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Cocaine- and Morphine-Induced Synaptic Plasticity in the Nucleus Accumbens

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Abstract

The critical brain areas and molecular mechanisms involved in drug abuse and dependence have been extensively studied. Drug-induced persistent behaviors such as sensitization, tolerance or relapse, however, far outlast any previously reported mechanisms. A challenge in the field of addiction, therefore, has been to identify drug-induced changes in brain circuitry that may subserve long-lasting changes in behavior. The present study examined behavioral changes and electron microscopic evidence of altered synaptic connectivity within the nucleus accumbens (NAc) following repeated administration of cocaine or morphine. The unbiased quantitative stereological physical disector method was used to estimate the number of synapses per neuron. Increases in synapse to neuron ratio were found in the NAc shell of cocaine-treated (49.1%) and morphine-treated (55.1%) rats and in the NAc core of cocaine-treated animals (49.1%). This study provides direct ultrastructural evidence of drug-induced synaptic plasticity and identifies synaptic remodeling as a potential neural substrate underlying drug-induced behavioral sensitization.

Keywords

addiction; basal ganglia; sensitization; electron microscopy; ultrastructure

INTRODUCTION

The critical brain areas and molecular mechanisms that underlie drug abuse and dependence have been well documented. Drug-induced persistent behaviors, which can last for several years even after drug cessation, far outlast any previously reported molecular mechanisms. A major challenge in the field of addiction, therefore, has been to identify long-lasting

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changes in the brain that may underlie such drug-induced persistent behaviors as sensitization, tolerance and relapse, which reportedly contribute to the development of addiction (Hyman et al, 2006; Kalivas and Volkow, 2005; Robinson and Berridge, 2003; Russo et al., 2010).

Long-term treatment with cocaine and morphine has previously been reported to induce changes in dendritic morphology in both the nucleus accumbens (NAc) and prefrontal cortex (PFC) of behaviorally sensitized animals. The NAc and PFC are two primary targets of the dopamine (DA) mesocorticolimbic pathway that have been implicated in the cognitive, hedonic and motor effects of drugs of abuse and transition to addiction. Golgi-Cox staining of these brain areas has revealed an increase in dendritic branching and spine density in the NAc shell following repeated intraperitoneal (i.p.) treatment with cocaine (Robinson and Kolb, 1999a) and a decrease in dendritic branching and spine density in the NAc shell following repeated i.p. treatment with morphine (Robinson and Kolb, 1999b). Increases in spine density in the NAc core have further been associated with the expression of cocaine-induced behavioral sensitization, a phenomenon whereby increased drug effects such as enhancement of locomotor activity or stereotypic behavior occur with repeated drug administration (Li et al., 2004). The present study, hence, examined ultrastructural synaptic changes, presumably concomitant with dendritic branching and spine density changes, in animals exhibiting drug-induced behavioral sensitization.

Changes in dendritic morphology and drug-induced persistent behaviors resulting from repeated treatment of cocaine and morphine suggest that changes in synaptic connections are also occurring. Direct evidence of synaptic plasticity and the precise pattern of synaptic restructuring can be obtained with electron microscopy (EM). Although a large body of literature supports the link between synaptic plasticity and experience or learning (Anderson et al., 1996; Black et al., 1990; Kleim et al., 1996; Turner and Greenough, 1985;), only recently have studies begun to examine changes at the ultrastructural level to explain the relationship between altered connectivity and drug treatment (Morrow et al., 2007). The present study employed EM and unbiased quantitative stereology to test the hypothesis that changes in synapses per neuron occur in the NAc following repeated cocaine and morphine treatment. The synapse-to-neuron ratio, calculated using the physical dissector method, has been reported to accurately reflect changes in synapse number when neuron number is stable as was the case in the present study. This ratio accurately reflects changes in synapse number irrespective of any changes in tissue volume and is not affected by tissue shrinkage that may occur during tissue processing. We further speculate that changes in synaptic connectivity underlie such persistent drug-induced sequela as behavioral sensitization.

MATERIALS AND METHODS

Subjects

Twenty-four adult female Sprague-Dawley rats were used in this study. Female rats were used in this study since they show more robust sensitization than male rats (Robinson, 1984), and because earlier published work reporting cocaine- and morphine-induced alterations in the morphology of dendrites and dendritic spines in the NAc used female rats (Robinson and Kolb, 1999a,b). Rats were randomly assigned to 3 groups, Cocaine (n=7), Morphine (n=7), and Saline (n=8). Two animals were excluded from this study due to the inadvertent administration of the wrong drug to one animal and observed tissue pathology in a second animal. All efforts were made to minimize animal suffering and the number of animals used. Experimental procedures conformed to National Institutes of Health guidelines and were carried out under an institutionally reviewed and approved research protocol.

Drug administration and behavioral procedures

Morphine sulfate (10 mg/kg) and cocaine hydrochloride (15 mg/kg) were dissolved in 0.9% saline and injected i.p. at 1 mL/kg of body weight. Drugs were administered in the testing room and animals were immediately placed into test cages (45 × 23.5 × 20.75 cm clear plastic cages) in the testing room following injections. On the first day of drug testing, rats were removed from their home cage and given an i.p. injection of cocaine, morphine, or saline followed by placement in the test cage. Behaviors in the test cage were recorded with a video camera for 60 minutes immediately following the injection. Animals were then returned to their home cage following the testing session. These procedures were repeated 5 days a week, followed by 2 consecutive drug-free days over a total of 4 weeks. After the last testing day, animals were left undisturbed in their home cage for an additional 21 days before their brains were removed. The additional 3 week drug-free period was included in the experimental design in order to measure persistent drug-induced synaptic changes rather than possible transient morphological changes (Robinson and Kolb (1999a,b)).

Behavioral analysis

Behavioral videotapes were coded and viewed by observers blind to group assignment. Behaviors quantified from the video footage for Days 1 and 28 included: line crosses, rearing, head bobbing, sniffing, immobility, chewing/gnawing, grooming and sleeping. Grid lines were made on the outside of the bottom of the cage dividing the cage into 6 quadrants. The lines were used to score line crosses following drug administration. Line crosses were counted when both forepaws crossed a line as the rat moved in the forward direction. Rearing activity was measured when the rat stood on its hind legs, either supported by the cage or unsupported. Head bobs were counted as head movements in the vertical direction (up to down), while stationary with all paws on the ground. Sniffing was timed while the rat was in a rearing position (sniffing up) or a standing position with all four paws on the ground (sniffing down). Grooming behavior was timed for only grooming of the face and head. Immobility was characterized by freezing behavior in the absence of head movement. Chewing/gnawing behavior was recorded when animals chewed either their claws (forepaws or hind paws) or the walls of the cage. Grooming, sniffing, immobility, and chewing/gnawing were measured and recorded in the number of bouts and in the number of seconds.

Light and electron microscopy tissue preparation

Following drug treatment and behavioral testing, animals were anesthetized with pentobarbital (100 mg/kg) and perfused transcardially with 4% paraformaldehyde/0.6% glutaraldehyde in 0.1M phosphate buffered saline, pH 7.4. The brains were removed, post-fixed in 4% paraformaldehyde for 2 hours, and sectioned with a vibratome (100 µm-thick). Sections were then processed for EM. Polymerized and osmicated EM processed tissue sections were initially inspected under a light microscope to locate the shell and core compartments of the NAc using the Paxinos and Watson Stereotaxic Coordinates for the Rat Brain (1998). Excised regions from the core and shell compartments for each animal (Fig. 1) were then attached to an epoxy block and trimmed into a small pyramid of tissue. For each trimmed tissue block, twenty semithin sections (~500 nm-thick) and sixteen ultrathin sections (~70 nm-thick) were cut on an ultramicrotome (Leica Ultracut UCT) using a diamond knife (Ted Pella). After the first five semithin sections were collected, 4 silver-gray serial sections (~70 µm thick) were taken using a diamond knife and sectioning continued alternating between semithin and ultrathin sections. All semithin sections were dried onto glass slides and all ultrathin sections were collected onto formvar-coated slot grids.

Unbiased quantitative stereology procedures

The present study used unbiased quantitative stereology to examine changes in the synapse-to-neuron ratio, a method described in 1979 (Mayhew) for measuring synaptic changes in brain tissue that has been widely used (Black et al., 1990; Braendgaard and Gundersen, 1986; Cooke and Woolley, 2005; Geinisman et al., 1996a,b; Gundersen, 1986; Gundersen et al., 1988; Kleim et al., 1996; Poe et al., 2001; Turner and Greenough, 1983). The physical disector method was used to estimate the synapse-to-neuron ratio calculated from neuronal density and synapse density (Gundersen et al., 1988; Sterio, 1984). Previous experiments have reported that experience can lead to an increase in the number of synapses as well as tissue volume where those synapse changes are occurring (Rosenzweig et al., 1962; Turner and Greenough, 1985). Synapse density alone, therefore, does not always accurately reflect changes in synapse number because of potential changes in tissue volume. The synapse-to-neuron ratio, however, accurately reflects changes in the number of synapses (made onto any part of the neuron) when neuron number is stable (Anker and Cragg, 1974). Thus, when using the ratio of synapses per neuron, volume is removed from the equation (Poe et al., 2001). Furthermore, variable tissue shrinkage can result from tissue processing. The synapse-to-neuron ratio overcomes this problem since it is unaffected by tissue shrinkage when both neuron density and synapse density are calculated from the same block of tissue, as done in this study (Geinisman et al., 1996a). The synapse to neuron ratio was therefore the most valid way to test the hypothesis that the number of synapses change in the NAc with drug treatment.

Neuron Density

Semithin sections were stained with toluidine blue and used to calculate neuronal density using a 40X lens on a computer-assisted light microscope. Neuronal density was calculated by dividing the number of cells counted by the volume of the tissue which involved comparing two serial sections in a series of ten sections (Gundersen, et al., 1988; Sterio, 1984). The first section is called the Reference section and the second is the Lookup section. Then the second section became the Reference section for the third section and so on, using a total of ten semithin sections. When a cell nucleus was found in the Reference section, but not the Lookup section, the nucleus was counted. Within an unbiased counting frame (A_{frame}) of a known area ($54,600 \mu\text{m}^2$ at 40x for semithin sections) the number of nuclei that were present in the Reference section and not in the Lookup section was counted (Q_{neuron}^-). Next the volume of tissue through which the cells were counted (V_{dis}) was calculated using the following formula: $V_{\text{dis}} = A_{\text{frame}} \times H$, where H is section thickness ($1 \mu\text{m}$) multiplied by the number of sections. The neuronal density, N_{Vneuron} , was then determined by dividing the number of cells counted by the volume of the tissue using the following formula: $N_{\text{Vneuron}} = Q_{\text{neuron}}^- / V_{\text{dis}}$ (using semithin sections).

Synapse Density

Ultrathin sections were collected on formvar-coated copper slot grids and stained with 2% aqueous uranyl acetate. Sections were then examined and photographed using a Philips EM208 transmission electron microscope (TEM) (FEI Company) at 80kv. Images, 1 Mb in size, were collected with an AMT Advantage HT camera. All electron photomicrographs were taken at a magnification of 18000X. Images were stored and analyzed by an observer blind to group assignment. Twenty electron photomicrographs were taken from two adjacent sections from the same positions within each section. Landmarks used to identify the correct location in each section typically consisted of cross-sections of small myelinated fibers and clusters of mitochondria transversing the section. A total of 40 micrographs were collected per NAc compartment per animal (twenty disector pairs), using one slot grid. Synapses were categorized as asymmetric, symmetric or undefined. Asymmetric synapses were identified by the presence of at least three round or spherical shaped synaptic (neurotransmitter)

vesicles in the presynaptic terminal, a thickened postsynaptic density and a synaptic cleft. Symmetric synapses were identified by the presence of at least three presynaptic synaptic vesicles with predominantly flattened or elongated appearance (i.e. pleomorphic), a synaptic cleft and two closely apposing parallel membranes with no prominent postsynaptic density. The postsynaptic target was identified and recorded for each synapse (i.e. soma, dendritic shaft or spine, and axon) whenever possible. For example, the dendritic shaft was identified by microfilaments and mitochondria visible in dendritic profiles, whereas dendritic spines were identified by their characteristic size, “fluffy” or “flocculent” material, absence of mitochondria and/or presence of spine apparatus (Peters et al., 1991; Peters and Palay, 1996).

The observers counted all synapses in the shell and core compartments of the NAc and the physical disector method was again used to calculate the synapse density, for which the number of synapses present in the reference section and not the look-up section were counted (Q^-_{synapse}). The total number of synapses within an unbiased counting frame of a known area (A_{frame}) was counted ($25.8\mu\text{m}^2$ at 18000x for the ultrathin sections). Again, the disector volume of tissue (V_{dis}) is calculated using the formula from above, where H is section thickness (70 nm) multiplied by the number of sections. Synapse density, Nv_{synapse} , was calculated using the following formula: $Nv_{\text{synapse}} = Q^-_{\text{synapse}} / V_{\text{dis}}$ (using ultrathin sections).

Synapse-to-Neuron Ratio

The number of synapses per neuron was determined by dividing the density of synapses per cubic millimeter by the neuronal density per cubic millimeter: $Nv_{\text{neuron}} / Nv_{\text{synapse}}$ (synapse to neuron ratio). Section thickness of ultrathin sections was verified using “Small-Fold’s” technique (Weibel, 1979). For this calculation an image of a small fold was captured from an ultrathin section and the width of the fold was estimated using the image scale bar. The following formula was then used: $2t=l$, where l is the width of the small fold (using the scale bar) and t is the estimated thickness of the ultrathin section.

Data Analysis

The statistical analyses of behavioral and anatomical data were designed to test whether changes in the synapse per neuron ratio in the NAc shell and core were significantly different in the drug-treated animals relative to control animals and whether rats showed behavioral sensitization to repeated drug treatment by comparing drug effect on day 1 to day 28. Statistical analyses of behavioral data were performed using SPSS (SPSS, Inc.) repeated-measures analysis of variance (ANOVAs) for the effects of Groups, Days, and Group by Day interaction followed by pairwise comparisons using Bonferroni’s correction. Significant changes in the synapse per neuron ratio were determined by a one-way ANOVA followed by pairwise comparisons using Bonferroni’s correction. Significance level was set at $p < .05$.

RESULTS

Cocaine- and morphine-induced behavior

Cocaine-treated animals showed significantly more locomotor activity (line crosses and rearing) relative to control animals both on days 1 and 28 (Fig. 2A, B). A significant decrease in the total number of line crosses (Fig. 2A) and rears (Fig. 2B) was observed in cocaine-treated animals by day 28. In contrast to cocaine, morphine administration on day 1 did not produce significant increases in locomotor activity relative to controls (Fig. 2A, B). Instead, morphine administration on day 1 produced motor immobility (opiate catalepsy) (Fig. 2C). Furthermore this drug-induced immobility was significantly higher on day 1 relative to day 28, suggesting evidence of motor tolerance.

A significant increase in the average number of stereotypic head bobs (Fig. 3A) and stereotypic sniffing (Fig. 3B), two reliable measures of cocaine-induced behavioral sensitization, was found in cocaine-treated animals by day 28, relative to controls. Morphine-treated rats did not exhibit significant behavioral levels of stereotypic head bobbing; however, a pronounced morphine-induced sensitization of stereotypic chewing/gnawing behavior was evident on day 28 (Fig. 3C).

Analysis of sleeping behavior revealed that control animals spent significantly more time sleeping on day 28 relative to the first day of testing. Drug-treated animals did not exhibit sleeping behavior during the 1 hour testing session. Therefore, all behavioral measures were adjusted for sleeping behavior observed in the control animals and re-evaluated (data not shown). All statistically significant behaviors reported in the present study, remained significant. Additionally, when grooming behavior was adjusted for the time animals were asleep on day 28, instead of the total 60 minute video, a significant increase in grooming bout duration (i.e. the number of seconds spent grooming per bout) and the total number of grooming bouts was found when day 1 was compared with day 28 (data not shown).

Altered synaptic connectivity measured by electron microscopy

A significant increase in the total number of synapses per neuron in the NAc shell of cocaine- (49.1%, $p < .05$) and morphine- (55.1%, $p < .05$, $F = 6.205$) treated animals was found, relative to controls (Fig. 4A). Neuron density was stable (i.e. not statistically different) in all three groups as indicated by the mean and standard deviation (SD): cocaine = 244,113 (SD 25,124) neurons per mm^3 ; morphine = 265,175 (SD 54,740.3) neurons per mm^3 ; and control = 279,215 (SD 51,940.2) neurons per mm^3 . Thus, the calculated number of synapses per neuron reflects a change in the number of synapses. Specifically, there was a significant increase in the number of asymmetric (presumably glutamatergic) synapses onto cells in the NAc shell in cocaine-treated animals (62.2%, $p < .05$) and morphine-treated animals (64.2%, $p < .05$, $F = 9.220$) (Fig. 4B).

A significant increase in the total number of synapses per neuron was also found in the NAc core of cocaine-treated animals (49.1%, $p < .05$, $F = 6.019$), but not morphine-treated animals, relative to controls (Fig. 5A). Again, a stable neuron density: cocaine = 208,647 (SD 47,076.2) neurons per mm^3 ; morphine = 209,167 (SD 30,166.9) neurons per mm^3 ; control = 203,962.13 (SD 30,711.5) neurons per mm^3 , reveals that this effect is driven by a change in synapse number. In the NAc core, the number of asymmetric synapses increased by 52.5% ($p < .05$, $F = 4.531$) relative to the control group (Fig. 5B). The percentage of asymmetric and symmetric synapses made onto dendritic shafts and spines (Fig. 6) are reported in Table 1. Although symmetric synapses were quantified in both the NAc shell and core, the number of symmetric synapses counted was not large enough to draw any definitive conclusions about changes related specifically to this category of synapses.

DISCUSSION

The present study provides ultrastructural evidence of drug-induced synaptic plasticity in the shell and core compartments of the NAc of animals. Because the drug-induced behaviors reported in the present study replicate previous work (Robinson and Kolb, 1999a,b), the behavioral data indicate that the new synaptic findings are occurring in a behaviorally validated context. Structural changes were observed following 4 weeks of drug treatment and a subsequent 3-week drug-free period. The NAc in addition to the amygdala, dorsal striatum, and PFC have been reported to play critical roles in the cognitive, hedonic and motor aspects of addiction. Functionally distinct subregions within those brain areas are further associated with specific drug effects. For example, the shell NAc plays a critical role in the reinforcing effects of drugs (DiChiara et al., 2004; Pontieri et al., 1995) whereas the

core is more closely associated with drug-induced behavioral sensitization (Cadoni and DiChiara, 1999; Ferrario et al., 2005; Li et al., 2004) and drug-seeking behavior (Kalivas and Volkow, 2005). Based on this evidence, we speculate that an increase in the number of synapses per neuron observed in the NAc shell may represent a neural substrate underlying the reinforcing effects of cocaine and morphine; whereas altered synaptic connectivity in the NAc core may underlie cocaine-induced behavioral sensitization.

Addiction, also known as substance dependence, is defined by the current American Psychiatric Association (APA), Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) as a cluster of cognitive, behavioral and physiological symptoms. It includes a pattern of repeated self-administration that can result in compulsive drug use (APA, DSM-IV, 2000). Compulsive drug-intake behavior has been hypothesized to be an important transition to addiction and can be studied through the phenomenon of behavioral and neural sensitization. The development of psychomotor sensitization has in fact been directly related to increases in cocaine-taking behavior and relapse (De Vries et al 1998, 2002; Zhao and Becker, 2009).

Cocaine sensitization is typically measured following a single high dose of cocaine followed by a challenge dose of cocaine 24 h later (Caster et al., 2007) or following 1 week of cocaine administration with effects that can last up to 3 weeks (Boudreau et al., 2007; Boudreau and Wolf, 2005; Pierce et al., 1996). A more extended cocaine sensitization paradigm (15mg/kg for 20 days), however, was reported by Robinson and colleagues to induce persistent structural changes in the NAc (Robinson and Kolb, 1999a,b) and more recently by Zhao and Becker (2009) to promote cocaine self-administration. The present study used the paradigm previously reported by Robinson and Kolb (1999a,b) and similarly used female rats. Extension of the present results to other findings in the literature is possible given that several recent studies investigating cocaine sensitization and cocaine self-administration also used female rats (Hu et al., 2004; Jackson et al., 2006; Nephew and Febo, 2010; Yang et al., 2007; Zhao and Becker, 2009). Altogether, the findings from these studies, which used female rats, are valuable since sex differences in drug abuse have been reported to exist in humans and animal models (for review see Becker and Hu, 2008).

Structural changes in the core and shell NAc of animals given limited access to cocaine self-administration (1 hour/day) and extended access to cocaine self-administration (6 hours/day) have been reported by Robinson and colleagues (Ferrario et al., 2005). Extended administration of cocaine followed by a cocaine challenge 1 month after the last self-administration test session, marked escalation in drug consumption, enhanced drug-seeking behavior, robust behavioral sensitization and increased density of dendritic spines on medium spiny neurons, specifically in the core of the NAc. Furthermore, the changes were specific to the NAc core since they were not observed in the shell or PFC. These findings suggest that the structural changes in the NAc core are associated with cocaine sensitization and not due to the pharmacological effects of high doses of cocaine. Consistent with these and other findings (Ferrario et al., 2005; Li et al., 2004), the changes in the number of synapses observed in the NAc core in the present study are therefore likely to be specific to cocaine sensitization.

The synaptic changes observed in the NAc in the present study may represent a neural substrate for cocaine-induced stereotypic behaviors. In general, cocaine-sensitized rats exhibit enhanced exploratory behaviors such as locomotor activity and rearing, as well as stereotyped sniffing and head bobbing minutes after drug administration (Broderick, 1991; O'Dell et al., 1996; Tzechenk and Schmidt, 1998). In the present study, a categorical shift occurred from locomotor activity to stereotypic behaviors with repeated administration of cocaine. Such changes in drug-induced behaviors with repeated administration of drugs have

been previously described as the inverted U-shaped function (Dews and Wenger, 1977; Lyon and Robbins, 1975). This theory describes a shift in the replacement of drug-induced motor and exploratory behaviors with the emergence of repetitive, stereotypic behaviors, presumably involving distinct neural circuits. The shift to stereotypic behaviors may represent the emergence of a critical and persistent component (i.e. compulsive drug-seeking state) of addiction. The NAc and dorsal striatum have previously been identified as neural correlates for psychostimulant sniffing and head bobbing (Broderick, 1991; Sharp et al., 1987; White et al., 1998).

Morphine-induced immobility (opiate catalepsy) was significantly higher on day 1 relative to day 28, suggestive of motor tolerance. The exact mechanism underlying opiate-induced catalepsy is not known, although it can be significantly prevented or reversed by phenytoin (antiepileptic) and naloxone (opioid receptor antagonist) (Cookson and Mann, 1980), and the development of tolerance to morphine-induced catalepsy can be delayed by lesioning the ventral tegmental area (VTA) (Hand and Franklin, 1985).

The synaptic changes observed in the NAc shell, in the present study, may underlie not only the reinforcing effects of drugs of abuse but also morphine-induced behavioral sensitization. Morphine-induced sensitization of stereotypic chewing/gnawing behavior was evident in the present study on day 28. Although this behavior has received some attention (Cadoni and Di Chiara, 1999; Kornetsky, 2004; Pollock and Kornetsky, 1989), the neurochemical mechanisms underlying morphine-induced oral stereotypy are not yet clear.

MK-801 (NMDA receptor antagonist), Fluoxetine (selective serotonin reuptake inhibitor), and SCH23390 (dopamine D1 receptor antagonist) all block the expression of this behavior, whereas only pretreatment with MK-801 blocks the development of sensitization to this oral stereotypy (Livezey et al., 1995; Wennemer and Kornetsky, 1999). Cerebral metabolic rates for glucose were measured in rats exhibiting morphine-induced oral stereotypy and the NAc was significantly more metabolically active in behaviorally sensitized animals than in controls (Kraus and Kornetsky, 2000). Furthermore, the metabolic changes occurred in the NAc shell in the presence or absence of a morphine-associated cue, but in the NAc core only when morphine was accompanied by a cue.

The increase in the number of synapses per neuron observed in the NAc shell in both cocaine and morphine-treated animals were due primarily to an increase in the number of asymmetric synapses. Asymmetric synapses in the accumbens are characteristic of glutamatergic synapses formed by inputs from the PFC, amygdala, hippocampus, or thalamus (Gerfen, 1988; Johnson et al., 1994; Meredith and Wouterlood, 1990; Pinto et al., 2003; Sesack and Pickel, 1990; Smith and Bolam, 1990; Torterdel and Smith, 1989). A proposed role for glutamatergic synaptic remodeling in the present study is consistent with the reported role of glutamate (GLU) in drug reinforcement (Todtenkopf et al., 2006) and cocaine and morphine-induced sensitization (Livezey et al., 1995; McFarland et al., 2003). In the present study, an overall increase in the number of asymmetric synapses per neuron was observed in the NAc shell of morphine-treated animals. Interestingly, this is in contrast to a reported reduction in spine density reported by Robinson and Kolb (1999b). One possible explanation is that the decrease in spine density shifts to an increase in the number of synapses made onto dendritic shafts due to spine retraction. Such a trend was observed in the present study (see Table 1). This notion is supported by a recent study using quantitative time lapse confocal imaging of cultured neurons, which reported that 40% of pruned spines kept their presynaptic innervation, which were transformed into shaft synapses (Ovtscharoff et al., 2008). These findings suggest that opiates and psychostimulants differentially affect neural inputs and synaptic connectivity within the shell and core regions of the NAc. Overall, the magnitude of synaptic changes reported in the present study was greater than the spine density changes previously reported by Robinson and colleagues (Li et al., 2004;

Robinson and Kolb, 1999a,b). A probable reason may be that a substantial proportion of asymmetric synapses as well as most symmetric synapses do not terminate on clearly identifiable spines and therefore would not have been identified by spine density counts. Future EM studies may shed light on the unique alterations in region and cell specific synaptic circuitry that mediate a variety of behavioral changes associated with distinct drug classes.

An increase in the number of asymmetric synapses observed in both the NAc shell and core in the present study supports the proposed role of glutamatergic signaling in behavioral plasticity to cocaine. Several studies have implicated GLU signaling and receptor plasticity in cocaine-related phenomena including cocaine administration, cocaine-seeking behavior, behavioral sensitization, withdrawal, reinstatement, craving and relapse. Although each of the GLU ionotropic and metabotropic receptors have been implicated in these phenomena, the majority of the work has shown the ionotropic glutamate AMPA receptor subunits GluR1 and GluR2 in the NAc, to be important in these cocaine-mediated processes (Anderson et al., 2008; Boudreau et al., 2007; Conrad et al., 2008; Kalivas and McFarland 2003; Kourrich et al., 2007; McFarland et al., 2003; Ping et al., 2008; Self et al., 2004; Sutton et al., 2003) including behavioral sensitization (Bachtell et al., 2008; Boudreau and Wolf, 2005; Ferrario et al., 2005; Zhang et al., 2007). Additionally, pretreatment with MK-801 prevents the induction of behavioral sensitization implicating the role of glutamatergic NMDA receptors in the induction of cocaine-induced sensitization (Wolf and Jeziorski, 1993). Moreover, application of this NMDA receptor antagonist also prevents molecular neuroadaptations associated with cocaine sensitization, such as DA autoreceptor subsensitivity and DA D1 receptor supersensitivity (Li et al., 1999). The activation of dopamine receptors, however, is not required for the development of behavioral sensitization to cocaine (White et al., 1998). Importantly, pharmacological and electrophysiological studies examining the effects of cocaine on behavioral sensitization consistently show involvement of the PFC. For example, repeated electrical stimulation of the PFC produces behavioral sensitization (Schenk and Snow, 1994), whereas lesions of the PFC block the development of this behavior (Tzschentke and Schmidt, 1998). Further work, however, is necessary to determine whether the changes in the number of asymmetric synapses per neuron, observed in the present study, involve glutamatergic prefronto-accumbal projections or inputs from other areas such as the amygdala, or hippocampus.

The precise mechanisms involved in mediating drug-induced synaptic remodeling will be of major interest to investigate in future studies. Glutamate NMDA receptor activation, for example, is known to mediate the effects of second messenger cascades and transcription factors such as cAMP response element binding protein (CREB) (Huang et al., 2008). CREB in the NAc is associated with tolerance and dependence and Δ FosB, which exerts opposite effects is associated with behavioral sensitization (Carlezon et al., 1998; McClung and Nestler, 2003; Nestler 2001, 2004; Self et al., 1998).

These transcription factors in turn affect gene expression of proteins that regulate drug-related neurotransmission. These molecular mechanisms, however, do not last as long as drug-induced behaviors, but instead are likely to trigger synaptic plasticity that underlies drug-induced persistent behaviors. Δ FosB, for example, accumulates and persists after repeated exposure to cocaine, for weeks or months even after the drug has been withdrawn (Nestler, 2001). Cyclin-dependent kinase 5 (Cdk5), a down-stream target of Δ FosB, in turn has been implicated in dendritic plasticity and synaptic restructuring (Hawasli et al., 2007; Hawasli and Bibb, 2007; Lai and Ip, 2009). Furthermore, site-specific administration of roscovitine, a Cdk5 blocker, has been shown to attenuate cocaine-induced spine outgrowth in the NAc core and shell (Norrholm et al., 2003). Roscovitine further augments the incentive-motivational effects of cocaine and the development and expression of cocaine-

induced locomotor sensitization (Bibb et al., 2001; Taylor et al., 2007). Additional studies are further necessary to demonstrate direct causal links between the mechanisms that underlie drug-induced synaptic plasticity and corresponding behavioral changes. Altogether, previous work together with our present findings provide the framework for future studies aimed at identifying the necessary and sufficient mechanisms that mediate such drug-related behaviors as sensitization or tolerance.

In conclusion, altered synaptic connectivity involving asymmetric synapses, observed in the present study, supports the proposed role of glutamatergic PFC and limbic inputs to the NAc in mediating long-lasting drug-induced behavioral changes such as sensitization that may contribute to the transition to addiction. Further work is necessary to identify the precise neuronal cell types within the NAc, such as medium spiny projection neurons or aspiny cholinergic interneurons previously reported to be affected by drug treatment (Berlanger et al., 2003; Camp et al., 2006), that contribute to this synaptic remodeling. The key intracellular signaling mechanisms involved in synaptic reorganization within these accumbal neuronal networks and other brain areas such as the dorsal striatum or PFC will also be important to define. Converging DA/GLU synapses made onto region and cell specific networks will provide further insight into the underlying neural basis of learning-associated compulsive drug intake, habit learning, and stress- or cue-induced relapse. Furthermore, identifying the interplay between prefrontal and subcortical circuits involved at different stages of drug treatment will be useful in the development of improved genetic, pharmacotherapeutic and behavioral treatments for addiction. Particular focus should be made on identifying novel treatments designed to strengthen the appropriate region and cell specific synaptic connections and alternatively weaken or eliminate maladaptive synapses that underlie sensitization, tolerance, compulsive drug intake, craving or relapse that may contribute to the switch to addiction.

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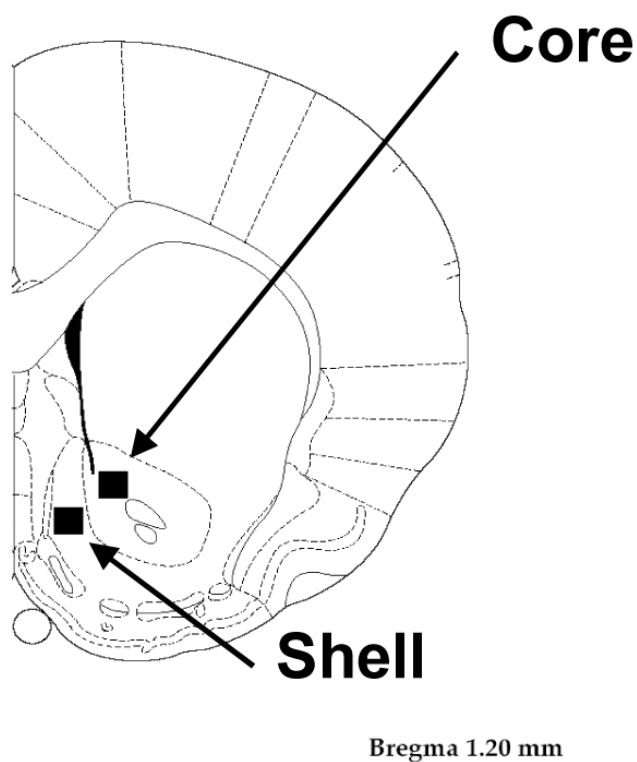


Figure 1. Coronal Section of the Rat Brain

Schematic representation of a coronal section (Bregma 1.20 mm) of the rat brain atlas (Paxinos and Watson, 1998). The boxes indicate the sample areas where neuron density and synapse density quantifications were obtained from the shell and core compartments of the NAc for each animal.

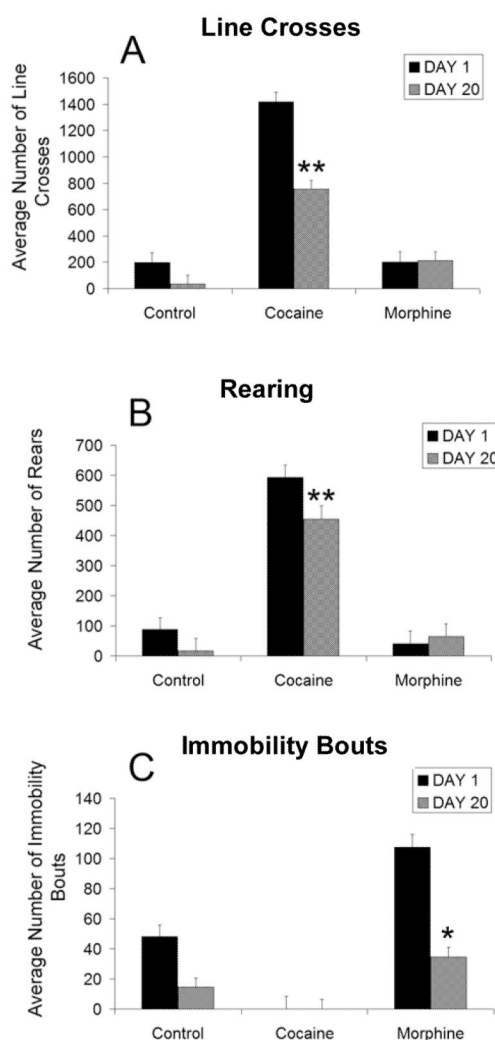


Figure 2. Non-stereotypic behaviors

The average (\pm SEM) number of line crosses (A) rears (B) and immobility bouts (C) during the 1-hour testing session, as a function of treatment and day. Significant decreases in line crosses and rears were observed in the cocaine treated animals compared to controls. A significant decrease in those behaviors, were observed in the cocaine group on day 28 compared to day 1. Morphine produced motor immobility and it was significantly higher on day 1 relative to day 28 indicative of motor tolerance. (** indicates statistical significance ($p < .05$) relative to control group and day 1; * indicates statistical significance ($p < .05$) relative to day 1).

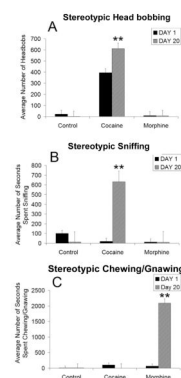


Figure 3. Stereotypic behaviors

The average (\pm SEM) number of head bobs (A) seconds spent sniffing (B) and seconds spent chewing/gnawing (C) during the 1-hour testing session, as a function of treatment and day. Sensitization of head bobbing and sniffing was evident in the cocaine group by day 28 relative to controls, whereas the morphine treated animals displayed sensitization of chewing and gnawing behavior by day 28 (** indicates statistical significance ($p < .05$) relative to control group and day 1; * indicates statistical significance ($p < .05$) relative to day 1).

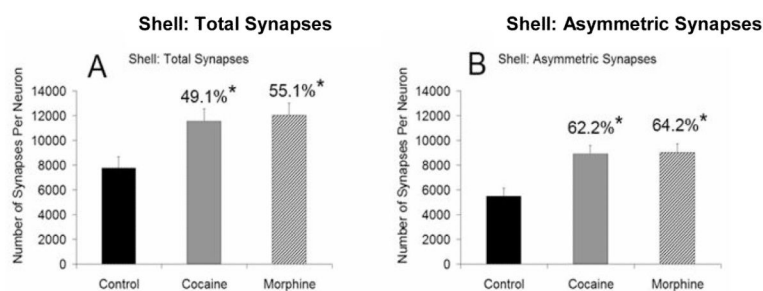


Figure 4. Total and asymmetric synaptic quantification in the NAc shell

The average (\pm SEM) number of total synapses (A) and asymmetric synapses (B), as a function of treatment in the NAc shell. A significant increase in the total number of synapses per neuron and asymmetric synapses per neuron in the NAc were found in the cocaine and morphine treated animals (* indicates statistical significance ($p < .05$) relative to control group).

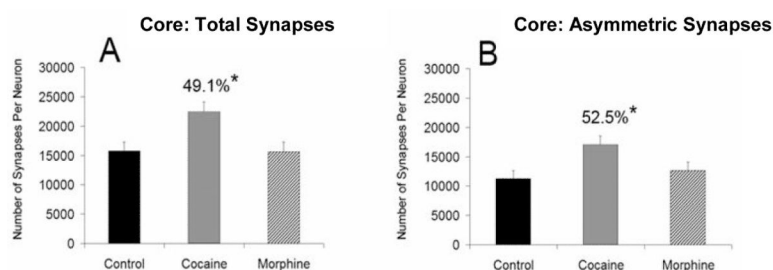


Figure 5. Total and asymmetric synaptic quantification in the NAc core

The average (\pm SEM) number of total synapses (A) and asymmetric synapses (B), as a function of treatment in the NAc core. A significant increase in the total number of synapses per neuron and asymmetric synapses per neuron in the NAc was found in the cocaine group (* indicates statistical significance ($p < .05$) relative to control group).

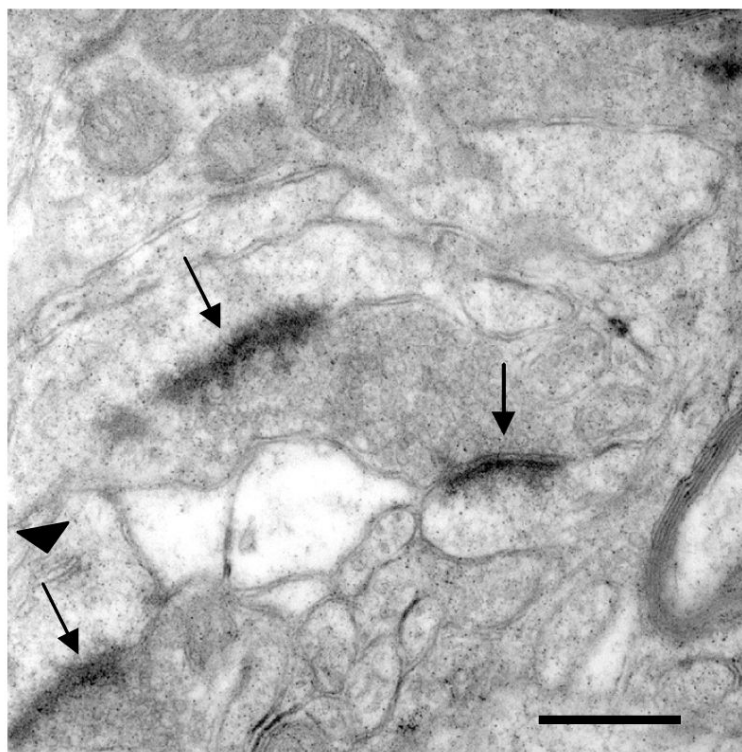


Figure 6. Electron micrograph of synapses in the NAc

Electron micrograph (18,000X) taken of asymmetric synapses (arrows) made onto spines in the NAc. A Spine apparatus can be seen in the lower left spine (arrowhead). Scale bar = 500 nm.

Table 1
Percentage of asymmetric and symmetric synapses onto dendritic spines and dendritic shafts in the nucleus accumbens shell and core

The values listed reflect the total group means and standard errors for each condition (Control, Cocaine, Morphine) for the percent of total synapses categorized as asymmetric and symmetric that made contact with either dendritic spines or dendritic shafts. Significant increases in the total number of synapses per neuron were found in the NAc shell of cocaine-treated and morphine-treated and in the NAc core of cocaine-treated animals.

	Control	Cocaine	Morphine
Shell Total Number of Synapses per Neuron	7759 ± 1286	11567 ± 2862 *	12036 ± 3363 *
Dendritic Spine	42.1% ± 4.4%	34.0% ± 4.4%	41.7 % ± 4.2%
Dendritic Shaft	44.3% ± 4.5%	53.5% ± 4.5%	42.8% ± 4.2%
Asymmetric	71.1% ± 2.9%	77.5% ± 3.1%	75.9% ± 3.1%
Dendritic Spine	54.1% ± 5.3%	49.5 % ± 5.7%	43.3% ± 5.7%
Dendritic Shaft	33.7% ± 4.8%	39.0% ± 5.2%	45.9% ± 5.2%
Symmetric	23.5% ± 2.5%	18.5% ± 2.6%	20.8% ± 2.6%
Dendritic Spine	9.9% ± 5.1 %	18.4% ± 5.5%	9.8% ± 5.5%
Dendritic Shaft	80.6% ± 5.2%	74.7% ± 5.5%	86.8% ± 5.5%
Core Total Number of Synapses per Neuron	15781 ± 3982	22477 ± 5877 *	15645 ± 2083
Dendritic Spine	43.2% ± 3.1%	39.3% ± 3.1%	42.8% ± 2.9%
Dendritic Shaft	26.8% ± 2.9%	32.4% ± 2.9%	29.1% ± 2.7%
Asymmetric	71.2% ± 3.5%	72.1% ± 3.7%	73.9% ± 3.7%
Dendritic Spine	53.8% ± 3.2%	55.3% ± 3.4%	48.7% ± 3.4%
Dendritic Shaft	22.8% ± 1.9%	20.2% ± 2.0%	25.8% ± 2.0%
Symmetric	24.2% ± 3.5%	21.8% ± 3.7%	20.1% ± 3.7%
Dendritic Spine	11.3% ± 3.2%	11.5% ± 3.2%	18.1% ± 3.0%
Dendritic Shaft	56.4% ± 6.8%	67.7% ± 6.8%	50.5% ± 6.4%

*
(indicates significance at $p < .05$).