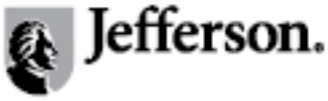

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Abstract

Cocaine's enhancement of dopaminergic neurotransmission in the mesolimbic pathway plays a critical role in the initial reinforcing properties of this drug. However, other neurotransmitter systems are also integral to the addiction process. A large body of data indicates that opioids and dopamine together mediate emotional and reinforced behaviors. In support of this, cocaine-mediated increases in activation of dopamine D1 receptors (D1R) results in a desensitization of **δ**-opioid receptor (DOR) signaling through adenylyl cyclase (AC) in striatal neurons. To further define cellular mechanisms underlying this effect, the subcellular distribution of DOR and D1R was examined in the rat dorsolateral striatum. Dual immunoperoxidase/gold-silver detection combined with electron microscopy was used to identify DOR and D1R immunoreactivities in the same section of tissue. Semi-quantitative analysis revealed that a subset of dendritic cellular profiles exhibited both DOR and D1R immunoreactivities. Of 165 randomly sampled D1R immunoreactive profiles, 43% contained DOR. Similarly of 198 DOR-labeled cellular profiles, 52% contained D1R. The present data provide ultrastructural evidence for co-existence between DOR and D1R in striatal neurons, suggesting a possible mechanism whereby D1R modulation may alter DOR function.

Introduction

Cocaine acts primarily by binding to transporters and inhibiting the re-uptake of monoaminergic neurotransmitters [33]. The initial reinforcing properties of cocaine are mainly attributed to the prolonged dopaminergic transmission caused by the inhibition of the dopamine transporter [4, 10]. The mesolimbic and mesocortical dopaminergic systems play a critical role in cocaine's reinforcing properties [1]. Cocaine also impacts other neurotransmitter systems including endogenous opioids [3, 16, 19, 39, 42, 43]. Dopamine neurons are known to interact with the endogenous opioid system and together they collectively modulate emotionally motivated behaviors, goal-directed behaviors, and locomotor activity [12, 34].

Dopaminergic activity has been implicated in modulating opioid peptides and receptors. Administration of drugs that alter dopaminergic neurotransmission can affect the opioid system by altering levels of enkephalins, endorphins, and dynorphins [9, 13, 17]. In addition, chronic cocaine administration results in an increase in μ-opioid receptors (MOR) in brain regions that are rich in dopamine receptors such as the striatum [16, 43, 44]. It has also been postulated that up-regulation of MOR is mediated by dopamine D2 receptor (D2R) activation [7]. These two receptors have been found to be co-expressed in a subset of striatal neurons[2] providing evidence for a potential molecular mechanism whereby cocaine can modulate opioid receptor expression through the dopamine system at a subcellular level.

Previous work supports a similar interaction with other members of the dopamine and opioid receptor families. Chronic cocaine administration results in attenuation of the ability of the DOR agonist D-Pen^2, D-Pen^5-enkephalin (DPDPE) to inhibit AC activity in the striatum [40]. This effect is thought to be mediated by D1R activation since the D1R agonist, SKF 82958, also attenuates DOR-inhibited cyclase activity. Further, SKF 82958 administration attenuates the ability of DPDPE to stimulate [35S] GTP S binding, indicative of an uncoupling of DOR from

G-proteins [41]. Interestingly, significant alterations in the levels of G-proteins do not play a role in this process [30]. Since the number of DOR is not changed following cocaine administration [44], the finding that cocaine modulates DOR-regulated cyclase activity suggests that exposure to cocaine results in a functional uncoupling of DOR from AC. The molecular mechanism by which cocaine induces DOR heterologous desensitization has not yet been elucidated.

A first step in addressing potential mechanisms underlying this effect is to examine the respective cellular distributions of DOR and D1R. There is compelling anatomical evidence provided by independent ultrastructural studies for possible co-localization of these two receptors. Previous studies indicate that opioid and dopamine receptors exist in close proximity to each other in the striatum [32]. 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 [38]. D1R have also been found on neuronal elements in direct contact with dopamine terminals and are expressed on medium spiny neurons in the shell of the accumbens [25, 38].

To contribute to the understanding of the mechanism involved in D1R regulation of DOR function, it is important to determine if D1R and DOR are co-expressed in individual neurons. The purpose of the present study was to determine whether D1R and DOR are localized to individual cellular profiles in the rat dorsolateral striatum using immunoelectron microscopy.

Methods

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. The methods used have been described extensively in a

previous publication [2]. Four male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN; 275-300 grams) were used in this study.

DOR was identified using a polyclonal antibody raised in rabbit (Immunostar, Inc., Hudson, WI) specific for amino acids 3-17 on the cloned mouse DOR1 [8]. For D1R a monoclonal antibody raised in rats (Sigma, St. Louis, MO) specific for a 97 amino acid sequence corresponding to the C-terminus of the human D1R was used [25]. Tissue sections were incubated overnight in a cocktail solution containing rabbit anti-DOR (1:500, Immunostar) and rat anti-D1R (1:500, Sigma) in 0.1M Tris-buffered saline containing 0.1% bovine serum albumin.

Sections were incubated in biotinylated donkey anti-rat IgG (1:400, Jackson ImmunoResearch Laboratories, West Grove, PA) followed by avidin-biotin complex (Vector Laboratories). For DOR, tissue sections were incubated in 1nm gold goat anti-rabbit IgG (1:50, Amersham Corp., Amersham, England) and intensified using a silver enhancement kit (Amersham Corp.). Sections were processed for electron microscopy following standard procedures [6]. Immunolabels were reversed in some sections to ensure that detection of antigens was not thwarted by immunolabeling techniques. Samples from the dorsolateral section of the caudate and putamen were cut and examined using a Morgagni 268 (FEI Company, Hillsboro, Oregon).

The classification of neuronal profiles was based on that of Peters et al. (1991) [31]. A profile was defined to be immunogold-silver labeled when two or more gold-silver particles were detected [2]. Grain counts were conducted over regions of tissue containing blood vessels and myelin, structures that should not exhibit immunoreactivity for dopamine or opioid receptors. Of 50 randomly sampled micrographs, three myelinated axons exhibited a gold particle. These data demonstrated minimal spurious gold-silver labeling 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. Adobe Photoshop (Adobe, San Jose, CA) was used to adjust brightness and contrast and to construct the final image.

Counting the same profile in adjacent ultrathin sections was avoided by analyzing non-overlapping sections separated by at least 500nm for each individual section. Due to known differences in sensitivity and resolution of gold and peroxidase markers, sections were also processed so that the labels for DOR and D1R were reversed. These data were pooled since it was determined that the semi-quantitative results obtained were independent of the labeling technique used.

Results

Our results confirm studies by others showing abundant D1R and DOR localization in the dorsal striatum [45, 46]. Forebrain sections containing the striatum that were processed for D1R and DOR immunoreactivity using light microscopy showed positive staining with a peroxidase reaction product (Fig. 1A and B). Specifically, peroxidase labeling for the D1R revealed a dense homogeneous distribution within the dorsolateral striatum (Fig. 1A). Similarly, the distribution of DOR immunoreactivity was homogeneous with a lateral to medial decrease in density within the dorsal striatum (Fig. 1B) [45]. Peroxidase labeling for the DOR was clearly distinguishable in somata and was present in both patch and matrix compartments of the dorsolateral striatum. This pattern stands in contrast to the known patchy distribution exhibited by the MOR in the dorsolateral striatum [26] [2]. The region of the striatum sampled for ultrastructural analysis is

shown in Figure 1C. Specifically, the dorsolateral region of the striatum was excised immediately adjacent to the corpus collosum for electron microscopic processing.

Immunoelectron microscopic labeling for D1R and DOR was detected within the dorsolateral striatum in both single and double-labeled profiles. Black deposits indicative of gold-silver immunolabeling were distinguishable from the electron dense peroxidase reaction product at the ultrastructural level (Fig. 2). Occasionally, if the diffuse peroxidase marker was dense along the plasma membrane, it was sometimes difficult to resolve the smaller gold-silver labeled particles in the same profile. However, examining an adjacent tissue section and sampling a large number of cellular profiles ensured that these labels were clearly distinguishable. To further circumvent this caveat, sections were processed where the immunolabels were reversed.

Peroxidase and immunogold labeling for DOR and D1R were detected primarily in spiny dendritic processes and neuronal perikarya (Fig. 2). DOR and D1R were less frequently observed in axon terminals. There was no evidence of immunoreactivity in glial cells or myelinated fibers of passage.

Of the total D1R-labeled profiles (n=198), 93% were somatodendritic processes that often contained spines. D1R labeling was mainly located in dendrites associated with the membranes of subcellular organelles and the internal plasma membrane. Labeled profiles were characteristic of medium sized spiny neurons.

Immunogold-silver labeling for the DOR was detectable in somatodendritic processes. Of the total DOR labeled profiles, 97% were characteristic of somatodendritic profiles often containing spiny processes. Within dendrites, labeling indicative of DOR could be identified along the plasma membrane and in the cytoplasm. At times, cytoplasmic DOR was found in

contact with membranes of subcellular organelles. Within perikarya, DOR immunoractivity was localized to profiles characteristic of spiny neurons.

Of two hundred eighty profiles, 43% of D1R-labeled profiles also contained DOR (n=198) and 52 % of the total number of DOR labeled profiles also contained D1R (n=165). Immunoreactivities were more commonly detected in cytoplasm but were occasionally seen along the plasma membrane. Labeling associated with the plasmalemma was localized along extrasynaptic portions of the cellular membranes.

Clusters of peroxidase labeling for D1R could be identified within dendrites that also contained gold-silver immunoreactivity for DOR. In some cases, unlabeled axon terminals could be seen forming synapses with dually labeled dendrites containing spines (Fig. 2A). Other unlabeled neuronal profiles or single labeled profiles were often found apposed or in close proximity to dual-labeled somatodentritic processes (Fig. 2). Axon terminals targeting single and double-labeled cellular profiles exhibiting immunolabeling for either D1R or DOR were heterogeneous in nature and included both symmetric and asymmetric synapses.

Discussion

This study provides evidence for dual localization of D1R and DOR in a subpopulation of striatal neurons. This data provides an anatomical substrate that may underlie the regulation of DOR function by D1R in the striatum [41] providing the first direct evidence for potential subcellular interactions underlying desensitization of DOR by D1R activation.

A potential limitation known to be associated with the preembedding immunolabeling technique is attributed to penetration of immunoreagents in thick Vibratome sections (~40 μm) [6]. Analysis of ultrathin sections was carried out exclusively on sections taken just beneath the tissue/plastic interface where penetration is maximal. Immunolabeling of antigens were routinely

reversed to ensure that patterns of immunolabeling were not attributed to differences in the detection of methods of reaction products. Artifactual enhancement of the peroxidase marker by the silver intensification procedure had been reported [6]. It is highly unlikely that artifactual enhancement of the peroxidase marker has taken place in the present study. Single-labeled profiles were clearly identified within the neuropil in fields containing dual-labeled cells. Thus, it is unlikely that false positives were identified. Control experiments were conducted where one primary antibody was omitted during the dual-labeling procedure. Following incubation in both secondary antibodies, only one reaction product was present.

Our study is in agreement with others showing a primarily postsynaptic distribution of the D1R in the dorsal striatum. Furthermore, our results are consistent with localization of D1R in medium sized spiny neurons [46] . Our results also confirm that DOR is present in both patch and matrix compartments of the striatum [26, 45]. Our data extend these findings by showing that individual striatal profiles contain both D1 and DOR.

A potential neurotransmitter in dually labeled striatal profiles is GABA. DOR is expressed by GABAergic medium spiny neurons [38] and medium spiny neurons composing the striatonigral pathway express D1R [15]. Because there is a low degree of co-localization with enkephalin, only a small number of the neurons expressing DOR are likely to be the enkephalin–containing striatopallidal neurons, whereas the majority might be the striatonigral neurons that also express D1R [15, 37]. Future studies are required to define the neurochemical phenotype of striatal neurons expressing both D1R and DOR.

Chronic binge-pattern cocaine administration causes increased density of MOR and KOR, however, the density of the DOR is not altered by cocaine [44]. This alteration in MOR

and KOR but not DOR expression has also been reported following chronic cocaine or during cocaine withdrawal in other rodent models [3, 16, 19, 20, 36] and in humans[18, 47].

Despite the unaltered density of DOR following chronic cocaine administration, there is evidence showing the attenuation of the ability of DOR agonists to inhibit AC activity in the striatum [40]. This effect is mediated by D1R as chronic administration of a D1R agonist also attenuates DOR-inhibited cyclase activity [41]. The cellular co-localization of these two receptors is a first step in determining the mechanism involved in the heterologous desensitization of DOR following D1R activation.

One implication of this finding is that both dopamine and opioid agonists together modulate the activity of single striatal neurons. Desensitization of DOR may underlie some of the behavioral consequences associated with cocaine abuse, including anxiety [27]. Interestingly, the DOR antagonist, naltrindole, has been shown to cause anxiogenic-like effects on the elevated-plus maze [29, 35]. Also, mutant knock-out mice lacking DOR demonstrated anxiogenic-like behaviors suggesting a modulatory role for DOR in anxiety [11].

The model presented in Figure 3 suggests one potential mechanism underlying direct interactions between D1R and DOR signaling systems. However, other interactions are potentially feasible and require further investigation. Both D1R and DOR are G protein-coupled receptors (GPCR) and may interact directly through hetero-oligomerization [23]. This type of interaction has been shown between other GPCR such as adenosine A2A receptors and D2R [5], as well as dopamine and serotonin receptors [24]. Fluorescence and bioluminescence resonance energy transfer are techniques that could be used to determine direct receptor-receptor interactions. As GPCRs are regulated by various molecules, involvement of such intracellular signaling elements should be investigated. These include GPCR kinases (GRKs), arrestins , and

regulators of G protein signaling (RGS) proteins. Specifically, RGS9 and RGS4 may be involved in dopamine and opioid signaling [14, 21]

The present study provides a cellular substrate in support of a potential mechanism for the heterologous desensitization of DOR following D1R activation due to cocaine or D1R agonist administration**.** As presynaptic actions of cocaine at monoamine uptake sites cause an increase in dopamine availability, it is tempting to speculate that our data provides a cellular substrate underlying the increases in D1R activation and DOR desensitization. Co-localization between D2R and MOR has been demonstrated [2], and may also play a role in cocaine's actions.

Neuroadaptations caused by drugs of abuse are thought to result in an increased likelihood for continued drug seeking and administration. As clinical observations indicate that cocaine addicts use opiates to reduce cocaine-induced anxiety and irritability [22, 27], continued investigations of dopamine-opioid interactions may elucidate novel targets for treatment of addiction.

Figure Legend

Figure 1:

Brightfield microscopy: D1R and DOR immunoreactivity in the rat dorsolateral striatum. A: Low magnification photomicrograph showing dense peroxidase labeling for D1R within the striatum. **B:** Peroxidase labeling for DOR reveals a lateral to medial decrease in density within the dorsal striatum. Individual neurons containing DOR can be seen at arrows. **C:** Schematic adapted from the rat atlas of Paxinos and Watson [28] showing the region sampled for ultrastructual analysis. Bar for A = 375 μm; B = 200 μm. Abbreviation: cc = corpus collosum; aca = anterior commisure; AcbC = nucleus accumbens core; AcbS = nucleus accumbens shell; CPu = caudate and putamen.

Figure 2:

Electron microscopy: Co-localization of D1R and DOR. A: Electron microscopy of a spiny dendrite exhibiting peroxidase labeling for D1R (arrows) and immunogold-silver labeling for DOR (arrowheads). An unlabeled axon terminal (ut) containing dense core vesicles (dcv) can be seen forming a symmetric synapse with the dual labeled dendrite. A single labeled dendrite

containing D1R and an unlabeled dendrite (ud) can also be identified. **B:** Electron micrograph showing peroxidase labeling for D1R (arrows) and immunogold-silver labeling for DOR (arrowheads) in a dendritic process. A single labeled dendrite containing D1R is also shown. **C:** Reverse labeling confirms co-localization. A dendrite contains immunogold-silver labeling for D1R (arrowheads) and peroxidase labeling for DOR (arrows). Bar = 500nm. Abbreviations: D1R + DOR-d = D1R and DOR labeled dendrite; D1R-d = D1R labeled dendrite; DOR-d = DOR labeled dendrite.

Figure 3:

Potential Mechanism Underlying Desensitization of DOR. At basal conditions D1R activation activates Gs which causes an increase in AC while activation of DOR activates Gi which inhibits AC activity. Interestingly, cocaine attenuates the ability of DOR to inhibit AC activity possibly through a D1R dependent mechanism. This co-existence provides a cellular substrate for the regulation of DOR function by D1R activation.

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