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Functional neuroanatomic analysis of the response of the nucleus accumbens to acute and chronic drugs of abuse

Approved by:

Michael Lehman, PhD
Ming Xu, PhD
James Fagin, MD
Lei Yu, PhD
Peter Stambrook, PhD
Functional neuroanatomic analysis of the response of the nucleus accumbens
to acute and chronic drugs of abuse

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Ryan R. Walsh
B.S., Georgetown University, 1995
Abstract:
The nucleus accumbens is a key limbic region implicated in mechanisms underlying reward and addiction. Evidence for involvement of the Nacc in responses to drugs of abuse (including cocaine and morphine) has partly come from studies using Fos as a marker for neuronal activation. While previous reports have consistently shown that Nacc neurons are activated following acute administration of cocaine, studies of morphine-induced activation have produced variable results. In the present study, we used two markers of neural activation, phosphorylation of the MAP-kinase, ERK, and expression of Fos, to investigate Nacc activation in response to either cocaine or morphine. In addition, we used a combination of retrograde tract tracing and immunocytochemistry to investigate whether activated Nacc neurons send projections to the ventral pallidum (VP), a major efferent target of the Nacc. Whereas cocaine induced neural activation at ten minutes, one hour, and two hours following drug administration, morphine only induced activation two hours following injection. Furthermore, while cocaine-activated neurons were present at all rostral-caudal Nacc levels, morphine-activated neurons were restricted to the rostral Nacc. Finally, cocaine induced activation of a subset of VP-projecting neurons in all rostral-caudal levels of the Nacc, while morphine did not. Thus, cocaine and morphine induce different temporal and regional patterns of activation in the Nacc, with cocaine producing more rapid and widespread activation than morphine. The only area of overlap in the patterns of activation produced by either drug was in the rostral Nacc, suggesting that this subdivision may be particularly important in reward and addiction.
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INTRODUCTION

Drug addiction is a major public health problem affecting populations worldwide. This disorder is primarily characterized by a compulsion to seek and take a drug, combined with a loss of control in limiting drug intake, despite negative consequences (Koob et al., 1998; Hyman and Malenka, 2001). Considerable effort has been made to elucidate the biological underpinnings of this uncontrolled and seemingly contradictory behavior. Many studies have demonstrated that specific brain regions appear to play key roles in the development of drug addiction. In particular, the mesolimbic dopamine system has been shown to be a critical neural substrate in the development of drug abuse (Koob et al., 1998).

The mesolimbic dopamine system and related limbic circuitry

The mesolimbic system is primarily comprised of a series of projections extending in the following order: ventral tegmental area (VTA) >> nucleus accumbens (Nacc) >> ventral pallidum (VP) >> thalamus >> prefrontal cortex (PFC) >> Nacc and VTA (Pierce and Kalivas, 1997; Bardo, 1998; see Figure 1). This system has been shown in numerous studies using animal models to be important for drug addiction. For example, lesions of the VTA, Nacc, thalamus, or VP diminish motivated responses to drugs (Wise and Bozarth, 1987; Pierce and Kalivas, 1997). Furthermore, pharmacologic antagonism of mesolimbic neurotransmitter function, particularly of its dopaminergic component, also impairs the development of reward to drugs of abuse (Wise and Bozarth, 1987; Hyman and Malenka, 2001). Importantly, recent functional-MRI brain-imaging studies demonstrate that the same neural circuitry is activated following drug injection in humans as has been observed in rodents (Hyman and Malenka, 2001). Furthermore, the same or
similar anatomy and connections found in the rodent mesolimbic system are present in
the human brain, giving credence to experiments conducted in rodent models of drug
abuse (Heimer et al., 1997b). Thus, the function of multiple regions included in the
mesolimbic system appears to be critical for the development of drug addiction in both
animal models and in humans.

Despite the strong evidence that implicates the mesolimbic system in the
development of drug addiction, precisely how the system works to produce this aberrant
behavior has been harder to define. The mesolimbic system is highly interconnected both
with itself and with other brain regions, and much effort has gone into determining
precisely what connections exist between brain regions and are important in the
development of drug addiction. Progress has been made in understanding this system,
particularly regarding the central role of the Nacc. Thus, the remainder of this
introduction will concentrate on the function of the Nacc in the response to drugs of
abuse, leading ultimately to the hypothesis and experiments pursued in this thesis.

The nucleus accumbens--overview

The Nacc represents an integrative center within the mesolimbic system,
receiving input from and projecting to numerous brain regions (see Figure 1). Within the
mesolimbic system itself, the Nacc receives projections from or provides output to the
VTA, PFC, thalamus, and VP (Pierce and Kalivas, 1997; Groenewegen et al., 1999). The
Nacc also has numerous important afferent or efferent projections with brain regions
outside the mesolimbic system including the hippocampus, amygdala, median raphe
nucleus, and the nucleus of the solitary tract (Groenewegen et al., 1999). Indeed, the
Nacc has connections with most regions of the brain, and these numerous and varied
projections hint at the central integrative role of the Nacc. Put into a general functional context, the connections with the mesolimbic system give the Nacc access to motivated behavior; interaction with the prefrontal cortex allows influence from cortical function; connectivity with the hippocampus allows involvement of memory; the connections with the amygdala demonstrate integration with emotional significance; and projections with the thalamus, raphe nucleus, and nucleus of the solitary tract allow influence of and on autonomic function. Thus, the Nacc is uniquely placed to have access to ‘information’ of many types via its interconnections with multiple brain regions. Numerous experiments have revealed this ability of the Nacc, through its many connections, to be involved in multiple types of behavior ranging from sexual activity to food-based reward to fear (Pecina and Berridge, 2000; Bradley and Meisel, 2001; Reynolds and Berridge, 2001). It is the importance of the Nacc in the response to drugs of abuse, however, that has received the greatest attention in the literature and is the focus of this thesis.

**The nucleus accumbens--role in drug addiction**

Strong evidence implicates the Nacc as a focal point in the brain’s response to all drugs of abuse, particularly regarding Nacc dopaminergic function. As previously mentioned, studies have shown that lesions of this region impair the development of addiction to multiple classes of drugs of abuse in animal models. In addition, animals will self-administer multiple types of drugs of abuse directly into the Nacc (Bardo, 1998). Further studies have demonstrated increases in synaptic dopamine within the Nacc in response to all classes of drugs of abuse (Koob et al., 1998). Additionally, pharmacologic inhibition of Nacc dopaminergic function and knock-outs of dopamine receptors, particularly the D1-subtype, diminish multiple types of behavioral responses to drugs of
abuse (Xu et al., 1994; Koob et al., 1998; Xu et al., 2000). Taken together, these numerous studies suggest that the Nacc is central to the response to all drugs of abuse, and that Nacc dopaminergic function in particular is critical in this process.

A caveat to these conclusions, however, arises from studies demonstrating the tremendous heterogeneity of neurons within the Nacc. From the distribution of expression of numerous proteins, to afferent and efferent connection patterns, to neurotransmitter responses (particularly dopamine), to involvement in specific behaviors, the Nacc demonstrates remarkable heterogeneity (specific examples will be discussed in the following sections). Thus, to refer to the Nacc as a single unit is increasingly incorrect and insufficient to understand the roles of specific divisions within this brain region.

Though a large and historic literature has demonstrated the importance of the Nacc as a whole in the response to drugs of abuse, less attention has been paid to the responses of Nacc sub-regions. These regional responses require a relatively detailed understanding of key aspects of Nacc anatomy.

**The nucleus accumbens--anatomy**

The heterogeneity of Nacc anatomy is evidenced even at its most fundamental anatomic level--the neuron. The cells that comprise the Nacc are organized primarily as clusters surrounded by more sparse populations of cells (Jongen-Releo et al., 1994; Pennartz et al., 1994). Furthermore, specific proteins are expressed in clusters as well. For instance, mu-opiate receptors, enkephalin, and substance P are each expressed in patches of intense expression surrounded by areas of low expression in the Nacc (Pennartz et al., 1994). Importantly, individual clusters of cells in the Nacc appear to maintain afferent and efferent connections unique to that given cluster. This clustering of
input and output projections may have functional implications. Projections from multiple
brain regions can influence the state of polarization of neurons in a specific cluster, which
in turn influences the ability of such neurons to fire an action potential in response to a
given input stimulus (Pennartz et al., 1994). Such a mechanism of convergent influence
on the firing of clusters of cells could be responsible for the ‘gating’ of the ability of
Nacc neurons to fire by hippocampal influence on excitatory PFC-to-Nacc input
(O'Donnell and Grace, 1995). It is difficult, however, to precisely determine the events
occurring in a cluster of Nacc neurons due to the limitations of current experimental
techniques. Nonetheless, this example serves to illustrate the importance of region-
specific functional analysis within the Nacc. Current techniques can, however, determine
what is occurring regionally within the Nacc at a more broad level of investigation than
that of the individual neuron or cluster of neurons.

The core and shell divisions are the most widely studied anatomic sub-regions of
the Nacc. These regions were initially identified by differences in patterns of
acetylcholinesterase activity and cholecystokinin immunoreactivity, but are best
categorized by differences in expression of calbindin (Zaborszky et al., 1985; Jongen-
Relo et al., 1994). Further studies have identified differences in functional responses and
connectivity in the Nacc core and shell. In particular, inputs to and outputs from the two
divisions suggest that the core may be more involved in motor function while the shell
may be more involved in motivation and reward (Heimer et al., 1997b). This presumed
functional difference has been substantiated in the response of levels of energy
metabolism and dopamine in the two divisions following drug administration.
Specifically, many drugs of abuse result in a preferential increase in dopamine levels and
energy metabolism in the shell division of the Nacc compared with the core (Di Chiara, 1999). Thus, functional distinctions seen between core and shell divisions of the Nacc further illustrate the need for studies of the Nacc to take into account regional anatomy.

In addition to core vs. shell distinctions, the functional anatomy of the Nacc also includes anterior-posterior (or rostral-caudal) specificity. Anterior-posterior differences in the specificity and topography of afferent and efferent Nacc connections have been noted (Phillipson and Griffiths, 1985; Oades and Halliday, 1987; Usuda et al., 1998). In addition, D1 and D2-dopamine-receptors, GAD_{67} (the enzyme important in production of the neurotransmitter GABA), and preproenkephalin are all expressed in a decreasing rostral-caudal gradient in the Nacc (Bardo and Hammer, 1991; Rogard et al., 1993). Recently, a functional difference between rostral and caudal Nacc has also been identified wherein injections of GABA_{A} agonist into the rostral Nacc induced eating behavior while the injections into the caudal Nacc elicited defensive behavior (Reynolds and Berridge, 2001). Interestingly, the apparent importance of rostral-caudal Nacc regional heterogeneity has not received much attention within the drug abuse field. This oversight has potential consequences regarding claims made about the central role of the Nacc in response to drugs of abuse. Thus, one of the goals of this thesis was to address this concern by examining the rostral-caudal spatiotemporal pattern of drug-induced Nacc stimulation using markers of neuronal activation.

**The nucleus accumbens--markers of neuronal activation**

Fos, the protein product of the immediate early gene (IEG) *c-fos*, has been used for some time as a marker for neuronal activation (for review, see (Sheng and Greenberg, 1990)). For example, Fos induction in neuron-like cell lines has been demonstrated
following application of neurotransmitters, growth factors, and electrical excitation.
Furthermore, Fos induction by seizures, convulsant drugs, and peripheral sensory stimulation has been demonstrated throughout the CNS. Taken together, these findings suggest that induction of Fos can indicate trans-synaptic neuronal stimulation.
Importantly, Fos induction has also been noted in the brain in response to systemic administration of multiple classes of drugs of abuse (for review, see (Harlan and Garcia, 1998)). In particular, such Fos induction by drugs of abuse has been widely observed in the Nacc, where dopamine appears to play a key role in this process. The previously detailed increase in Nacc dopamine following the administration of any class of drug of abuse leads to activation of post-synaptic dopamine receptors. This dopaminergic agonism, particularly of the D1-subtype receptor (D1R), has been shown to be crucial in induction of Nacc Fos expression by multiple drug classes (Liu et al., 1994; Harlan and Garcia, 1998; Xu et al., 2000). Once the D1R is activated by dopamine, a second messenger cascade involving stimulatory G-proteins, adenylyl cyclase, cAMP, and protein kinase A leads ultimately to Fos induction (Koob et al., 1998). Thus, the induction of Fos expression within the Nacc has provided a useful tool by which drug-induced neuronal stimulation can be assayed.

Despite the extensive literature using Fos as a marker for Nacc neuronal activation, there are specific problems with this assay in the context of drug responsiveness. First, studies have reported conflicting levels of Nacc Fos responses following acute injection of drugs of abuse, particularly regarding morphine (Liu et al., 1994; Nye and Nestler, 1996; Harlan and Garcia, 1998). Furthermore, the temporal pattern of Nacc Fos expression after acute drug injection can be variable, again
particularly regarding morphine (Graybiel et al., 1990; Garcia et al., 1995; Nye and Nestler, 1996). In addition, relatively few studies have compared Fos induction following acute or chronic administration of different classes of drugs of abuse in the same study. As mentioned previously, few studies have taken rostral-caudal regional anatomy into account in analysis of Nacc activation. Very few studies have analyzed whether Fos is induced in Nacc projection neurons (i.e. whether Nacc output neurons are activated following administration of drugs of abuse). And finally, induction of Fos is desensitized over time following chronic administration of drugs of abuse, and thus can no longer be used as a marker for neuronal stimulation. Thus, in spite of the insights provided by using Fos as a marker of Nacc activation following administration of drugs of abuse, several important questions remain unanswered in this field. In particular, a detailed understanding of precisely which Nacc sub-regions are commonly activated by different classes of drugs is lacking. In an effort to answer some of these questions, we performed a series of experiments to analyze regional responses of the Nacc to systemic drug administration.

**The nucleus accumbens--regional analyses of response**

Regional stimulation of the Nacc by acute or chronic systemic administration of either cocaine or morphine was examined to accurately map the region-specific response of the Nacc to different classes of drugs. Several markers of acute or chronic neuronal activation were used to identify stimulated cells throughout rostral-caudal regions of the Nacc core and shell divisions. First, we analyzed rostral-caudal Nacc stimulation at several time-points following acute systemic administration of cocaine or morphine. Next, we compared the pattern of Nacc stimulation to that of other mesolimbic regions
following acute systemic administration of cocaine or morphine. In addition, we
examined the cellular phenotype of Nacc neurons stimulated by acute or chronic cocaine
or morphine. Finally, we explored the rostral-caudal pattern of Nacc stimulation
following chronic administration of cocaine or morphine.
CHAPTER 1

The nucleus accumbens (Nacc) is a key limbic region involved in affective and motivational aspects of behavior, and has been implicated in mechanisms underlying reward and addiction (Robinson and Berridge, 1993; Pierce and Kalivas, 1997). The role of the Nacc in mediating behavioral responses to drugs of abuse is based on evidence from a variety of studies (Nestler et al., 1993; Koob et al., 1998; Hyman and Malenka, 2001), including reports demonstrating increased activity of Nacc neurons following administration of drugs. Fos, the protein product of the immediate-early gene (IEG) c-fos, has been used in many of these studies as a marker for neuronal activation. Multiple drugs have been shown to induce Fos expression in neurons within Nacc sub-regions, including core and shell (Graybiel et al., 1990; Young et al., 1991; Liu et al., 1994; Garcia et al., 1995; Barrot et al., 1999).

While previous studies using Fos as a marker of activation suggest that different drugs act through common pathways, there are several caveats to this conclusion, particularly regarding Nacc activation by morphine. Studies have reported conflicting levels of Nacc Fos responses following acute injection of morphine (Liu et al., 1994; Garcia et al., 1995; Nye and Nestler, 1996; Barrot et al., 1999). Furthermore, the temporal pattern of Nacc Fos expression following morphine is unclear (Garcia et al., 1995; Nye and Nestler, 1996; Barrot et al., 1999). Thus, some controversy exists regarding Nacc activation following acute morphine administration. In addition, relatively few studies have directly compared opiate and psychostimulant-induced Nacc activation using the same drug-delivery paradigm.
To attempt to resolve some of these contradictions, the first goal of this study was to conduct a detailed examination of the pattern of activation of Nacc neurons in response to different classes of drugs of abuse. Activation throughout the rostral-caudal extent of the Nacc was examined following systemic administration of either of two classes of drugs, psychostimulant (cocaine) or opioid (morphine), at three time intervals following drug injection (ten minutes, one hour, or two hours). In this study, two markers of neuronal activation were used: expression of Fos, and phosphorylation of the MAP-kinases, ERK1 and ERK2. Phosphorylation of ERK (PERK) occurs rapidly in the Nacc in response to systemic cocaine (Valjent et al., 2000) and in culture in response to morphine (Kramer and Simon, 2000; Trapaidze et al., 2000). In addition, PERK has been implicated in the signaling pathways controlling expression of Fos, both in vivo (Valjent et al., 2000) and in vitro (Impey et al., 1999). Therefore, PERK provided a complementary marker to Fos to study Nacc neural activation shortly after administration of either cocaine or morphine.

A second goal of the current study was to determine whether cocaine or morphine activates Nacc neurons that project to the ventral pallidum (VP), a major efferent target of the Nacc (Groenewegen et al., 1996; Heimer et al., 1997a; Pierce and Kalivas, 1997) important in mediating behavioral responses to drugs of abuse (Hubner and Koob, 1990; Gong et al., 1996; Fletcher et al., 1998; Johnson and Napier, 2000). Previous work has shown that the psychostimulant, amphetamine, can induce Fos expression in VP-projecting neurons of the Nacc (Robertson and Jian, 1995). It is not clear, however, whether other classes of drugs, such as opioids, also induce activation of VP-projecting Nacc neurons. To address this question, we combined retrograde tract tracing from the
VP with immunocytochemical detection of Nacc Fos or PERK in rats following cocaine or morphine administration.

METHODS

Animals. Male Sprague-Dawley rats (Harlan) weighing 240-300g at the time of experiment were housed two animals per cage on a 12:12hr light:dark cycle (lights on at 6am) with food and water available ad libitum. All animal surgeries, drug injections, and perfusions were performed during light phase of the light:dark cycle. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Cincinnati, in accordance with NIH guidelines.

Ctb retrograde tracer injection. Rats were deeply anesthetized with 87% ketamine/13% xylazene (0.15ml/100g; i.p.) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). The retrograde tracer Ctb (1% in 0.1M PB, pH7.4; List Biological Laboratories, Campbell, CA) was injected unilaterally into VP (coordinates: AP -0.4mm, ML +2.2mm from bregma; DV -7.6mm from skull surface). A glass micropipette (tip diameter 15-20µm) filled with Ctb was lowered into the brain and the tracer was applied by iontophoresis (positive pulse of 5µA, 5sec on/off) for a period of 7.5min. After an additional period of 3min, the micropipette was removed and the scalp incision was closed with wound clips.

Protocol for drug administration. Animals were housed as described above for five to seven days following Ctb-injection surgery to allow for sufficient transport of tracer. Rats were then divided into one of nine treatment groups. The treatment groups differed by type of injection (saline, cocaine, or morphine) and time to sacrifice following injection.
Thus, the nine treatment groups consisted of animals that were sacrificed 10min after injection of saline (Saline-10min, n=8), cocaine (Cocaine-10min, n=4), or morphine (Morphine-10min, n=5); animals that were sacrificed 1hr after injection of saline (Saline-1hr, n=4), cocaine (Cocaine-1hr, n=4), or morphine (Morphine-1hr, n=4); and animals that were sacrificed 2hrs after injection of saline (Saline-2hr, n=3), cocaine (Cocaine-2hr, n=4), or morphine (Morphine-2hr, n=4).

Cocaine (cocaine hydrochloride; Sigma, St. Louis, MO; 20mg/kg; dissolved in 0.9% sterile saline), morphine (morphine sulfate; Spectrum Laboratory Products, Gardena, CA; 10mg/kg; dissolved in 0.9% sterile saline), or saline (0.9% sterile saline) were administered i.p. while the animal was in its home cage. Each animal was carefully observed following injection to confirm an appropriate behavioral response to the administered substance (for cocaine, increased locomotion; for morphine, sedation). At 10min, 1hr, or 2hrs following injection, animals were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and transcardially perfused with saline (100ml) followed by 4% paraformaldehyde in 0.1M PB (500ml). Brains were removed and post-fixed in the 4% paraformaldehyde preparation at room temperature for 1hr and subsequently stored in 20% sucrose at 4°C.

Immunoocytochemical (ICC) procedures. Coronal sections through the forebrain were cut with a freezing microtome at a thickness of 35µm, collected in four parallel series in cryoprotectant (Watson et al., 1986), and stored at -20°C until further processing. All incubations were performed at room temperature under gentle agitation.

PERK/Ctb dual immunofluorescence. For each animal sacrificed 10min post-treatment, one series of every fourth section through the Nacc and VP was processed for PERK
expression and Ctb labeling using a dual immunofluorescent protocol. The sections were washed extensively in 0.1M phosphate-buffered saline (PBS) between incubation steps. Sections were incubated 10min in 1% H₂O₂ in PBS and for 1hr in PBS with 0.4% Triton and 0.1% BSA (all incubations with were performed using reagents dissolved in this incubation solution). Next, sections were incubated with anti-PERK rabbit polyclonal antibody (1:400; 16hr incubation; Cell Signaling Technology, Beverly, MA), with biotinylated donkey anti-rabbit IgG (1:400; 1hr incubation; Jackson ImmunoResearch Laboratories, West Grove, PA), with avidin-biotinylated peroxidase complex (ABC Elite kit, 1:500; 1hr incubation; Vector Laboratories, Burlingame, CA), with biotinylated tyramine amplification solution (TSA Biotin System, 1:250; 10min incubation; NEN, Boston, MA), and with CY3-conjugated streptavidin (1:200; 30min incubation; Jackson). Subsequently, sections were incubated with anti-Ctb goat polyclonal antibody (1:2000; 18hr incubation; List) and with donkey anti-goat Alexa-Fluor-488 IgG (1:100; 30min incubation; Molecular Probes, Eugene, OR). Sections were then mounted on Superfrost slides (Fisher Scientific, Pittsburgh, PA), air dried, and coverslipped with cover glass (Corning, Big Flats, NY) using mountant containing DABCO (Sigma) to prevent fading of fluorescence.

**Fos/Ctb dual immunoperoxidase labeling.** For each animal sacrificed 1hr or 2hrs after injection, one series of every fourth section containing Nacc and VP was processed for Fos expression and Ctb labeling using the dual immunoperoxidase technique described below. Sections were incubated with H₂O₂ and with incubation solution as described above, and then incubated with anti-Fos rabbit polyclonal antibody (1:15,000; 16hr incubation; Santa Cruz Biotechnology, Santa Cruz, CA). Sections were incubated with
biotinylated donkey anti-rabbit IgG and with avidin-biotinylated peroxidase complex (1:1000) as described above. Sections were incubated with DAB (0.02%; 10min incubation; Sigma) in PB containing 0.08% nickel ammonium sulfate and 0.01% H2O2, blocked with 1% H2O2 in PBS, and incubated with anti-Ctb goat polyclonal antibody (1:30,000; 18hr incubation; List). Sections were incubated with biotinylated donkey anti-goat IgG (1:400) and with avidin-biotinylated peroxidase complex (1:1000) as described above. Sections were incubated 10min with 0.02% DAB in PB containing 0.01% H2O2, mounted on Superfrost slides (Fisher), dehydrated, and coverslipped with cover glass (Corning) using DPX mountant (Electron Microscopy Sciences, Washington, PA).

Calbindin (CalB) immunolabeling and Nissl-stain. In order to delineate the boundaries of the Nacc, and its core and shell divisions (Jongen-Relo et al., 1994), an adjacent series of sections from each animal was processed for CalB immunolabeling (1:40,000 mouse monoclonal antibody; Sigma; see above for DAB-staining protocol), mounted on Superfrost slides (Fisher), counterstained with Nissl-stain, and then dehydrated and coverslipped as described above.

*Data Analysis.*

Location of Ctb injection sites. To determine whether injection sites were limited to the VP, we analyzed the series of sections containing fluorescent or peroxidase-labeled Ctb, as well as the adjacent series of CalB/Nissl stained sections. The rostrocaudal placement of each injection site was evaluated using as landmarks the anterior commissure, corpus callosum, caudate putamen, globus pallidus, third ventricle, stria terminalis, fornix, and internal capsule. The core of an individual injection site was defined as that area in which tracer completely filled the neuropil.
Quantification of single and double-labeled cells in Nacc.

**Confocal microscopic analysis of PERK/Ctb dual immunofluorescence.** Three brain sections from each animal processed for PERK and Ctb immunofluorescence were chosen to represent rostral, middle, and caudal levels through Nacc. These sections corresponded approximately to +2.15mm, +1.45mm, and +0.95mm anterior to bregma, according to the rat brain maps of Swanson (Swanson, 1998). Multiple images covering, in aggregate, the entire Nacc for each section were captured with a laser-scanning confocal microscope system (Zeiss LSM510, Carl Zeiss Microimaging, Thornwood, NY) at 10x magnification. CY3-fluorescence (PERK) was imaged with a 567nm emission filter and a HeNe laser, and Alexa 488-fluorescence (Ctb) was imaged with a 505nm emission filter and an Argon laser (488nm). Adjacent sections processed for CalB immunoreactivity and Nissl-staining were used to identify core and shell regions within Nacc and their boundaries (Figure 1). Fluorescence images were printed and a collage was made of all Nacc images for a given section. PERK-IR cells were identified by red CY-3 fluorescence; Ctb-IR cells were identified by green Alexa-488 fluorescence; and PERK+Ctb-IR dual-labeled cells were identified by colocalization of red and green fluorescence in the same cell (Figure 2). The number of PERK-IR, Ctb-IR, and PERK+Ctb-IR cells was counted within core and shell regions for each Nacc section.

**Light microscopic analysis of Fos/Ctb chromogenic dual immunolabeling.** Three brain sections from each animal processed for Fos and Ctb were chosen to represent rostral, middle, and caudal levels through Nacc according to the same guidelines listed above. Camera lucida drawings were made at 20x magnification using a drawing tube attached to a Leica DMR microscope (Leica Microsystems, Germany) to map Fos-IR, Ctb-IR, and
Fos+Ctb-IR neurons (Figure 3). Fos-IR cells were identified by the presence of dark-purple nuclear staining; Ctb-IR cells were identified by brown cytoplasmic staining; Fos+Ctb-IR dual-labeled cells were identified by presence of both nuclear Fos and cytoplasmic Ctb (Figure 4). As in the analysis of PERK/Ctb, adjacent CalB/Nissl-stained sections were used to help in the identification of core and shell subdivisions within the Nacc. The number of Fos-IR, Ctb-IR, and Fos+Ctb-IR cells was determined within core and shell regions for each Nacc section.

_Digital Images._ Digital images of immunostained sections were captured using a digital camera (MagnaFire, Optronics, Goleta, California) attached to a Leica DMR microscope (Leica). Images were imported into Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA) to compose the figures. Images were not altered, except for minor adjustment of brightness.

_Statistical analysis._ All analyses were conducted using StatView (V4.5, Abacus Concepts, Cary, NC). For each treatment group, the mean total number of single-labeled cells was calculated for each Nacc sub-region (core and shell in each rostral, middle, or caudal section). In addition, for each treatment group, the mean percentage of double-labeled cells (PERK+Ctb or Fos+Ctb) was calculated in each Nacc subdivision as a percentage of Ctb-labeled cells. Means for each treatment group were also summed over rostral, middle, or caudal Nacc regions (sum of core+shell means within rostral, middle, or caudal regions for a treatment group) and over core or shell Nacc regions (sum of rostral+middle+caudal means within each core or shell region for a treatment group).

Analyses of the mean number of PERK-IR cells and the percentage of double-labeled PERK+Ctb-IR cells were performed using one-way ANOVA with drug as the
factor and Fisher’s least significant difference (LSD) *post-hoc* test. Analyses of the mean number of Fos-IR cells and the percentage of double-labeled Fos+Ctb-IR cells were performed using two-way ANOVA, with drug and time as factors, and Fisher’s LSD *post-hoc* test. Significance for all comparisons was set at *p*<0.05.

**RESULTS**

**Tracer injection sites**

Based on examination of Ctb and Nissl-stained series, 40 of 67 rats that received Ctb injections had sites that were limited almost entirely to the VP with minimal spread to adjacent areas (Fig. 5). The number of animals in each treatment group that had injections limited to the VP is shown in Table 1. While individual injections varied in their precise rostrocaudal placement, a majority of them were located at levels corresponding to −0.26 to −0.83 mm posterior to bregma, according to the rat brain maps of Swanson (Swanson, 1998).

The mean total number of Ctb-labeled neurons found within the Nacc following these tracer injections is summarized in Table 1. Few statistically significant differences in the absolute numbers of Ctb-labeled neurons were found between compared treatment groups; however, to account for this variation, all results regarding PERK and Fos expression in projection neurons were analyzed as percentages of Ctb-labeled cell populations in Nacc.

**PERK expression in Nacc**

There was a significant increase in the mean number of PERK-positive cells in the Nacc following injection of cocaine, but not following administration of morphine
Cocaine increased PERK expression in all divisions of the Nacc analyzed: rostral, middle, and caudal Nacc, in both core and shell regions (Figure 6; Table 2A).

Furthermore, cocaine, but not morphine, significantly increased the percentage of double-labeled PERK/Ctb cells in the Nacc (Figure 7). Specifically, cocaine induced PERK expression in VP-projecting neurons in the core region of Nacc (Figure 7, p<0.05; Table 2B). While there was tendency toward increased percentages of double-labeled PERK/Ctb cells in the rostral and middle Nacc for cocaine-injected animals, these differences did not reach statistical significance (p=0.15 rostral Nacc, p=0.055 middle Nacc).

**Fos expression in Nacc**

Cocaine induced Fos expression in the rostral and middle Nacc regions at one hour following injection, and in all three rostrocaudal subdivisions at two hours (Figure 8, p<0.05; Table 3A). Fos induction two hours post-cocaine-treatment was significantly higher than at one hour post-treatment in middle and caudal Nacc (Fig. 8, p<0.01), but not in the rostral Nacc. Furthermore, cocaine induced Fos expression in both core and shell Nacc regions at two hours post-treatment (Figure 9, p<0.01). There was a trend toward increased Fos expression at one hour following cocaine injections in both the core (p=0.056) and shell (p=0.056), but this difference did not reach statistical significance. Finally, morphine induced Fos expression in the Nacc, but only in rostral Nacc and only at two hours following injection (Figure 8, p<0.05). There was a trend toward increased Fos expression at two hours after morphine injection in both core and shell Nacc regions,
but this did not reach statistical significance (Figure 9, core Nacc $p=0.079$ and shell Nacc $p=0.088$).

Cocaine, but not morphine, induced Fos expression in VP-projecting cells of the Nacc (Figures 10 and 11; Table 3B). Administration of cocaine resulted in an increase in the percentage of VP-projecting neurons expressing Fos in the rostral and caudal Nacc at one hour following injection, and in the middle and caudal Nacc at two hours (Fig 10, $p<0.05$). When double-labeled cells were analyzed by their location in either the core or shell divisions of the Nacc, cocaine injections increased the percentage of VP-projecting neurons in both core and shell at one hour following injection and in the core region at two hours (Fig 11, $p<0.05$). By comparison to cocaine, morphine failed to increase the percentage of VP-projecting Nacc neurons that expressed Fos in any Nacc subdivision (Figs. 10 and 11), even in the rostral Nacc at two hours where morphine increased the mean number of Fos-positive cells (Fig. 8, panel A).

**DISCUSSION**

The results of the present study demonstrate that two classes of drugs, i.e. psychostimulants and opioids, result in different temporal and spatial patterns of neural activation in the Nacc. Specifically, cocaine induced neural activation at all time intervals studied, namely ten minutes, one hour, and two hours. In contrast, morphine only induced activation at two hours following administration. Moreover, cocaine induced activation in all subregions of the Nacc, including core and shell in all rostral-caudal subdivisions. Morphine, on the other hand, only induced activation in rostral Nacc. Finally, cocaine, but not morphine, activated a subset of Nacc neurons that project to the VP, one of the
major efferent targets of the Nacc. Together, these results demonstrate that cocaine and morphine administration differentially activate the Nacc, with the exception of neurons in its rostral subregion.

In general, the patterns of Fos expression in the Nacc we observed following cocaine administration are similar to those reported in previous studies. For example, we observed cocaine-activated neurons in both shell and core divisions of the Nacc at one and two hours following administration; similarly, previous studies report cocaine-induced Fos expression in both divisions of the Nacc as early as 45 minutes following injection, with an increase at two hours (Graybiel et al., 1990). The temporal pattern of morphine-induced Fos in the Nacc that we observed is similar to previous reports of morphine-induced Fos in the Nacc at two hours (Barrot et al., 1999) but not at one hour following injection (Nye and Nestler, 1996). Other studies, however, have either failed to observe Fos induction (Nye and Nestler, 1996), or report minimal induction (Liu et al., 1994), two hours following a single morphine injection. Since we detected morphine-induced Fos only in the rostral Nacc, it is possible that the failure of some earlier studies to observe significant changes in Fos expression following a single morphine injection was due either to analyses that grouped all rostral-caudal levels of the nucleus, or to samples that excluded rostral levels through the Nacc.

While there have been multiple previous reports of cocaine and morphine-induced Fos in the rat Nacc, the present results provide a first demonstration of acute cocaine-induced PERK in rat Nacc, and parallel a previous report of cocaine-induced PERK in mice (Valjent et al., 2000). In contrast to the effects of cocaine, morphine failed to induce PERK in any Nacc region. Given the presence of mu-opioid receptors in the Nacc
(Mansour et al., 1995; Moriwaki et al., 1996; Svingos et al., 1996), it might be questioned as to whether phosphorylation of ERK is a normal consequence of mu-opioid receptor activation. However, studies in cell culture have shown that activation of mu-opioid receptors by morphine and other mu-opioid receptor agonists results in significant increases in phosphorylation of ERK (Kramer and Simon, 2000; Schmidt et al., 2000; Trapaidze et al., 2000). Taken together, the failure of morphine to induce PERK ten minutes after injection, and the absence of morphine-induced Fos at one hour, suggests that morphine may not be directly activating the Nacc, despite the presence of mu-opioid receptors within Nacc. Instead, morphine may activate Nacc neurons indirectly by influencing dopaminergic afferents in the VTA. There is strong evidence suggesting that the increase in dopamine seen in the Nacc following systemic injection of morphine (Barrot et al., 1999; Szumlinski et al., 2000) is due to mu-opioid receptor activation in VTA. Specifically, morphine, acting upon mu-opioid receptors on GABAergic interneurons of the VTA, leads to the disinhibition of dopaminergic neurons projecting to Nacc (Di Chiara and North, 1992). The VTA-mediated increase in Nacc dopamine release activates D1-receptors in the Nacc leading to the induction of Fos expression (Liu et al., 1994). Thus, opiates are capable of activating Nacc neurons by indirectly activating dopaminergic VTA projection neurons.

Cocaine, on the other hand, blocks dopamine uptake directly in the Nacc (Izenwasser et al., 1990) leading to local increases in dopamine levels at synapses throughout this nucleus (Wise and Bozarth, 1987). Thus, systemic cocaine injection leads to induction of Fos and PERK expression through activation of Nacc D1-receptors (Pierce and Kalivas, 1997; Valjent et al., 2000). Unlike morphine, cocaine mediates these
effects by acting within the Nacc. In addition, the increase to peak Nacc dopamine levels following cocaine administration is more rapid than following morphine (Kalivas and Duffy, 1993; Barrot et al., 1999; Szumlinski et al., 2000). Thus, the early induction of PERK and Fos by cocaine, and the lack of such induction by morphine, could be a result of differences in the temporal pattern of Nacc dopamine release following drug administration. Therefore, it is possible that the window for PERK induction following morphine injection was missed in this study, and that a later time-point of analysis is necessary to observe this induction.

A growing number of studies suggest that the core and shell subdivisions of the Nacc possess distinct functional properties (Heimer et al., 1997b). In the present study, no qualitative differences were observed in the trends of neural activation between core and shell following drug administration. In contrast, a rostral-caudal distinction within the Nacc was observed in that the rostral Nacc was the only subregion activated by morphine. Indeed, several earlier findings have suggested that the rostral Nacc is functionally distinct from caudal regions. For example, D1 and D2-receptors are distributed more densely in rostral than caudal portions of the Nacc (Bardo and Hammer, 1991). Moreover, neurons in the rostral Nacc exhibit higher levels of GAD₆₇ and preproenkephalin mRNAs than those in the caudal Nacc (Rogard et al., 1993), and they display an altered c-fos RNA-induction pattern following chronic v. acute injection of psychostimulants and opiates (Curran et al., 1996). In addition, naturally-occurring motivated behaviors such as food intake appear to be preferentially mediated by the rostral Nacc (Reynolds and Berridge, 2001). Finally, the rostral Nacc may receive distinct projections, including from rostral VTA (Oades and Halliday, 1987), that could
contribute to its function. Any or all of these differences may account for the regional specificity of the rostral Nacc with respect to its response to morphine in this study. For example, the greater density of D1 and D2-receptors in the rostral Nacc may make this subdivision more responsive to the lower levels of DA released by morphine compared to cocaine (Barrot et al., 1999). Finally, the fact that the rostral Nacc was the only subdivision of this nucleus to be activated by both cocaine and morphine, albeit with different time course, suggests that it may be an important mediator for common behavioral or rewarding effects of drugs and natural behaviors.

The final result from this study is that cocaine, but not morphine, induced PERK and Fos in a subpopulation of Nacc neurons that project to the VP. Activated VP-projecting neurons were present in all rostrocaudal subregions of the Nacc, and in both core and shell. Nonetheless, cocaine activated a relatively small subset of VP-projecting neurons (between 2-9%). The percentage of Nacc VP-projecting neurons activated by cocaine in the present study closely approximates the percentage of VP-projecting neurons activated by amphetamine (Robertson and Jian, 1995). Thus, acutely injected psychostimulants may require only limited activation of the Nacc-VP connection to elicit their behavioral effects. Alternatively, the absence of PERK and/or Fos may not necessarily indicate the lack of participation of VP-projecting neurons in these effects, and may simply reflect a limitation of the use of these markers. It is unlikely that the low percentage of activated projection neurons in the present study was a result of the use of tract tracer Ctb since the uptake of this tracer does not appear to alter neuronal activation as measured by the use of Fos (Coolen et al., 1998). In addition, the prior study demonstrating amphetamine-induced activation in VP-projecting neurons showed similar
percentages of double-labeling, even though a different retrograde tract tracer (Fluorogold) was used. Finally, given that cocaine activated only a small percentage of VP-projecting neurons, the phenotypic identity and projections of the majority of cocaine-activated neurons in Nacc remains to be determined. Some of these cells may be cholinergic interneurons of the Nacc (Consolo et al., 1999; Hikida et al., 2001), while others may be D1-receptor-containing Nacc neurons that project to the VTA (Robertson and Jian, 1995; Lu et al., 1998). Additional combined tract tracing and immunocytochemical studies will be needed to address these and other possibilities.

In summary, our results indicate that cocaine and morphine produce different temporal and regional patterns of neuronal activation in the Nacc, with cocaine producing a much more rapid and widespread pattern of activation after acute systemic injection than morphine. The only area of overlap observed between the patterns of activation produced by either type of drug was in the rostral Nacc, suggesting that this subdivision may be particularly important as a mediator of reward and addiction. Finally, similar to previous findings for amphetamine, we observed cocaine-induced activation in only a small percentage of Nacc neurons that project to the VP. Thus, the activated neurons revealed by Fos or PERK in the Nacc may be cells that are either predominantly interneurons, or possess projections to other targets of the Nacc besides the VP.
Figure 1. Core vs. shell divisions in rostral, middle, and caudal Nacc regions distinguished by calbindin D28K (CalB) expression and Nissl counterstain. The three photomicrographs are representative coronal sections through rostral (panel A), middle (panel B), and caudal (panel C) Nacc. The core is distinguished by intense brown CalB labeling, while shell is distinguished by little or no CalB labeling (see Methods). Core and shell divisions are outlined with dashed lines. ac, anterior commissure; Co, core; LV, lateral ventricle; Sh, shell. Scale bar, 200 μm.
**Figure 2.** PERK and Ctb dual immunofluorescence. Confocal images (2μm optical section) showing neurons labeled for either PERK (panel A, CY3 immunofluorescence), Ctb (panel B, Alexa-488 immunofluorescence), or both (panel C) in the core of the Nacc in a cocaine-treated animal. The arrow indicates a cell that is double-labeled for both PERK and Ctb; adjacent single-labeled cells are also seen. Scale bar, 20μm.
Figure 3. Camera lucida drawings of Fos and Ctb dual labeling. Three representative drawings made at 20x magnification are from cocaine-1hr subjects and depict rostral (panel A), middle (panel B), and caudal (panel C) levels of the Nacc. Black dots are Fos-IR cells, gray dots are Ctb-IR cells, and open circles are Fos+Ctb-IR dual-labeled cells. Core and shell divisions were determined by processing adjacent sections for CalB and Nissl (see Methods and Fig. 1), and these areas are labeled in each drawing. Similar drawings were made for PERK-Ctb dual immunofluorescence directly on printed 10x magnification confocal image-collages of Nacc sections (see Methods). ac, anterior commissure; Co, core; Sh, shell. Scale bar, 200\(\mu\)m.
Figure 4. Fos and Ctb chromogenic dual immunolabeling. A, Low-magnification (10x) photomicrograph of dorsomedial Nacc core from a cocaine-1hr subject. B, Higher power (40x) image of same section shown in panel A. Single-labeled Fos-IR cells are indicated by dark-blue nuclear labeling (e.g., black arrowhead); Ctb-IR cells are indicated by a brown ring of cytoplasm (e.g., white arrow); Fos+Ctb-IR dual-labeled cells (e.g., solid arrow) contain both dark-blue nuclear and brown cytoplasmic labeling. ac, anterior commissure. Scale bars: panel A, 200 μm; panel B, 50 μm.
Figure 5. VP Cfo-injection sites. The three photomicrographs are representative coronal sections of Cfo-injections from subjects that received saline (panel A), cocaine (panel B), or morphine (panel C), and were sacrificed 1 hr or 2 hrs following treatment. ac, anterior commissure; fx, fornix; LV, lateral ventricle; ST, stria terminalis; V3, third ventricle. Scale bar, 500µm.
Figure 6. Mean numbers of PERK-IR cells (±SEM) in Nacc after saline, cocaine or morphine injection. The data were summed across rostral, middle, or caudal Nacc (panel A) and core or shell Nacc (panel B) for each 10 min post-treatment group (see Methods). Statistical comparisons: * $p<0.01$, significantly different from both saline and morphine-treated groups; Fisher’s LSD test.
Figure 7. Mean percentage of Ctb-labeled cells (±SEM) in Nacc that co-expressed PERK after saline, cocaine, or morphine injection. The data were summed across rostral, middle, or caudal Nacc (panel A) and core or shell Nacc (panel B) for each 10min post-treatment group (see Methods). Statistical comparisons: * p<0.05, significantly different from both saline and morphine-treated groups; Fisher’s LSD test.
Figure 8. Mean numbers of Fos-IR cells (±SEM) in rostral, middle, and caudal Nacc after saline, cocaine, or morphine injection. The data were summed across rostral (panel A), middle (panel B), or caudal (panel C) Nacc for each 1hr or 2hr post-treatment group (see Methods). Statistical comparisons: * p<0.05, significantly different from the saline-treated group for the same time-point; ** p<0.01, significantly different from the cocaine-1hr group; *** p<0.01, significantly different from the morphine-treated group for the same time-point; Fisher’s LSD test.
Figure 9. Mean numbers of Fos-IR cells (±SEM) in core or shell Nacc after saline, cocaine, or morphine injection. The data were summed across core (panel A) or shell (panel B) Nacc for each 1hr or 2hr post-treatment group (see Methods). Statistical comparisons: * $p<0.01$, significantly different from the saline-treated group for the same time-point; ** $p=0.01$, significantly different from the cocaine-1hr group; *** $p=0.05$, significantly different from the morphine-treated group for the same time-point, Fisher’s LSD test.
Figure 10. Mean percentage of C-fos-labeled cells (±SEM) in rostral, middle, or caudal Nacc that co-expressed Fos after saline, cocaine, or morphine injection. The data were summed across rostral (panel A), middle (panel B), or caudal (panel C) Nacc for each 1 hr or 2 hr post-treatment group (see Methods). Statistical comparisons: * $p<0.05$, significantly different from the saline-treated group for the same time-point; ** $p<0.05$, significantly different from the cocaine-1 hr group; *** $p<0.05$, significantly different from the morphine-treated group for the same time-point; Fisher’s LSD test.
Figure 11. Mean percentage of C-fos-labeled cells (±SEM) in core or shell Nacc that co-expressed Fos after saline, cocaine, or morphine injection. The data were summed across core (panel A) or shell (panel B) Nacc for each 1hr or 2hr post-treatment group (see Methods). Statistical comparisons: * p<0.05, significantly different from the saline-treated group for the same time-point; ** p<0.01, significantly different from the cocaine-1 hr group; *** p<0.05, significantly different from the morphine-treated group for the same time-point; Fisher’s LSD test.
CHAPTER 2

The nucleus accumbens (Nacc) is thought to be a common mediator of responses to drugs of abuse, but little is known about the cellular identity of drug-sensitive target neurons in the Nacc. Furthermore, few studies have explored whether multiple classes of drugs activate the same Nacc cells. In the previous study, we used Fos as a marker for neuronal activation to specifically map cellular patterns of Nacc stimulation in response to either cocaine or morphine. We found that while acute systemic administration of cocaine resulted in widespread activation of neurons within all divisions of the Nacc, morphine produced a pattern of activation that was restricted to parts of the rostral Nacc. In addition, morphine did not activate Nacc neurons that send axonal projections to the ventral pallidum (VP), a major output of the Nacc. In contrast, cocaine induced Fos expression in some VP-projecting neurons in all divisions of the Nacc. The percentage of Fos-expressing neurons that projected to the VP was very small (<10%), however, suggesting that many of the Nacc cells we observed to be activated by cocaine could be interneurons rather than projection neurons.

Two questions arose from these initial findings. First, since a majority of cocaine-activated cells in the Nacc were not projection neurons, what is their neurochemical phenotype (and is this common between drugs)? And second, is the difference in the pattern of Nacc activation by acute cocaine and morphine unique to this brain region, or is this difference also seen in other limbic brain regions?

As an approach to the first question, we examined whether Fos was induced in Nacc cholinergic interneurons by acute cocaine or morphine. Several recent lines of
evidence indicate that Nacc cholinergic interneurons are involved in the behavioral response to drugs of abuse. Ablation of cholinergic cells in Nacc leads to increased behavioral sensitivity to both acute and chronic cocaine (Hikida et al., 2001). Furthermore, acute and chronic cocaine and morphine treatments lead to alterations in the release of Nacc acetylcholine (ACh) (Wilson et al., 1994; Consolo et al., 1999; Fiserova et al., 1999). Finally, rats will self-administer a cholinergic agonist directly into the Nacc (Ikemoto et al., 1998), indicating the potential of a direct involvement of ACh in reward processes. Thus, to see if any of the Nacc cells activated by acute cocaine and morphine are cholinergic interneurons, we used dual immunocytochemistry for Fos and choline acetyltransferase (ChAT), the enzyme required for ACh synthesis from the dietary precursor, choline. Induction of Fos in ChAT-immunoreactive (ChAT-IR) cells was analyzed using brain sections from animals that had received acute injections of cocaine or morphine in our previous study. In addition to determining whether acutely-activated Nacc cells might be cholinergic, we also examined Nacc cells from animals that had received chronic treatment with cocaine or morphine. In the case of chronic responses to these drugs, we used Fos Related Antigens (FRAs) as markers for Nacc stimulation (in the chronic situation it is FRAs, but not Fos, that are induced--see Chapter 3 for extensive discussion of FRA v. Fos induction following chronic drug administration). Thus, in chronically treated animals we asked whether FRA-expressing cells of the Nacc also contained ChAT.

The second question we addressed was whether cocaine and morphine produced different anatomical patterns of activation in other limbic system structures, as they did in the Nacc. Five brain regions were chosen for analysis based on their interconnections
with Nacc: prefrontal cortex (PFC), central amygdala (CEA), basolateral amygdala (BLA), and ventral tegmental area (VTA). These regions, like the Nacc, are thought to be involved in various aspects of limbic brain function, and specifically in the behavioral response to drugs of abuse (Bardo, 1998; Koob et al., 1998). In addition, each of these regions either receives dopaminergic innervation and expresses dopaminergic receptors (PFC, CEA, BLA) or contains dopaminergic cell bodies projecting to other brain regions (VTA) (Weiner et al., 1991; Lazarov et al., 1998). The dopaminergic influence in these regions is important since the limbic dopaminergic system is known to be involved in mediating both behavioral responses (Joel and Weiner, 2000) and the Fos-induction response to drugs of abuse (Harlan and Garcia, 1998). Thus, immunocytochemistry was used to analyze Fos expression in the PFC, CEA, BLA, and VTA of rats that received acute systemic injections of either cocaine or morphine in our previous study.

**METHODS**

*Animals.* Male Sprague-Dawley rats (Harlan) weighing 240-300g at the time of experiment were housed two animals per cage for acute studies, and housed individually for chronic studies, on a 12:12hr light:dark cycle (lights on at 6am) with food and water available ad libitum.

*Protocol for drug administration.* The rats were divided into one of nine treatment groups. The treatment groups differed by type of injection (saline, cocaine, or morphine), the acute or chronic nature of injection, and the time to sacrifice following acute injection. Thus, the nine treatment groups consisted of animals that were sacrificed: 1hr after acute injection of saline (acute-saline-1hr), cocaine (acute-cocaine-1hr), or
morphine (acute-morphine-1hr); animals that were sacrificed 2hrs after acute injection of saline (acute-saline-2hr), cocaine (acute-cocaine-2hr), or morphine (acute-morphine-2hr); and animals that were sacrificed 2hrs after chronic injection of saline (chronic-saline), cocaine (chronic-cocaine), or morphine (chronic-morphine). Cocaine (acute=20mg/kg, chronic=10mg/kg; dissolved in 0.9% sterile saline), morphine (10mg/kg for acute and chronic; dissolved in 0.9% sterile saline), or saline (0.9% sterile saline) were administered i.p. while the animal was in its home cage. Animals were sacrificed and perfused in the same manner as detailed in Chapter 1.

*Immunocytochemical (ICC) procedures and data analysis.*

Fos or FRA/ChAT dual immunoperoxidase labeling. To analyze induction of Fos in cholinergic interneurons of acutely treated rats and induction of FRA in cholinergic interneurons of chronically treated rats, a dual immunoperoxidase ICC protocol was used for Fos or FRA/ChAT double-labeling. The procedure was similar to the dual immunoperoxidase protocol described in Chapter 1, with the exception of the use of the anti-ChAT antibody (1:10,000; Chemicon) as the second primary antibody in this protocol, and the use of the anti-FRA antibody (Santa Cruz), which was used at a 1:10,000 dilution for the chronic animals as detailed in Chapter 3. One Nacc section (including core and shell) was examined per rostral-caudal level per animal, following the same anatomical guidelines described in Chapter 1. One animal per acute treatment group (n=1 animal each of acute-cocaine-1hr, acute-saline-1hr, acute-morphine-1hr, acute-saline-2hr, acute-cocaine-2hr, and acute-morphine-2hr groups) and per chronic treatment group (n=1 animal each of chronic-saline, chronic-cocaine, and chronic-morphine groups) was used for a total of n=9 animals included in this portion of the
study. Fos/ChAT double-labeling was examined in the acute animals, while FRA/ChAT double-labeling was analyzed in the chronic animals. Sections were analyzed at 20x magnification using the same microscope described in Chapter 1. Digital images of immunostained sections were captured using a digital camera (Optronics) attached to a Leica DMR microscope (Leica). Images were imported into Adobe Photoshop 6.0 (Adobe) to compose the figures. Images were not altered, except for minor adjustment of brightness.

**Fos single immunoperoxidase labeling.** To analyze induction of Fos in brain regions other than Nacc following acute systemic injection of drugs of abuse, we processed alternate sections for single-label detection of Fos (first part of the immunoperoxidase ICC protocol detailed in Chapter 1). Only animals in the acute-1hr treatment groups from Chapter 1 were analyzed. Thus, $n=4$ animals from each acute-saline-1hr, acute-cocaine-1hr, and acute-morphine-1hr treatment group (for a total of $n=12$) were used in this portion of the study. Immunopositive cells were counted within a standard area for each brain region using the same camera lucida setup described in Chapter 1. For each treatment group, the mean total number of Fos-IR cells was calculated for each brain region analyzed. Analysis of the mean numbers of Fos-IR cells was performed using one-way ANOVA with drug as the factor and Fisher’s least significant difference (LSD) *post-hoc* test. Significance for all comparisons was set at $p<0.05$. 
RESULTS

Lack of Fos/FRA induction in Nacc cholinergic interneurons

Although numerous ChAT-IR cells were seen throughout the Nacc in all cocaine and morphine-treated animals, no Fos or FRA immunoreactivity was detected in ChAT-IR cells of the Nacc following administration of either drug (Figure 1). This finding was true for both the acute (Fos-immunoreactivity; Figure 1A&C) and chronic (FRA-immunoreactivity; Figure 1B&D) drug administration settings, despite that overall induction of Fos and FRA was observed as expected. Furthermore, this same finding was observed at all rostral-caudal Nacc levels, and in both core and shell subdivisions (data not shown). In addition, there were no obvious differences in the intensity of ChAT staining within the Nacc, or in the numbers of ChAT-positive cells, related to either the type of drug or treatment.

Fos induction in brain regions other than the Nacc

Acute administration of cocaine significantly increased the number of Fos-expressing cells in CEA and VTA, while acute morphine failed to induce significant changes in Fos expression in any brain region tested (Figure 2). Although acute injection of cocaine produced a trend toward Fos induction in PFC, this change did not reach statistical significance (Figure 2A; \( p=0.09 \)).
DISCUSSION

Neither Fos nor FRA immunoreactivity was detected in ChAT-IR cells in the Nacc. This finding was true following both acute and chronic drug administration for both cocaine and morphine. Furthermore, the same results were observed throughout the Nacc in both core and shell divisions, and in rostral, middle, and caudal regions. In the analysis of drug-induced Fos expression in limbic regions other than the Nacc, acute cocaine induced Fos expression in the CEA and VTA, while acute morphine did not induce Fos in any brain region outside the Nacc.

Stimulation of Nacc cholinergic interneurons

The lack of Fos or FRA induction in Nacc ChAT-IR cells in response to any drug treatment suggests that these IEG protein products may not be suitable markers of stimulation for Nacc cholinergic interneurons. Given that previous studies examining drug-induced Fos expression in these interneurons are limited and have led to contradictory results, this finding is not necessarily surprising. Only one previous study has demonstrated induction of Fos in cholinergic interneurons, and this was observed only following acute injection of combined direct D1 and D2-dopamine-receptor agonists rather than drugs of abuse (Svenningsson et al., 2000). Thus, although Fos may be induced in cholinergic interneurons following application of dopaminergic agonists, the known increase in Nacc dopamine levels following administration of drugs of abuse (in combination with the complex and numerous other brain responses to drugs) does not appear to lead to the same mechanism of induction. Furthermore, to the best of our knowledge, no previous studies have examined the induction of FRAs in Nacc cholinergic interneurons following chronic drug administration. It is interesting that,
Despite the lack of expression of either FRA or Fos in cholinergic cells in this study, studies have shown changes in Nacc cholinergic function following drug administration. For example, previous studies have demonstrated an increase of Ach in the Nacc following acute injection of cocaine, and a decrease following acute morphine (Consolo et al., 1999; Fiserova et al., 1999). Furthermore, Nacc Ach release appears to diminish directly following chronic drug injection, indicating a shift in the Ach response to long-term drug administration (Wilson et al., 1994; Fiserova et al., 1999). Thus, previous findings combined with the present results indicate that neither an increase nor a decrease in drug-induced Nacc Ach release involves the induction of Fos or FRA in cholinergic interneurons.

**Differences in drug-induced activation of limbic system**

It is interesting that acute cocaine induced activation in limbic regions outside the Nacc while acute morphine did not. This finding is similar to the Fos patterns seen in the Nacc in Chapter 1 in response to the two drugs (with the exception of induction of Fos in rostral-Nacc by morphine). It must be noted that, in the present examination of brain-region activation outside the Nacc, only animals sacrificed one hour following drug administration were analyzed. If these brain regions are secondary targets for the effects of drugs, then it is possible that activation may not be observed until a later time point. Despite this caveat, the similarity in the Fos-responses observed throughout the limbic system demonstrates that these brain regions respond similarly to the same acute drug, but differently to different acute drugs.

A possible explanation for the observed differences in limbic activation by cocaine and morphine relates to the role played by the neurotransmitter dopamine. As
previously mentioned, each limbic region analyzed expresses dopaminergic receptors and has either efferent (VTA) or afferent (PFC, CEA, BLA) dopaminergic projections. Furthermore, it seems that dopamine is crucial in the drug-induced expression of Fos in several of these brain regions, though studies examining the pharmacology of Fos induction in brain regions other than the Nacc and striatum are limited (Harlan and Garcia, 1998). Thus, it is possible that the dopaminergic component of the mesolimbic system is more highly activated by acute cocaine administration than following acute morphine. This speculation has precedent in previous studies of dopamine levels in the Nacc where cocaine administration results in greater and more rapid increases in dopamine than does administration of morphine (Kalivas and Duffy, 1993; Barrot et al., 1999). While neurotransmitters other than dopamine are certainly involved in the activation of these limbic brain regions, it does seem likely that dopamine plays a key role in the observed differences in activation by acute cocaine and morphine.
Figure 1. Lack of acute-Fos/ChAT or chronic-FRA/ChAT dual immunolabeling. A&B, Low-magnification (10x) photomicrographs of dorsomedial Nacc core from an acute-cocaine subject (A, Fos/ChAT) and a chronic-cocaine subject (B, FRA/ChAT). C&D, Higher power (20x) images of the sections shown in panels A and B respectively. Single-labeled Fos or FRA-IR cells are indicated by dark-blue nuclear labeling (e.g., dashed arrows); single-labeled ChAT-IR cells (e.g., solid arrows) contain brown cytoplasmic labeling. Scale bars: panels A&B, 200μm; panels C&D, 100μm.
Figure 2. Mean percentage of Fos-labeled cells (±SEM) in PFC (panel A), CEA, BLA, or VTA (panel B) after acute saline, cocaine, or morphine injection. Statistical comparisons: * $p<0.05$, significantly different from the saline-treated group, Fisher’s LSD test.
CHAPTER 3

The nucleus accumbens (Nacc) has been recognized as a key brain area mediating behavioral responses to acute and chronic drug administration (Koob et al., 1998). For example, Nacc neurons exhibit changes in drug-induced immediate early gene (IEG) expression associated with both acute and chronic administration (Nestler et al., 1993; Hyman and Malenka, 2001; Nestler, 2001). Furthermore, differences between the specific types of IEGs expressed in the acute vs. chronic drug setting are thought to reflect the processes involved in drug sensitization and addiction. In particular, several studies have demonstrated a shift in the members of the AP-1 family of IEGs expressed following chronic drug administration. Specific Fos-Related-Antigens (FRAs), particularly isoforms of delta-FosB, become the predominant AP-1 IEGs expressed in the chronic drug setting (Nestler, 2001). These delta-FosB ‘chronic FRAs’ are more stable and longer lasting than their acute counterparts (Nestler et al., 2001), and mice overexpressing delta-FosB demonstrate increased sensitivity to the rewarding aspects of cocaine in (Kelz et al., 1999). Importantly, chronic FRAs (particularly delta-FosB isoforms) have been observed in the Nacc in response to chronic administration of multiple classes of drugs of abuse, including cocaine and morphine (Moratalla et al., 1996; Nye and Nestler, 1996). Thus, induction of chronic FRAs may represent a common ‘molecular switch’ in the response of Nacc neurons to chronic drug administration (Nestler, 2001).

The spatial pattern of expression of FRAs following chronic administration of drugs of abuse has been examined in the dorsal striatum (Moratalla et al., 1996), but few detailed anatomic analyses of chronic FRA expression in the Nacc have been carried out.
This is a potentially important oversight in light of the growing awareness of the regional complexity and heterogeneity of the Nacc (Pennartz et al., 1994; Groenewegen et al., 1999). For example, multiple studies have shown that the Nacc core and shell regions are anatomically and functionally distinct: the core is closely aligned with the striatal sensorimotor complex; the shell seems more involved in limbic function, and caudally is closely linked with the extended amygdala (Brog et al., 1993; Heimer et al., 1997b; Bardo, 1998). In addition, rostral-caudal differences within the Nacc have been observed. For example, D1 and D2-receptors are distributed more densely in rostral than caudal portions of the Nacc (Bardo and Hammer, 1991). Furthermore, rostral-caudal differences in Nacc afferent and efferent projections have been demonstrated (Phillipson and Griffiths, 1985; Oades and Halliday, 1987; Zahm and Heimer, 1993). Finally, motivated behaviors such as fear and food intake are differentially influenced along a rostral-caudal gradient in the Nacc (Reynolds and Berridge, 2001). Together, these studies suggest the need for detailed anatomic analysis of the Nacc FRA response to chronic drugs of abuse. Furthermore, few studies have directly compared the spatial pattern of FRA expression in the Nacc induced by different classes of drugs of abuse, i.e., both psychostimulants and opiates.

Thus, the primary goal of this study was to compare the pattern of FRA expression in the Nacc following chronic systemic administration of either of two different drugs of abuse, cocaine or morphine. In order to provide a detailed map of this response, the location of FRA-expressing cells was examined in the both core and shell divisions of rostral, middle, and caudal Nacc.
METHODS

Animals. Male Sprague-Dawley rats (Harlan) weighing 240-300g at the time of experiment were housed individually on a 12:12hr light:dark cycle (lights on at 6am) with food and water available ad libitum. All drug injections and perfusions were performed during light phase of the light:dark cycle. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Cincinnati, in accordance with NIH guidelines.

Protocol for drug administration. Three treatment groups (n=5 per group) were included in the study. Animals were injected i.p. once daily for five consecutive days in their home cage with either cocaine (cocaine hydrochloride; Sigma, St. Louis, MO; 10mg/kg; dissolved in 0.9% sterile saline), morphine (morphine sulfate; Spectrum Laboratory Products, Gardena, CA; 10mg/kg; dissolved in 0.9% sterile saline), or saline (0.9% sterile saline). Two hours following the injection on the last day, animals were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and transcardially perfused with saline (100ml) followed by 4% paraformaldehyde in 0.1M PB (500ml). Brains were removed and post-fixed in the same fixative at room temperature for 1hr and subsequently stored in 20% sucrose at 4°C.

Immunocytochemical (ICC) procedures. Coronal sections through the forebrain were cut on a freezing microtome at a thickness of 35µm, collected in four parallel series in cryoprotectant (Watson et al., 1986), and stored at -20°C until further processing. All incubations were performed at room temperature under gentle agitation.
For each animal, sections through the rostral-caudal extent of the Nacc were washed extensively in 0.1M phosphate-buffered saline (PBS). Sections were rinsed extensively in PBS between each incubation step. Sections were incubated 10min in 1% H$_2$O$_2$ in PBS and for 1hr in PBS with 0.4% Triton and 0.1% BSA (all subsequent incubations were performed using reagents dissolved in this incubation solution). Next, a series of every fourth section was incubated (1:10,000; 16hr incubation) either with an antibody specific for c-Fos (SC-52; Santa Cruz Biotechnology, Santa Cruz, CA) or with antibody recognizing all Fos-Related-Antigens or FRAs (SC-253; Santa Cruz). Following incubation with primary antibody, sections were thoroughly washed and then incubated with biotinylated goat anti-rabbit IgG (1:400; 1hr incubation; Vector Laboratories, Burlingame, CA). Following additional washes, sections were placed into a solution containing avidin-biotin HRP complex (ABC Elite kit, 1:1000; 1hr incubation; Vector). After another series of washes, sections were incubated with DAB (0.02%; 10min incubation; Sigma) in PB containing 0.08% nickel ammonium sulfate and 0.01% H$_2$O$_2$. Sections were then mounted on Superfrost slides (Fisher Scientific, Pittsburgh, PA), dehydrated, and coverslipped using DPX mountant (Electron Microscopy Sciences, Washington, PA).

Quantification of immunopositive cells in Nacc. For each animal, three brain sections processed for either Fos or FRA ICC were chosen to represent rostral, middle, and caudal levels through the Nacc. These sections corresponded approximately to +2.15mm, +1.45mm, and +0.95mm anterior to bregma, according to the rat brain maps of Swanson (Swanson, 1998). Camera lucida drawings were made at 10x magnification using a drawing tube attached to a Leica DMR microscope (Leica Microsystems, Germany) to
map immunopositive neurons, which were identified by the presence of nuclear staining. In order to delineate the boundaries of the Nacc, and its core and shell divisions (Jongen-Relo et al., 1994), sections at equivalent rostral-caudal levels from a separate series of rat brain sections processed for calbindin ICC and counterstained with Nissl were used (see Chapter 1). The total number of immunopositive cells was determined within the core and shell regions for each rostral, middle, and caudal Nacc section processed for either Fos or FRA ICC. Sections were analyzed by an observer blind to the treatment group identity of individual animals.

Statistical analysis. All analyses were conducted using StatView (V4.5, Abacus Concepts, Cary, NC). For the Fos and FRA ICC datasets in each treatment group, the mean number of labeled cells was calculated for each Nacc sub-region (core and shell in each rostral, middle, or caudal level). Analysis of statistical differences among treatment groups in the mean number of Fos or FRA immunopositive cells within each Nacc sub-region was performed using one-way ANOVA with drug as the factor followed by Fisher’s least significant difference (LSD) post-hoc test. Significance for all comparisons was set at \( p<0.05 \).

RESULTS

Both chronic cocaine and morphine increased FRA expression in most rostral-caudal levels of the core and shell regions of the Nacc (Figure 1, \( p<0.05 \)). Specifically, cocaine injections increased the number of FRA-positive cells in each Nacc sub-region, and induced greater numbers of FRA-positive cells than morphine in all sub-regions except for the rostral shell (Figure 1B). Morphine injections produced significant
increases in the number of FRA-positive cells in all Nacc sub-regions with the exception of the rostral core (Figure 1A, \( p=0.42 \)) and middle shell (Figure 1B, \( p=0.10 \)). Overlap between the patterns of FRA-positive cells induced by both cocaine and morphine was particularly striking in the caudal portion of the shell (Figure 2). Dense clusters of FRA-positive cells in cocaine and morphine treated animals were seen in the region of the caudal shell previously defined as the ‘septal pole’ (Figure 2, insets), the dorsomedial tip of the caudal Nacc shell (Voorn et al., 1986).

In contrast to the effects on FRA expression, more limited induction of Fos expression was seen following chronic drug treatment (Figure 1C&D). Specifically, morphine failed to significantly increase the number of Fos-positive cells in any Nacc sub-region. Cocaine, on the other hand, significantly increased the number of Fos-positive cells in all rostral-caudal levels of the Nacc core (Figure 1C, \( p<0.05 \)), but not in the shell.

**DISCUSSION**

The results of this study indicate that chronic systemic administration of either cocaine or morphine induced widespread FRA expression in the Nacc. FRA induction was observed throughout the rostral-caudal extent of the Nacc, as well as in both core and shell subdivisions. In addition to FRA induction, limited Fos expression was also observed, but only following cocaine and only in Nacc core. Finally, the overlap in the pattern of FRA induction by the two separate classes of drugs was most striking in the septal pole of the dorsocaudal Nacc shell.
Identity of FRAs

It should be noted that, since the anti-FRA antibody used in this study recognizes all Fos family members (c-Fos, FosB, delta-FosB, FRA-1, and FRA-2), it is not possible to know precisely which FRAs were detected. However, several lines of evidence support that these FRAs are probably largely comprised of isoforms of delta-FosB. For example, the present results demonstrate that induction of Fos cannot account for the induction of FRAs seen in this study. The number of FRA-IR cells was much larger (approximately three times larger, on average) than the number of Fos-IR cells for each sub-region and treatment group analyzed (Figure 1; note y-axis values). This result is supported by previous studies that have demonstrated little or no striatal or Nacc Fos induction following chronic cocaine administration paradigms similar to the one used in this study (Hope et al., 1992; Nye et al., 1995; Moratalla et al., 1996). In addition, these prior studies demonstrated that the chronic FRAs are primarily the 35-37kD delta-FosB isoforms, with a ‘significantly desensitized’ induction both of other acute FRAs (Nye et al., 1995) and of AP-1 binding (Hope et al., 1992). Thus, it is likely that a majority of the FRAs detected in this study are the chronic delta-FosB isoforms.

It is interesting to note, however, that statistically significant Fos induction was observed in this study in the Nacc core following chronic cocaine. This result resembles previous findings by Western analysis of minor Fos induction in striatum following a similar chronic cocaine injection paradigm (Moratalla et al., 1996). The core region of the Nacc is thought to be closely linked with the striatum (Zahm and Heimer, 1993), and indeed can be thought of as part of the sensorimotor striatal complex (Bardo, 1998). Thus, perhaps the induction of Fos observed in both the Nacc core and the striatum
represents the activation of neurons specifically involved in the locomotor response to the psychostimulant cocaine. Further experiments would obviously be needed to test this hypothesis.

**Chronic v. acute Nacc FRA induction**

The main result from this study is that chronic systemic administration of either of two classes of drugs of abuse, psychostimulants and opiates, induced similar patterns of FRA expression throughout the rostral-caudal extent of the Nacc. In addition, this similarity in induction pattern extended to the Nacc core and shell subdivisions. Although a greater number of FRA-IR cells were observed throughout the Nacc sub-regions following chronic cocaine, the pattern of FRA induction was similar to that observed for chronic morphine. This result extends the findings of previous studies that relied primarily on Western or Northern analysis to identify changes in Nacc FRAs without reference to rostral-caudal or core-shell anatomy. In addition, the comparable pattern of FRA induction by chronic cocaine and morphine differs markedly from observations of FRA expression following acute cocaine or morphine administration. For example, multiple studies have shown strong induction of Nacc Fos (reviewed in (Harlan and Garcia, 1998)) as well as other acute FRAs (Nye et al., 1995) following acute cocaine injection. Studies of the Nacc following acute morphine injection, however, generally report either little or no Fos/acute-FRA induction (Nye and Nestler, 1996), minor Fos induction (Harlan and Garcia, 1998), or rostral-specific Fos induction (see Chapter 1). Thus, the present results indicate a shift from differential patterns of Fos/acute-FRA induction by acute cocaine or morphine to common patterns of FRA induction in the chronic drug administration setting. Furthermore, few studies have directly compared the
regional induction of FRAs in the Nacc following chronic injection of different classes of drugs of abuse. A previous study has, however, demonstrated common FRA induction patterns in the Nacc following chronic self-administration of cocaine or nicotine (Pich et al., 1997). Thus, the present study confirms and extends the observation that chronic administration of multiple classes of drugs of abuse results in common patterns of FRA induction throughout the Nacc.

Growing evidence suggests that the presumed chronic FRAs (delta-FosB isoforms) induced in this study may function as ‘molecular switches’ in the Nacc in response to chronic drugs of abuse (for review, see (Nestler et al., 2001)). Some of these studies have shown that delta-FosB can regulate AP-1-mediated transcription, and thus could mediate long-term changes in Nacc protein expression. These studies also suggest that such protein expression changes could, in turn, alter the physiology of Nacc neurons. Such changes in Nacc neuronal electrophysiological properties could be manifest as changes in Nacc responses to chronic drugs. For example, mice overexpressing delta-FosB in the Nacc show increased sensitivity to the rewarding effects of cocaine, increased locomotor response to chronic cocaine, and increased motivation to self-administer cocaine (Kelz et al., 1999; Nestler et al., 2001). Thus, it is possible that following chronic administration of drugs of abuse, the induction of FRAs in the Nacc observed in this study is an important component in mediating long-term changes in protein expression that could ultimately be manifest as behavioral changes leading to addiction.
Anatomy of FRA induction

The most striking overlap in the pattern of Nacc FRA induction following chronic cocaine or morphine was observed in the clusters of intensely labeled cells in the septal pole of the dorsocaudal shell. This is an intriguing finding given the unique features of this Nacc region. The region was first defined by Voorn, et al., in a study demonstrating the high density of dopaminergic innervation of the septal pole (Voorn et al., 1986). Further studies have shown that the septal pole also receives heavy noradrenergic innervation (Berridge et al., 1997), receives dense projections from caudal amygdala (Johnson et al., 1994), and can be included in the ‘extended amygdala’ functional anatomic system (Brog et al., 1993; Heimer et al., 1997b). Thus, the septal pole is uniquely situated to be a potential site for integration of input from multiple brain regions. The striking common induction of FRAs in this Nacc region could indicate the importance of the septal pole in aspects of Nacc-mediated responses to chronic drugs of abuse. Functional studies specifically aimed at the septal pole’s involvement in the response to chronic drugs would shed light on this possibility.

Summary

This study provides evidence for the convergence of the anatomic pattern of induction of Nacc FRAs following chronic administration of different classes of drugs of abuse. This finding extends previous observations of chronic-drug-induced FRA expression in Nacc by identifying induction throughout the sub-regions of this heterogeneous structure. Growing evidence indicates that different regions of the Nacc, both rostral v. caudal and core v. shell, maintain different protein expression patterns, have different afferent and efferent projections, and are differentially activated in
response to various stimuli. It is, therefore, important to identify Nacc responses in accordance with such region specificity. Thus, the current finding of the striking overlap of the FRA expression pattern in the septal pole reinforces that attention must be paid to regional specificity of Nacc responses to drugs of abuse.
Figure 1. Mean numbers of FRA-IR cells (A,B) or Fos-IR cells (C,D) (±SEM) in core and shell divisions of rostral, middle, and caudal NAcc after chronic saline, cocaine, or morphine injection. Statistical comparisons: + \(p<0.05\), significantly different from chronic saline group; * \(p<0.05\), significantly different from chronic morphine group, Fisher’s LSD test.
Figure 2. Camera lucida drawings of FRA labeling. Three representative drawings made at 10x magnification are from chronic saline (panel A), chronic cocaine (panel B), or chronic morphine (panel C) subjects and depict caudal levels of the Nacc. Core and shell divisions were determined by processing adjacent sections for CalB and Nissl (see Methods), and these areas are to the left and right of the dividing line in each drawing. Insets, higher magnification (40x) of labeling in the 'septal hook' (within red box) of the dorsomedial shell for each panel. ac, anterior commissure; LV, lateral ventricle. Scale bar, 200 μm.
SUMMARY AND CONCLUSIONS

The key overall observation of this thesis is that administration of separate classes of drugs of abuse result in different patterns of activation of the Nacc acutely, but activate common areas of the Nacc following chronic injections. Whereas cocaine induced neural activation rapidly and continually following acute administration, morphine only induced activation at the latest time-point examined following acute injection. Furthermore, while cocaine-activated neurons were present at all rostral-caudal Nacc levels following acute injection, morphine-activated neurons were restricted to the rostral Nacc. Additionally, in the acute setting, cocaine induced activation of a subset of VP-projecting neurons at all rostral-caudal levels of the Nacc, while morphine did not. In limbic regions outside the Nacc, similar differences in the pattern of stimulation by cocaine v. morphine were also observed. That is, after acute injection, cocaine induced Fos expression in the CEA and VTA, while morphine did not. In contrast to the responses seen following acute administration, chronic injections of either cocaine or morphine resulted in widespread stimulation in the Nacc. FRA induction was observed throughout the rostral-caudal extent of the Nacc, as well as in both core and shell subdivisions. The overlap in the pattern of chronic-drug-induced FRA expression was most striking in the septal pole of the dorsocaudal Nacc shell. The limited current understanding of rostral-caudal functional differences in the Nacc makes it difficult to precisely determine why a shift from differential to convergent stimulation of the Nacc was observed in the acute v. chronic drug settings. As will be described in the following section, it does seem likely that the neurotransmitter dopamine is intimately involved in the mechanism underlying these results.
The characteristics of the patterns of Nacc activation seen after both acute and chronic drug administration suggest a role for dopamine in the transition to convergent activation that accompanies chronic drug exposure. In the acute setting, cocaine induced Fos expression rapidly and all times examined, while morphine produced a delayed pattern of Fos induction. These differences in the time course of activation parallel the ability of these two drugs to increase dopamine release in the Nacc (see Chapter 1 Discussion). In particular, cocaine produces a rapid and sustained increase in Nacc dopamine following acute systemic administration; by contrast, morphine produces a delayed and smaller increase in Nacc dopamine following acute injection. Furthermore, as previously discussed, the only region of the Nacc where morphine did induce Fos (the rostral subdivision) is also the area of highest concentration of D1-subtype dopamine receptors (D1R), the subtype that is critical for dopamine-induced Fos following acute administration of either cocaine or morphine. Thus, differences in the time course of dopamine release following acute injection, together with regional heterogeneity in D1R expression, may account for the observed difference in the patterns of acute Fos induction between cocaine and morphine.

Following chronic administration of either cocaine or morphine, neuronal stimulation was observed throughout the rostral-caudal extent of both the core and shell of the Nacc. Using FRA expression as a marker (see Chapter 3 discussion), it was shown that both cocaine and morphine induced similar spatial patterns of Nacc stimulation in the chronic drug setting. Three lines of evidence support the involvement of dopaminergic neurotransmission in the convergence of activation within the Nacc: 1) drug-stimulated dopamine levels in the Nacc are increased following chronic administration of either
drug, compared to those levels seen in the acute setting, 2) the sensitivity of post-synaptic
dopaminergic signaling pathways is increased following chronic drug administration, and
3) D1R are required for FRA induction following chronic drug administration.

Drug-induced dopamine transmission and basal dopamine levels, as assayed by
microdialysis, are enhanced in the Nacc following chronic administration of either
cocaine or morphine, though there is some controversy regarding basal dopamine (Vezina
et al., 1992; Johnson and Glick, 1993; Kalivas and Duffy, 1993). Furthermore, chronic
administration of either cocaine or morphine has been shown to result in increased D1R
sensitivity as measured by single-cell recording following chronic drug injection or
following post-injection challenge using microiontophoretic application of D1-agonist
(Henry and White, 1991; Vanderschuren et al., 1997). Post-synaptic intracellular changes
downstream in the D1R-signaling cascade have also been observed in response to chronic
cocaine and morphine, including up-regulation of adenlyate cyclase and protein kinase A,
and decreases in inhibitory G-proteins (Nestler et al., 1993; Hyman, 1996). Additionally,
D1R antagonists have been shown to diminish behavioral responses to both chronic
morphine and cocaine (Jeziorski and White, 1995; Koob et al., 1998). Finally, similar to
the previously discussed dopaminergic mechanism of acute-FRA/Fos induction, FRA
induction in the Nacc following chronic cocaine administration is blocked by D1R
antagonists (Nye et al., 1995). Taken together, the increases in drug-induced dopamine
levels and transmission seen following chronic injection of either cocaine or morphine,
combined with the augmented D1R responsiveness after chronic exposure, may lead to
the similar patterns of FRA observed in the Nacc. This possibility is further supported by
the striking overlap in FRA induction noted in the dorsocaudal septal pole of the Nacc
following chronic, but not acute, injection of cocaine or morphine. This overlap, following multiple exposures to cocaine or morphine, may reflect the accumulation of dopamine in a known dopamine-rich and dopamine-reuptake-transporter-poor region (Voorn et al., 1986; Marshall et al., 1990). Thus, the increased dopaminergic transmission in the Nacc following chronic drugs of abuse may be particularly elevated in the septal pole, resulting in the striking overlap of FRA induction seen in this region.

Further studies are necessary to test the role of these chronic changes in dopaminergic transmission in patterns of neuronal Nacc activation. For example, it is not currently known if increases in drug-induced dopamine release and neurotransmission in the chronic drug setting occur in all subdivisions of the Nacc. To test this, microdialysis of dopamine and its metabolites throughout the rostrocaudal extent of the Nacc would need to be performed at several time points during the chronic treatment in order to accurately map the spatial Nacc dopamine response. The identification of Nacc subregions that share a common dopaminergic response to chronic drugs could then be compared to the patterns of convergent activation within the Nacc seen using IEGs as markers.

Accumulating evidence, including that presented in this thesis, indicates that studies of the Nacc need to take into account the regional specificity inherent in this anatomically and functionally heterogeneous brain region. In particular, studies concentrating on the rostral-caudal differences in the Nacc response to drugs of abuse will be crucial in determining which Nacc regions respond in a common manner. Such studies will further the basic understanding of Nacc function, and will provide a framework for refined functional manipulations of the Nacc. For example, studies of
rostral-caudal Nacc release and transmission of dopamine (and other neurotransmitters) following drug administration could identify which regions are crucial in the initiation and maintenance of the Nacc response to all drugs of abuse. Functional manipulations, including restricted lesions, pharmacologic manipulations, and localized manipulations resulting in gene/protein overexpression (i.e., delivery of transgenes via viral vectors), will be necessary to determine if specific Nacc regions are important in certain aspects of the drug response. These studies will assist in developing a more precise functional anatomic map of the Nacc during the development of drug addiction. Such maps, in turn, could potentially be used in the clinical setting in treating drug addicts. For example, high-resolution functional-MRI could be used in patients to monitor how Nacc sub-regions (and other mesolimbic areas) are responding to treatment for addiction. These data could be compiled to construct a functional anatomic map of the mesolimbic response to various treatments for addiction in an effort to define subcircuits that may be more or less responsive to such treatments. Thus, an increased understanding of the anatomic and functional heterogeneity of the Nacc, and its response to drugs of abuse, may have far-reaching implications for our understanding of the processes mediating drug addiction.

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