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Cocaine withdrawal-induced trafficking of delta-opioid receptors in rat nucleus accumbens

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Abstract

Interactions between the opioidergic and dopaminergic systems in the nucleus accumbens (NAcb) play a critical role in mediating cocaine withdrawal-induced effects on cell signaling and behavior. In support of this, increased activation of striatal dopamine-D1 receptors (D1R) results in desensitization of δ -opioid receptor (DOR) signaling through adenylyl cyclase during early cocaine withdrawal. A potential cellular substrate underlying receptor desensitization is receptor internalization. The present study examined the effect of cocaine withdrawal on subcellular localization of DOR in dendrites of the NAcb core (NAcbC) and shell (NAcbS) using immunoelectron microscopy. Female and male rats received binge-pattern cocaine or saline for 14 days and subsequently underwent 48 hour withdrawal. Animals were transcardially perfused and tissue sections were processed for immunogold-silver localization of DOR. Semi-quantitative analysis revealed that cocaine withdrawal caused an increase in the percentage of DOR localized intracellularly in the NAcbS of male and female rats and the NAcbC of male rats compared to saline controls. In contrast, in the NAcbC of female rats, there was an increase in DOR associated with the plasma membrane following cocaine withdrawal. To determine whether modulation of D1R could directly impact DOR containing neurons, the hypothesis that DOR and D1R co-exist in common neurons of the NAcb was examined in naïve rats. Semi-quantitative analysis revealed a subset of profiles containing both DOR and D1R immunoreactivities. The present findings demonstrate a redistribution of DOR in the NAcb following cocaine withdrawal and provide anatomical evidence supporting D1R regulation of DOR function in a subset of NAcb neurons.

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Classification terms

opioid receptors; catecholamine receptors; regional localization of receptors and transmitters; Drugs of abuse: cocaine; **Section:** Structural Organization of the Brain

Keywords

nucleus accumbens; ventral striatum; opioid; dopamine; electron microscopy; cocaine

1. Introduction

The nucleus accumbens (NAcb), a component of the ventral striatum, is composed of core and shell regions involved in motor and limbic functions, respectively. Interactions between the dopaminergic and opiodergic systems in the NAcb play a critical role in cocaine-induced effects by collectively modulating emotionally-motivated and goal-directed behaviors as well as locomotor activity (Fink and Smith, 1980; Roberts and Koob, 1982). Administration of drugs that alter dopaminergic neurotransmission can affect the opioid system by altering levels of enkephalins, endorphins, and dynorphins as well as opioid receptors (George and Kertesz, 1987; Hurd et al., 1992; Daunais and McGinty, 1994, 1995). Additionally, opioid receptor activation enhances extracellular dopamine levels in the NAcb (Hirose et al., 2005).

The striatum, including the caudate putamen and NAcb, is a major site of action for cocaine's effects on the dopamine and opioid systems. There is compelling anatomical evidence provided by independent ultrastructural studies for co-localization of dopamine-D1 receptors (D1R) and δ -opioid receptors (DOR) in neurons. Expression of both D1R and DOR mRNA has been detected in the NAcb (Weiner et al., 1991; Mansour et al., 1995; Lu et al., 1998). Furthermore, a potential co-existence of DOR and D1R is supported by ultrastructural studies demonstrating that dendritic spines labeled for DOR are in direct contact with dopamine transporter-immunoreactive terminals (Svingos et al., 1999). D1R has also been found on medium-sized spiny dendrites in dorsal and ventral striatum (Levey et al., 1993; Hara and Pickel, 2005; Dumartin et al., 2007; Hara and Pickel, 2007). In addition, DOR and its endogenous ligand, enkephalin, have been localized to the NAcb where they co-exist in a subset of profiles (Svingos et al., 1998). Members of the opioid and dopamine receptor families have been found to be co-expressed in a subset of striatal neurons in naïve rats (Ambrose et al., 2004; Ambrose et al., 2006), providing a potential mechanism for these two systems to interact at the subcellular level.

Both dopamine and opioid systems are altered in the presence of drugs of abuse, such as cocaine. Chronic cocaine administration and early withdrawal from chronic cocaine result in attenuation of the ability of the DOR agonist D-Pen², D-Pen⁵-enkephalin (DPDPE) to inhibit adenylyl cyclase activity in the striatum (Unterwald et al., 1993; Unterwald and Cuntapay, 2000; Perrine et al., 2008). This effect is mediated by D1R activation since the D1R agonist, SKF 82958, also attenuates DOR-inhibited adenylyl cyclase activity. Moreover, chronic SKF 82958 administration attenuates the ability of DPDPE to stimulate [35S] GTP γ S binding, indicative of an uncoupling of DOR from G-proteins (Unterwald and Cuntapay, 2000). Interestingly, significant alterations in the levels of G-proteins do not play a role in this process (Perrine et al., 2005). Since DOR expression is not changed following cocaine administration (Unterwald et al., 1994), the finding that cocaine modulates DOR-regulated adenylyl cyclase activity suggests that exposure to cocaine results in a functional uncoupling of DOR from adenylyl cyclase in striatal regions. Neuroadaptations in cell signaling pathways following chronic cocaine administration may contribute to an increased likelihood of relapse (Nestler, 2005). In rats, early cocaine withdrawal resulted in desensitization of striatal DOR in addition

to increased anxiety and depression-like behaviors (Perrine et al., 2008). Administration of the DOR agonist SNC-80 was able to ameliorate these cocaine-withdrawal induced behaviors (Perrine et al., 2008), supporting a role for DOR in the regulation of anxiety and depression during this period.

The molecular mechanism by which cocaine induces DOR heterologous desensitization has not yet been elucidated. Desensitization and internalization are two mechanisms known to regulate the function of the G-protein coupled receptor (GPCR) family to which DOR belongs. Phosphorylation by GPCR kinases and binding of β -arrestin results in an uncoupling of GPCR from associated G-proteins leaving receptors desensitized and unable to signal upon agonist binding. Following desensitization, cell surface receptors may undergo internalization with subsequent degradation or recycling to the cell surface. Examples of DOR trafficking in response to drugs of abuse have been demonstrated. For instance, following prolonged treatment with morphine, there is a translocation of DOR from intracellular compartments to the plasmalemma in dendrites of both the NAcB and striatum (Lucido et al., 2005). The trafficking patterns of DOR in the NAcB in response to cocaine withdrawal have yet to be reported. Therefore, the present study investigated the effects of withdrawal from chronic cocaine administration on DOR receptor trafficking in the NAcB core (NAcB_C) and shell (NAcB_S) of both male and female rats using immunoelectron microscopy. Since previous studies report DOR desensitization, our hypothesis postulated that early cocaine withdrawal would induce internalization of DOR. The cellular distributions of DOR and D1R in the NAcB_C and NAcB_S of naïve rats was also examined using fluorescence and electron microscopy. Since D1R activation mediates DOR desensitization, we tested the hypothesis that a subset of NAcB profiles would co-express D1R and DOR, providing an anatomical substrate for their interaction.

2. Results

DOR trafficking

Regions sampled for electron microscopic analysis are depicted in Figure 1A. Immunogold-silver detection for DOR was employed to examine the subcellular distribution of DOR in dendritic profiles in the NAcB_C and NAcB_S of both saline and cocaine-treated male and female rats that underwent two day withdrawal from drug or vehicle treatment. In chronic saline-treated rats, DOR was localized to both plasmalemmal and intracellular compartments of dendritic profiles in both the core and shell regions in both male (data not shown) and female rats (Figure 1 B,C and Figure 2 A,B). In the NAcB_S of female rats undergoing cocaine withdrawal, the majority of DOR was localized to intracellular compartments (Figure 1D, 1E). Within intracellular compartments, immunogold-silver labeling for DOR was often associated with endosome-like structures. Interestingly, DOR was localized to both the plasma membrane and intracellular compartments in the NAcB_C of cocaine-treated females (Figure 2 C, D). In males, cocaine withdrawal resulted in a decrease in plasma membrane DOR in both core and shell regions (Figure 3 C,D).

Semi-quantitative analysis was conducted to determine the percentage of DOR localization to the plasma membrane of dendritic profiles in the NAcB_C and NAcB_S of both male (n=2) and female (n=3) rats (Figure 3). In females, withdrawal from chronic cocaine resulted in a decrease in the percentage of DOR localized to the plasma membrane in NAcB_S and an increase in the percentage of plasma membrane DOR in NAcB_C. Thirty-five percent of DOR in the NAcB_S of saline-treated females was localized to the plasma membrane (n=185 labeled profiles) compared to only 24% (n=245) in cocaine-treated rats (Figure 3A). This represents ~ 31% overall reduction in plasma membrane DOR after two days of withdrawal from chronic cocaine. Twenty percent (n=199) of DOR in the NAcB_C of saline-treated females was localized to the

plasma membrane compared to 26% (n=213) in cocaine-treated females representing ~ 30% overall increase in DOR localization to the plasma membrane (Figure 3B).

In males, withdrawal from chronic cocaine resulted in a decrease in plasma membrane DOR in both core and shell regions. In the NAcS, 29% of DOR in dendritic profiles (n = 103) from saline-treated males compared to 19% of DOR (n=103) from cocaine-treated males was localized to the plasma membrane (Figure 3C). In the NAcC, 22% of DOR (n = 114) was localized to the plasma membrane in saline-treated males compared to only 17% (n=103) in cocaine-treated males (Figure 3D). Overall this represents a ~34 % and ~23% decrease in DOR localization to the plasma membrane in core and shell regions, respectively.

Western blot analysis was used to examine DOR expression levels in the NAc two days following saline or cocaine administration (n=4 rats per group) (Figure 4). Incubation of the primary antibody with its blocking peptide resulted in a loss of immunoreactivity for DOR at the expected molecular weight of 50 kDa (Figure 4A) (Persson et al., 2003; Persson et al., 2005). Densitometric analysis of DOR protein expression in the NAc indicated that DOR was not different between saline-treated vs. cocaine-treated rats (Figure 4B, 4C). There was also no difference in male vs. female rats (data not shown).

Co-localization of DOR and D1R

To determine the degree of co-existence between DOR and D1R both immuno-fluorescence and electron microscopy were employed. Single labeling for D1R and DOR was present within the NAcC and NAcS subregions. Confocal microscopy revealed that a subset of neurons in both the NAcS and NAcC exhibited both DOR and D1R immunoreactivities (Figure 5A,B). These are clearly distinguishable in merged images that show singly labeled DOR and D1R neurons as well as dually labeled DOR and D1R cells (Figure 5C).

Electron microscopy allowed for further analysis of DOR and D1R co-localization. At the ultrastructural level, immunogold-silver labeling for D1R and DOR was detected within the NAcC and NAcS in both single and dual-labeled profiles. Black deposits indicative of gold-silver immunolabeling were distinguishable from the electron dense peroxidase reaction product. To circumvent potential caveats caused by the visualization of these two different labeling techniques, immunomarkers were reversed in a separate set of tissue run simultaneously to ensure that the localization of each antigen was not thwarted by the detection method used. Results were similar irrespective of the labeling approach used.

In both the NAcC and NAcS, single-labeled profiles were visualized within similar portions of the neuropil. Often, single-labeled D1R and DOR somatodendritic processes were in close proximity to one another. In some cases, single-labeled profiles were apposed to one another. Localization of D1R and DOR to the same cellular profiles was evident in both the shell and core regions (Figure 6A, B). Clusters of immunoperoxidase labeling for D1R could be identified within dendrites (Figure 6A) and somata that also contained gold-silver immunoreactivity for DOR. Furthermore, co-localization was also present when labels were reversed (Figure 6B).

Semi-quantitative analysis was conducted to determine the percentage of co-existence between DOR and D1R in the NAc (Figure 6C). In the NAcS, the percentage of co-existence was similar with 33% of D1R labeled profiles (n=215) containing DOR immunolabeling and 34% of DOR labeled profiles (n=211) containing D1R immunoreactivity. In the NAcC, D1R labeled profiles (n=316) contained DOR immunoreactivity in 24% of sampled neurons. Interestingly, a greater percentage of total DOR labeled profiles (n=198) showed immunolabeling for D1R (n=75, 38%). As both of these receptors were primarily localized to somatodendritic processes, co-existence was predominantly seen in these cellular profiles.

3. Discussion

This study provides ultrastructural evidence for trafficking of DOR during early withdrawal from chronic cocaine treatment. In addition, these results demonstrate sex differences in DOR trafficking during early phases of cocaine withdrawal. Our findings also indicate that a subset of neurons express both D1R and DOR, providing an anatomical substrate in support of potential dopamine-opioid interactions in this region. Finally, our data suggest that alterations in DOR signaling following withdrawal from cocaine are not readily explained by changes in receptor expression levels but are more likely related to alterations in receptor availability on the plasma membrane.

Methodological Considerations

A potential limitation known to be associated with the pre-embedding immunolabeling technique is attributed to penetration of immunoreagents in thick Vibratome sections (~40 µm) (Chan et al., 1990). To circumvent this caveat, analysis of ultrathin sections was carried out exclusively on sections near the tissue/plastic interface where penetration is maximal. Further, immunolabeling of antigens was reversed to ensure that patterns of immunolabeling were not attributed to differences in the detection methods (see below).

The specificity of the D1R and DOR primary antibodies are discussed in the section entitled “Experimental Procedure” and we have performed the requisite control experiments to ensure antibody specificity here and in our previous studies (Commons et al., 2001; Ambrose et al., 2006). Our findings are in agreement with others that used the same D1R primary antibody and reported a primarily postsynaptic distribution of D1R along the plasma membrane of neurons in both subregions of the NAc (Hara and Pickel, 2005). Furthermore, our results are consistent with localization of D1R in medium-sized spiny neurons (Yung et al., 1995; Hara and Pickel, 2005; Dumartin et al., 2007; Hara and Pickel, 2007).

Both receptors were primarily localized to somatodendritic processes in both the core and shell of the NAc. In both the core and shell region of NAc, DOR was predominantly intracellular as previously shown using immunohistochemistry in other CNS regions (Cheng et al., 1995; Zhang et al., 1996). This pattern of prominent somatodendritic labeling for the DOR is consistent with previous studies using antibodies specific for this region of the N-terminus (amino acids 3–17 on the cloned mouse DOR1) (Cahill et al., 2001; Lucido et al., 2005). For example, Lucido et al. demonstrated DOR membrane recruitment in NAc dendritic profiles following morphine treatment and reported that most of DOR immunoreactivity was localized to neuronal somata and dendrites, with low to moderate labeling of synaptic terminals. However, studies using antibodies raised to different sequences of the receptor have reported different patterns of labeling. For example, Svingos and colleagues (using an antibody raised to amino acids 34–47 of the N-terminal domain) reported that 60% of DOR-immunoreactive profiles in the NAcS were axon terminals and small unmyelinated axons with 35% of DOR profiles characterized as dendrites and dendritic spines (Svingos et al., 1998). This discrepancy could be due to multiple factors. It is possible that the inclusion criteria used in the present analysis for immunogold labeling may have led to an under-representation of presynaptic DOR labeling. In addition, variations in antibody recognition could cause differences in detection. Although this caveat remains, the present study is consistent with previous reports and extends these findings by showing cocaine withdrawal-induced trafficking of DOR and co-existence of D1R and DOR in the NAc.

The present study compared NAcC and NAcS but these regions can be further subdivided medially and laterally. Although the majority of samples were taken from the medial aspect of the core and shell of the NAc, we did not investigate subdivisions of the core and shell here. Anatomical differences between medial and lateral accumbens shell have been reported in

regards to both neuronal morphology and connectivity (Meredith et al., 1992; Voorn et al., 2004; Ikemoto, 2007). Additional studies focusing on medial and lateral divisions of the core and shell will be required to determine if any differences exist.

Lastly, we used both males and females in the present analysis and uncovered some potentially important sex differences. Sex differences in response to cocaine and other drugs of abuse are emerging [for reviews, see (Lynch et al., 2002; Carroll et al., 2004; Becker and Hu, 2007)]. In the NAcB, there are sex differences in dopaminergic function as well as regulation of cell signaling in both naïve and cocaine-treated animals (Becker, 1999; Lynch et al., 2006). The present study did not investigate the influence of hormonal variations associated with the estrous cycle on DOR trafficking. It is possible that varying hormone levels may influence DOR modulation in female rats. Future studies investigating therapeutic agents targeting dopamine and opioid systems will need to consider potential sex differences.

DOR trafficking in NAcB and NAcC during early cocaine withdrawal

Receptor trafficking can regulate the number of receptors available at the cell surface and can therefore influence cell reactivity to neurotransmitters and drugs. Under normal conditions, only a small percentage of DOR are available at the plasma membrane. The majority of DOR is localized intracellularly (Arvidsson et al., 1995; Cheng et al., 1995; Cheng et al., 1997; Cahill et al., 2001; Commons et al., 2001). Consistent with previous evidence, 20–35% of DOR was localized to the plasma membrane of saline-treated controls. In the NAcC of males and the NAcB of males and females, early cocaine withdrawal-induced internalization of DOR resulted in a reduction in the number of receptors at the cell surface. This condition did not lead to a decrease in the overall expression of receptors since Western blot analysis did not reveal any change in expression levels. The present findings are consistent with previous evidence reporting no change in DOR receptor number using quantitative autoradiography on tissue obtained immediately following 14 day binge-pattern cocaine administration (Unterwald et al., 1994). These data suggest that the difference in DOR during early cocaine withdrawal is more likely due to redistribution of DOR and may not result from changes in protein expression.

Desensitization of DOR has been reported immediately following chronic cocaine and during early withdrawal (Unterwald et al., 1993; Unterwald and Cuntapay, 2000; Perrine et al., 2008). The results of the present study demonstrate cocaine withdrawal-induced trafficking of DOR which may contribute to the increased anxiety and depression associated with withdrawal from chronic cocaine. Although the reported difference in subcellular localization of DOR did not achieve conventional statistical significance, it is likely that the change in receptor localization at the plasma membrane represents an important change in the number of receptors at the cell surface available for agonist binding. Decrease in cell surface DOR could lead to alterations in emotional state as DOR has been shown to be involved in anxiety and depression, conditions that are both linked to cocaine withdrawal (Filliol et al., 2000; Perrine et al., 2008). Since the NAcB communicates with many limbic regions, cocaine-withdrawal induced neuroadaptations in this region have an influence on behavioral state. The NAcB is most associated with limbic functions and therefore, it is possible that alterations in DOR in the NAcB could modulate emotional behavior. Interestingly, there was a slight increase in the localization of DOR to the plasma membrane in the NAcC of female rats. The core is known to be more closely related to motor functions. Future studies are needed to determine if cocaine-induced changes in this region may be involved in the underlying mechanisms involved in the increased behavioral sensitization to cocaine exhibited by female rats (Hu and Becker, 2003).

Our data indicate that alterations in DOR signaling following withdrawal from cocaine are not readily explained by changes in receptor expression levels as Western blot analysis did not show any difference in DOR expression levels when comparing cocaine withdrawn rats to

saline-treated controls. Interpretation of these data should consider, however, that both the core and shell subregions of the NAc were sampled together and that regional differences may exist that were not readily identified in this study. Therefore, *in situ* hybridization analysis of mRNA for DOR should be conducted in future studies to unequivocally establish the presence or lack of DOR receptor downregulation.

Co-existence of D1R and DOR in NAcS and NAcC

Previous work from our laboratory has demonstrated co-existence of D1R and DOR in a subset of neuronal profiles in the dorsolateral striatum (Ambrose et al., 2006). Since the NAc is a major target for cocaine, the present study sought to address whether a similar organization exists in the NAc core and shell regions. Co-localization of DOR and D1R in accumbal neurons is a first step in elucidating the cellular mechanisms involved in the heterologous desensitization of DOR. This effect is mediated by D1R as chronic administration of a D1R agonist also attenuates DOR-induced inhibition of adenylyl cyclase activity (Unterwald and Cuntapay, 2000). It is interesting to note that although the NAcS had an almost equal percentage of DOR profiles containing D1R and D1R profiles containing DOR, this was not the case in the NAcC. In addition, although the co-expression of these two receptors was evident in the ventral striatum, it occurs to a lesser extent compared to the dorsolateral striatum which demonstrated about 50% co-localization (Ambrose et al., 2006). A recent study investigating the subcellular localization of D1R in the dorsal striatum, NAcC and NAcS also reported differences among these regions (Dumartin et al., 2007). Potentially, this regional heterogeneity could affect the contribution of each of these regions to the interaction between the dopaminergic and opioidergic systems following cocaine.

Further investigation is essential in order to determine other factors that may be involved and the degree to which this co-existence plays a role in the interaction between these two receptor systems. In a recent study utilizing a novel strategy where the nuclear translocation pathway was adapted to visualize opioid-dopamine receptor hetero-oligomers, data suggested that DOR and D1R did not form hetero-dimers while μ -opioid receptor (MOR) and D1R did form hetero-dimers (Juhász et al., 2007). Although this does not support a direct interaction between DOR and D1R, there is evidence demonstrating the ability of MOR and DOR to form functional hetero-dimers (George et al., 2000; Gomes et al., 2000; Gomes et al., 2004) at the cell surface (Law et al., 2005). This could be a mechanism by which D1R can directly interact with both MOR and DOR receptors. Electron microscopy demonstrates co-localization of DOR and MOR in the striatum (Wang and Pickel, 2001) but dual-labeling in the NAc has not been examined. However, separate studies using single labeling for MOR and DOR have shown these receptors to be localized to accumbal neurons [present results; (Svingos et al., 1996; Svingos et al., 1998)].

DOR agonists as potential therapeutic agents during cocaine withdrawal

Both desensitization and trafficking of DOR are possible mechanisms underlying anxiety and following abstinence from chronic cocaine treatment. Receptor trafficking can regulate the number of receptors available at the cell surface therefore influencing the availability of receptor for stimulation by ligands. Interestingly, results from manipulation of the DOR system suggest a modulatory role for DOR in anxiety (Filliol et al., 2000; Perrine et al., 2005; Saitoh et al., 2005). Moreover, a recent study demonstrated that cocaine-induced anxiety- and depression-like behaviors during early cocaine withdrawal accompanied DOR desensitization (Perrine et al., 2008). Administration of DOR agonist SNC-80 was able to ameliorate these cocaine-induced behaviors (Perrine et al., 2008). The present data report a decrease in plasma membrane DOR available for binding, however, it is possible that DOR agonists are able to activate the functional DOR remaining on the plasma membrane.

Since the NAcB plays a key role in the rewarding aspects of drugs of abuse, continued investigations of dopamine–opioid interactions may elucidate novel targets for the treatment of addiction. Clinical observations indicate that cocaine addicts use opiates to reduce cocaine-induced anxiety and irritability (Kosten et al., 1986; O'Brian, 2001). Moreover, DOR agonists are reported to have fewer negative side effects and lower abuse potential compared to MOR agonists such as morphine (Jutkiewicz, 2006). DOR agonists may be beneficial as a therapeutic for both addiction and other opioid-related disease states (i.e. pain).

Conclusions

These results support potential sex and regional differences in cocaine withdrawal-induced trafficking of DOR in the NAcB. In addition, these results support the existence of a subset of NAcB neurons co-expressing D1R and DOR. Based on these data, it is tempting to speculate that co-existence of opioid and dopamine receptors in common neurons may mediate DOR trafficking during cocaine withdrawal.

4. Experimental Procedure

Animals

All animal care and procedures were conducted in compliance with the guidelines set forth by the Institutional Animal Care and Use Committee of Thomas Jefferson University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. In addition, adequate measures were taken to minimize pain or discomfort. Nine male and fourteen female Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN; age ~ 60 days) were used in this study. Animals were housed three to a cage and maintained on a 12 hour light/dark cycle (lights on at 7 am) with free access to food and water.

Drug Administration

Cocaine HCl (generously provided by NIH/NIDA) was dissolved in saline (0.9%) and injected in a volume of 1 ml/kg body weight. Male and female Sprague-Dawley rats were administered binge-pattern (3 injections/day at 1 hour intervals) cocaine (15 mg/kg i.p.) or saline (1ml/kg i.p.) for 14 days followed by a 48 hour withdrawal period. In humans, addicts generally self-administer cocaine repeatedly over a short time period, “binging”, typically in the early evening hours followed by a period of abstinence (Gawin, 1991). This dosing schedule in animals mimics this pattern in terms of temporal pattern and also in relation to circadian rhythm. Following the 48 hours of withdrawal from drug administration, animals were euthanized and brains were processed for either immunohistochemistry or Western blot analysis.

Tissue Preparation for Immunohistochemistry

Electron Microscopy—The methods used for fixation and immunolabeling have been described in previous publications (Ambrose et al., 2004; Ambrose et al., 2006). Naïve and drug treated rats were initially anesthetized with isoflurane and subsequently received an intraperitoneal injection of sodium pentobarbital (60mg/kg). The rats were perfused transcardially through the ascending aorta with 60ml of heparinized saline (1000 U/ml) followed by either 3.8% acrolein in 2% paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.4), followed by 2% paraformaldehyde in 0.1M PB or 1.5% glutaraldehyde in 4% paraformaldehyde in 0.1M PB (for fluorescence microscopy). For electron microscopic analysis, following perfusion-fixation, brains were removed and post-fixed overnight at 4°C. After vibratome (Technical Products International, Inc., St. Louis, MO) sectioning at 30–40 µm through the rostrocaudal extent of the NAcB [plates 10–14 of the rat brain atlas (Paxinos, 1998)], sections were placed in 1% sodium borohydride in 0.1M PB. Tissue sections were then

rinsed in 0.1M PB, followed by 0.1M Tris-buffered saline (TBS; pH 7.6) and blocked in 1% bovine serum albumin (BSA) in TBS before incubation in primary antibody solution.

DOR was identified using a polyclonal antibody raised against a region of the N-terminus on the cloned mouse DOR1 (Immunostar, Inc., Hudson, WI) (Dado et al., 1993). D1R was identified using a monoclonal antibody raised against the C-terminus of the human D1R (Sigma, St. Louis, MO) (Levey et al., 1993). Tissue sections were incubated overnight in primary antibody solution containing either rabbit anti-DOR (1:500, Immunostar) in 0.1M TBS containing 0.1% BSA (single labeled sections for analysis of trafficking) or a cocktail containing both anti-DOR (1:500, Immunostar) and rat anti-D1R (1:500, Sigma) in 0.1M TBS containing 0.1% BSA (dual-labeled sections for co-localization studies).

For dual co-localization studies, immunoperoxidase labeling for D1R was obtained by incubating sections in biotinylated donkey anti-rat IgG (1:400, Jackson ImmunoResearch Laboratories, West Grove, PA) followed by avidin-biotin complex (Vector Laboratories). The D1R was visualized by reacting the tissue with 22 mg of 3–3' diaminobenzidine (DAB) (Aldrich, Milwaukee, WI) containing 10 µl of 30% hydrogen peroxide in 100ml of 0.1M TBS. For immunogold detection of DOR in both trafficking and co-localization studies, sections were incubated in 1nm gold-conjugated goat anti-rabbit IgG (1:50, Amersham Corp., Amersham, England), fixed in 2% glutaraldehyde in PBS, and intensified using a silver enhancement kit (Amersham Corp.). Alternate sections were processed so that DOR was labeled with immunoperoxidase using biotinylated donkey anti-rabbit IgG (1:400, Jackson ImmunoResearch Laboratories) secondary antibody and the D1R was labeled using 1nm gold anti-rat IgG (1:50, Ted Pella, Inc.) to ensure that detection of antigens was not thwarted by immunolabeling techniques. Ultrathin sections were cut through the NAcB (~70 nm) with a diamond knife, collected on copper mesh grids, and viewed using a Morgagni 268 (FEI Company, Hillsboro, Oregon) digital electron microscope. Adobe Photoshop (Adobe, San Jose, CA) was used to adjust brightness and contrast and to construct the final image.

Fluorescence microscopy—For fluorescence microscopy, brains were incubated post-fixation in a gradient of sucrose solutions (containing 0.1% sodium azide) of 10% and 20% for 1 hr each, followed by 30% sucrose for 48–72 hrs. Brain sections were placed in TBS Tissue Freezing Medium (Fisher Scientific, Pittsburgh, PA) and frozen in liquid CO₂. Brains were placed in a Microm HM550 cryostat (Richard-Allan Scientific, Kalamazoo, MI) for thirty minutes before the sectioning. Thirty µm thick coronal sections were cut through the rostrocaudal extent of the NAcB and collected in chilled 0.1 M PB. Briefly, sections were incubated in a primary antibody cocktail containing rat anti-D1R (1:500, Sigma) and rabbit anti-DOR (1:500, Immunostar). Sections were rinsed and incubated in fluorescein isothiocyanate-conjugated donkey anti-rat (1:100, Jackson Laboratories) and tetramethyl rhodamine isothiocyanate-conjugated goat anti-rabbit (1:100, Jackson Laboratories) antibodies. Sections were then mounted from 0.05 M PB onto gelatinized slides, dried, and coverslipped using Krystalon mounting medium (EM Industries, Gibbstown, NJ). Slides were stored in dark containers at 4 °C until sections were visualized using a confocal microscope (Zeiss LSM 510 Meta, Carl Zeiss Inc., Thornwood, NY, USA) and digital images were obtained and imported using the LSM 5 image browser.

Antibody Specificity—DOR was identified using a polyclonal antibody raised in rabbit against a synthetic peptide sequence corresponding to amino acids 3–17 predicted from the cloned mouse DOR1 (Immunostar, Inc., Hudson, WI) (Dado et al., 1993). Previous studies using this DOR antibody have shown that immunolabeling is abolished following preadsorption with the antigenic peptide (Arvidsson et al., 1995). We have replicated this in our laboratory with both immunohistochemistry and Western blotting techniques. In addition, control studies in CHO cells lines transfected with DOR, MOR or KOR, demonstrated that

this antibody was able to label DOR transfected cells but not MOR or KOR transfected cells lines (generously provided by Dr. Lee-Yuan Liu Chen, Temple University).

For D1R immunolabeling, a monoclonal antibody raised in rats (Sigma, St. Louis, MO) specific for a 97 amino acid peptide sequence corresponding to the C-terminus of the human D1R was used (Levey et al., 1993). The region to which this antibody binds has little to no sequence homology with other dopamine receptor subtypes. The specificity and lack of cross-reactivity of this antibody has been described in previous studies using the following techniques: immunoprecipitation, immunocytochemistry, and Western blot analysis (Levey et al., 1993; Smiley et al., 1994; Hersch et al., 1995).

To determine specificity of the secondary antibodies used in the present study, experiments were conducted where the primary antibodies were omitted during immunocytochemical processing. Following incubation in secondary antibody, no immunoreactivity was detected indicating that the secondary antibodies used did not yield any non-specific labeling.

Western Blot Analysis—The NAcb was micro-dissected from the brains of both treatment groups. Tissue was extracted in RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA). Samples containing equal amounts of total protein (21 µg) were separated on 4–12% Tris–Glycine polyacrylamide gels (Invitrogen) followed by electrophoretic transfer to Immobilon-P PVDF membranes (Millipore, Bedford, MA). Membranes were probed using DOR primary antibody (1:1000, Immunostar, Inc., Hudson, WI) and alkaline phosphatase-conjugated secondary antibody (WesternBreeze® Chemiluminescent Kit) followed by incubation with a chemiluminescent substrate and film exposure. Exposed films were scanned and the density of each band was quantified using Un-Scan-It blot analysis software (Silk Scientific Inc., Orem, Utah). In order to control for protein loading, each blot was stripped using Restore Stripping Buffer (Pierce) to disrupt previous antibody–antigen interactions and re-probed for β-actin (1:5000, Sigma). Blot quantification was normalized using inter and intra-blot controls and an unpaired t-test was conducted to compare treatment groups.

Data Analysis

For electron microscopy, the classification of neuronal profiles was based on that of Peters et al. (Peters et al., 1991). A profile was defined as immunogold-silver labeled when two or more gold-silver particles were detected (Ambrose et al., 2004). To determine spurious gold-silver deposits in the neuropil, blood vessels and the myelin sheath of myelinated axons, structures not expected to exhibit immunoreactivity for dopamine or opioid receptors, were used as an indicator of the degree of spurious labeling. Of fifty randomly sampled micrographs from tissue processed for the co-localization study, only three randomly sampled myelinated axons contained a gold particle. This demonstrates that minimal spurious gold-silver labeling was present in the tissue used for ultrastructural analysis. A profile was defined as immunolabeled with peroxidase when electron dense deposits were detected in the cytoplasm or along the plasma membranes. These were more electron dense than that of morphologically similar profiles observed within adjacent portions of the neuropil. Gold particles were identified as plasmalemmal if they were associated with the plasma membrane, and cytoplasmic if they were not in contact with the plasma membrane.

Four different treatment groups were used for the analysis of DOR trafficking: Male Cocaine, Male Saline, Female Cocaine, and Female Saline. Each treatment group included 2–3 rats and both core and shell regions of the nucleus accumbens were analyzed. At least forty labeled dendritic profiles were quantified from at least two grids from each brain region of each animal. Unpaired t-tests were used for statistical analysis. For co-localization studies, tissues from three naïve male rats were analyzed. At least 2 grids from each brain region were examined.

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Figure 1. Subcellular distribution of DOR in the NAcS in both saline and cocaine-treated females

A: Schematic of a coronal hemisection of rat forebrain illustrating the regions of the NAc sampled for electron microscopic analysis. Trapezoids indicate the NAc core and shell regions sampled for ultrastructural analysis. ac, anterior commissure; cc, corpus callosum; CPu, caudate putamen; NAcC, nucleus accumbens core; NAcS, nucleus accumbens shell. Modified from Paxinos and Watson (Paxinos, 1998). Scale bar = 3 mm. **Saline-treated. B:** In the NAcS of saline-treated rats DOR immunolabeling was visualized in association with the plasma membrane (arrows) and the cytoplasm (arrowheads) in dendritic profiles. **C:** An unlabeled terminal was presynaptically apposed to a dendrite containing DOR along the plasmalemma. **Cocaine-treated. D:** Withdrawal from chronic cocaine administration resulted in internalization of DOR (arrowheads). Often internalized receptors were associated with endosome-like structures (arrowhead in circle) **E:** A DOR-labeled dendrite containing cytoplasmic DOR (arrowheads) was postsynaptic to an unlabeled terminal. The large arrow indicates DOR associated with an endosome-like structure. Boxes include enlarged portions of D (arrowhead in circle) and E (large arrow) representing DOR association with endosome-like structures. Abbreviations: ut = unlabeled terminal; ma= myelinated axon; m=mitochondria.

Figure 2. Subcellular distribution of DOR in the NAcS in both saline and cocaine-treated females
Saline-treated

In the NAcC of saline treated rats, DOR was localized to both membrane (arrow) and intracellular compartments (arrowheads). **A:** An unlabeled terminal forms a synapse with a spinous process of a DOR-labeled dendrite. **B:** A dendrite with DOR associated with the outer plasma membrane and intracellular compartments. In the NAcC, withdrawal from chronic cocaine administration resulted in a slight increase in DOR at the plasma membrane. **Cocaine-treated.** **C:** A DOR-labeled dendrite containing plasma membrane and cytoplasmic DOR post-synaptic to an unlabeled terminal. **D:** Two DOR-labeled dendrites were visualized in the same field. Both contained plasma membrane (arrows) and cytoplasmic (arrowheads) immunogold labeling for DOR. DOR was associated with endosome-like structures (arrowhead in circle). Abbreviations: ut = unlabeled terminal; m=mitochondria.

Figure 3. Quantification of DOR localization to plasma membrane

Bar graphs representing the percentage of DOR on the plasma membrane in saline and cocaine-treated male and female rats. **A:** In the NAcS, 35% of DOR was localized to the plasma membrane of saline-treated females compared to 24% in cocaine-treated rats. **B:** In the NAcC, 20% of DOR was localized to the plasma membrane of saline-treated females compared to 26% of cocaine-treated females. **C:** In the NAcS of male rats, 29% of DOR from saline-treated rats were localized to the plasma membrane compared to 19% in cocaine-treated males. **D:** In the NAcC, 22% of DOR was localized to the plasma membrane in saline-treated males compared to 17% in cocaine-treated males. Abbreviations: PM= plasma membrane. Female (n=3 rats/group); Male (n=2 rats/group)

Figure 4. Analysis of Protein Expression

Western blot analysis of NAcB tissue from female cocaine and saline-treated animals. **A:** Preincubation of DOR antibody with control peptide prior to immunoblot probing resulted in a loss of immunoreactivity (arrow) at 50kDa, the expected molecular weight of the DOR. **B:** Following two day withdrawal from chronic binge-pattern cocaine, DOR expression was unchanged in NAcB compared to saline controls (C=cocaine; S=saline). An unpaired t-test revealed no significant difference in DOR band density in NAcB tissue extracts between treatment groups following withdrawal from binge-pattern cocaine administration (n=4 rats/group).

Figure 5. Immunofluorescence microscopy showing localization of the D1R and DOR in the NAcS
 Confocal images of a coronal section through the NAcS dually labeled for visualization of
A: DOR (TRITC) and **B: D1R (FITC)**. **C: Merged image** showing neurons expressing both
 DOR and D1R indicated by arrowheads in A, B, and C. Scale bar = 25 μm .

Figure 6. Co-localization of the D1R and the DOR in the NAcS and NAcC

A: A dendrite co-expressing immunoperoxidase labeled D1R (arrows) and immunogold labeled DOR (arrowheads) in the NAcS. D1R is visualized along the plasma membrane while DOR is located intracellularly. **B:** Reverse labeling illustrating a dendrite in the NAcC exhibiting co-localization of immunoperoxidase labeled DOR (arrows) and immunogold labeled D1R (arrowheads). An unlabeled axon terminal containing vesicles is apposed to the co-labeled dendrite. Bar = 500nm. Abbreviations: D1R + DOR-d = D1R and DOR labeled dendrite; ut = unlabeled terminal; D1R-d = D1R labeled dendrite; ma= myelinated axon; m=mitochondria. **C:** Bar graphs representing the percentage of co-localization between DOR and D1R. In the NAcS 33% of D1R labeled profiles contained DOR immunolabeling and

34% of DOR labeled profiles contained D1R immunoreactivity. In the NAcB, 24% of D1R labeled profiles contained DOR immunoreactivity and 38% of DOR labeled profiles contained immunolabeling for D1R.