensitized to cocaine
Rats were injected with saline or cocaine (20 mg/kg, i.p.) once daily for three days and sacrificed on day 8. Brain regions were isolated, homogenized and assayed for the level of OFQ/N-IR as described under legend to figure 2. Data are mean ($\pm$ SEM) of 6 rats/group. *indicates a significant increase as compared to saline group ($p < 0.05$).

Table 1

The level of OFQ/N-IR in different brain regions of naïve rats measured by RIA.

Brain region	Level of OFQ/N-IR $\pm$ S.E.M.
Hypothalamus	29.59 $\pm$ 3.76*
Midbrain	15.17 $\pm$ 2.16
Thalamus	12.65 $\pm$ 0.84
Brainstem	9.25 $\pm$ 0.60
Prefrontal Cortex	6.46 $\pm$ 0.48
Parietal cortex	6.42 $\pm$ 0.62
Hippocampus	5.88 $\pm$ 0.58
Striatum	3.09 $\pm$ 0.28
Cerebellum	2.46 $\pm$ 0.32

Rats (n = 6) were deeply anesthetized with isoflurane, then sacrificed and their brain regions isolated and processed for the measurement of OFQ/NIR using a commercially available RIA kit (Phoenix Pharmaceuticals, Inc; Belmont, California, USA).

* indicates a significantly greater level of OFQ/N-IR as compared to other brain regions (p<0.05).