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Differential subcellular distribution of rat brain dopamine receptors and subtype-specific redistribution induced by cocaine

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Abstract

We investigated the subcellular distribution of dopamine D₁, D₂ and D₅ receptor subtypes in rat frontal cortex, and examined whether psychostimulant-induced elevation of synaptic dopamine could alter the receptor distribution. Differential detergent solubilization and density gradient centrifugation were used to separate various subcellular fractions, followed by semi-quantitative determination of the relative abundance of specific receptor proteins in each fraction. D₁ receptors were predominantly localized to detergent-resistant membranes, and a portion of these receptors also floated on sucrose gradients. These properties are characteristic of proteins found in lipid rafts and caveolae. D₂ receptors exhibited variable distribution between cytoplasmic, detergent-soluble and detergent-resistant membrane fractions, yet were not present in buoyant membranes. Most D₅ receptor immunoreactivity was distributed into the cytoplasmic fraction, failing to sediment at forces up to 300,000g, while the remaining portion was localized to detergent-soluble membranes in cortex. D₅ receptors were undetectable in detergent-resistant fractions or raft-like subdomains. Following daily cocaine administration for seven days, a significant portion of D₁ receptors translocated from detergent-resistant membranes to detergent-soluble membranes and the cytoplasmic fraction. The distributions of D₅ and D₂ receptor subtypes were not significantly altered by cocaine treatment. These data imply that D₅ receptors are predominantly cytoplasmic, D₂ receptors are diffusely distributed across the cell, whereas D₁ receptors are mostly localized to lipid rafts within the rat frontal cortex. Dopamine receptor subtype localization is susceptible to modulation by pharmacological manipulations that elevate synaptic dopamine. Given the molecular and functional interactions among dopamine receptor, the present pharmacological modulation of subcellular receptor distribution probably has functional implications that warrant further investigation.

Keywords

D₁ dopamine receptor, D₅ dopamine receptor, D₂ dopamine receptor, cellular subdomains, receptor translocation, lipid rafts, cocaine

1. Introduction

Dopamine receptor sensitivity and selectivity are modulated by the magnitude and duration of synaptic dopamine concentrations. Prolonged elevation of synaptic dopamine produces receptor desensitization (Cho et al., 2006; Memo et al., 1982), whereas persistent reduction of synaptic dopamine can lead to the development of postsynaptic dopamine receptor supersensitivity (Gerfen, 2003; Henry and White, 1991; Klawans et al., 1975; Mishra et al., 1974). While numerous studies have documented dramatic changes in dopaminergic function as a consequence of experimental or disease-induced dopamine imbalance in the brain (Anderson and Pierce, 2005; Berthet et al., 2009; Guigoni et al., 2007; McGinty et al., 2008; Stanwood and Levitt, 2007), the mechanisms underlying such changes do not always involve changes in cell-surface receptor expression. To explain how function may change without a change in receptor number, some propositions have been advanced, including the mobilization of extrasynaptic receptors (Balfour et al., 2000; Korf and Loopuijt, 1988; Paspalas and Goldman-Rakic, 2004) as well as receptor internalization or receptor phosphorylation. More recently, receptor trafficking via signaling endosomes (Hanyaloglu and von Zastrow, 2008; Sadowski et al., 2009), receptor heterodimerization (Agnati et al., 2003; Lee et al., 2004; Lee et al., 2000; Pin et al., 2007), and plasma membrane receptor segregation across lipid rafts (Ostrom and Insel, 2004) have been explored as novel mechanisms that modulate dopamine or other G protein-coupled receptor signaling.

The association of diverse receptors with lipid rafts – specialized nanoscale assemblies of cholesterol, sphingolipid and proteins – has raised the speculation that dopamine receptor subtypes may be differentially distributed within and outside these specialized structures, and that such distribution may have relevance to the pharmacology of this catecholamine. While it is clear from receptor trafficking experiments that synaptic dopamine concentration drives the subcellular location or endosomal transport of dopamine receptors (Dumartin et al., 1998;

Dumartin et al., 2000; He et al., 2009; Sun et al., 2003), it is still unknown if transmitter concentrations or exposure duration might influence the intra-membrane distribution of specific receptor subtypes, and how such influences may relate to physicochemical or functional receptor interactions or chronic alterations in receptor function.

In this study we used differential detergent solubilization and gradient centrifugation with subsequent immunodetection to examine dopamine receptor subtype localization in rat cortical tissues under basal conditions. We also induced alterations in synaptic dopamine activity via repeated administration of cocaine, and then re-examined the receptor subtype distribution profiles among the subcellular compartments. Since distinct signaling molecules can be enriched in specific subcellular compartments or plasma membrane subdomains, we tested the hypothesis that the intensity and duration of synaptic dopamine can drive dopamine receptor redistribution and thus direct the nature of receptor coupling to signal transduction cascades.

2.0 Results

2.1 Distribution of dopamine receptor subtypes among subcellular compartments

Previous ligand-binding studies by our group and others suggested that dopamine receptor oligomerization and function can be modulated by exposure to reducing agents (Panchalingam and Undie, 2005; Sidhu, 1990; Sidhu et al., 1994). In the present study, we used rat cerebrocortical slices prepared through a process that was identical to conditions used in the prior functional studies examining various aspects of dopamine receptor-mediated biological activities (Panchalingam and Undie, 2005; Sidhu, 1990; Sidhu et al., 1994). Inclusion of a reducing agent (β -mercaptoethanol or dithiothreitol) in the sample loading buffer was essential

for visualization of monomeric and dimeric forms of the D₁ subtype (Fig. 1A). The D₁ receptors (~66kDa) were predominantly located in the detergent-resistant membrane (DRM) fraction, at more than 20 times the concentration found in the detergent-soluble membrane (DSM) fraction.

FIGURE 1 HERE

Under reducing conditions, the majority of D₅ receptor immunoreactivity was observed as monomers in the cytoplasmic fraction with significantly less receptor protein in the DSM fraction; D₅ receptors were undetectable in the cortical DRM fraction (Fig 1C). A monoclonal D_{1B} (D₅) receptor antibody also exhibited greatest immunoreactivity in the cytoplasmic fraction (Fig 1G). The D₂ receptor monomer (~55kDa) was observed predominantly in the DSM fraction, with less localized to the DRM fraction (Fig 1E). Under these conditions, where 20 µg of protein extract was loaded per lane, no D₂ receptor immunoreactivity was detected in the cytoplasmic fraction. Nevertheless, the cytoplasmic D₂ receptors appeared to be labile, with respect to cellular localization (Supp Fig 2). A correlation existed between the rapidity with which the tissue was removed and processed, and the ability to observe D₂ receptors in the cytoplasmic fraction. Likewise, the duration of slice incubation was correlated with the ability to visualize D₂ receptors in the cytoplasmic compartment. Finally, the data shown in Figure 1 were derived from membranes solubilized with 1% Triton X-100; however, the results were identical when 1% deoxycholate was used as the solubilizing agent (deoxycholate data not shown).

Under non-reducing conditions the D₁ receptor resisted solubilization as evidenced by the absence of immunoreactivity on Western blots. This observation was interpreted as support for the formation of a protein complex that was either too large or too insoluble to enter the gel matrix, resulting in the lack of detectable D₁ receptor signal on Western blots (Fig 1B). This contrasted with the D₅ and D₂ receptors, where monomeric and oligomeric forms could readily be discerned under non-reducing conditions in the DSM and cytoplasmic fractions (Fig 1D, 1F). The strength of association of the non-migrating D₁ receptor complexes was tested by exposing DRMs to urea or potassium iodide – reagents that can disrupt hydrophobic protein-protein

interactions (Brady et al., 2004). Neither denaturant was capable of dissociating D₁ receptors from the insoluble complexes, indicating that the association was probably of a covalent nature (data not shown).

To determine the specificity of the dopamine receptor antibodies used in this study, extracts from HEK293 cells expressing D_{2L} receptors, and Sf9 cells expressing either D_{2L} or D₃ receptors were screened by Western blot. The Santa Cruz D₂ receptor antibody elicited immunoreactivity only in cells that were transfected with D_{2L}, but not D₃ receptor (Supp. Fig 1A, B). The specificity of the rabbit polyclonal D₅ receptor antibody was demonstrated by comparing the immunoreactive profile of mouse hippocampal and liver tissue extracts. Immunoreactive proteins with molecular weights similar to that seen in rat cortex and striatum were observed in hippocampal, but not liver, extracts (Supp. Fig 1C); these observations correspond to previously published reports (Bergson et al., 1995; Luedtke et al., 1999). Blots were reprobed with β -actin to confirm equal loading of protein. Vascular tissue extracts (Cy, DSM, DRM) are shown to emphasize that β -actin comprises a smaller percentage of total protein in the DSM compared to cytoplasm and DRM, as noted in hippocampal and liver extracts (Supp Fig 1D).

We next sought to characterize the cytoplasmic, DSM and DRM fractions by reprobing blots with antibodies for Rap1, G_{olf}, G_{i1}, G_{q/11} and PLC β 1 – dopamine receptor-related signaling components whose subcellular localization has previously been characterized (Canobbio et al., 2008; Donati and Rasenick, 2005; Kelly et al., 2005). Rap1, G_{olf}, G_{q/11}, G_{i1} and PLC β 1 were all found in the DSM (Fig 2). Rap1 was the only protein tested that was not detected in DRM fractions; PLC β 1 was the only molecule detected in the cytoplasmic fraction. The non-receptor tyrosine kinases Src and Fyn have not been linked to direct activation by dopamine receptors, but have been found in both DSM and DRM (Patra, 2008; Yasuda et al., 2002). Src and Fyn were present in nearly equal amounts in both the DSM and DRM (Fig 2). Notably, Fyn, but not Src, was also found in the cytoplasmic fraction. The metabotropic glutamate receptor 5

(mGluR5) is another G protein-coupled receptor that was not expected to be present in the cytoplasmic fraction, and indeed was found to localize exclusively to DSM and DRM fractions (Fig 2).

FIGURE 2 HERE

2.2 Dopamine receptor localization among cellular subdomains

In order to further characterize the DRM in which the D₁ receptor was predominantly located, subcellular fractionation was carried out, adding a detergent step to separate DSM from DRM in each fraction. In these experiments, extracts were prepared from acutely isolated rat prefrontal cortex tissues. The data revealed that the D₁ receptor was consistently localized to the DRM fraction, while residual amounts of the receptor could be detected in membrane subfractions sedimenting at each tested centrifugation step, including the 300,000g step (Fig 3A). The D₅ receptor was again found primarily in the cytoplasmic fraction comprised of the supernatant from centrifugation at 300,000g (Fig 3B).

FIGURE 3 HERE

In the case of the D₂ receptor, the plasma membrane was the predominant fraction in which the receptor was found, with approximately equal distribution between DSM and DRM (Fig 3C). The remaining minor component of D₂ receptors was distributed between the synaptosomal, microsomal and ribosomal membrane fractions. Interestingly, the ratio of D₂ receptor in DSM and DRM was variable among these substructures. Synaptosomal DSM contained more of the D₂ receptor than synaptosomal DRM, whereas for microsomal and ribosomal membranes the D₂ receptor tended to locate more in the DRM fraction than the DSM. In this experiment and in the density gradient experiments, cross-reacting proteins of 60 – 70kDa were noted in the cytoplasmic fraction. These species have been described in the past as representing differing extents of glycosylation of the D₂ receptor (Fishburn et al., 1995). Consistently, the predominant D₂ receptor band was that which possessed a relative migration of approximately 55kDa. In these analyses, the controls included PSD95, which is detected only in the synaptosomal DRM

fraction (Fig 3D), and calnexin, which is enriched in the microsomal fraction, but can also be observed in the synaptosomal fraction (Archibald et al., 1999)(Fig 3E).

2.3 Dopamine receptors display distinct buoyancies in sucrose gradients

To further explore the idea that dopamine receptor subtypes partition differentially into membrane fractions within the cell, we carried out discontinuous sucrose density gradient fractionation using acutely isolated cortical membranes. This method has been used to isolate membrane subdomains that have distinct biochemical compositions or properties, such as lipid rafts and caveolae. We found a significant proportion of D₁ receptors in buoyant membranes (Fig 4A), in fractions where flotillin (Fig 4D) and caveolin (Fig 4E) are observed. D₁ receptors were distributed throughout the lower portion of the gradient, including the insoluble pellet (Fig 4A). As predicted, PSD95 was restricted to the insoluble pellet (Fig 4F). Similar to what was observed in the crude fractionation experiments, the D₅ receptors preferentially partitioned into the cytoplasmic phase while D₂ receptors were found both in the cytoplasmic and membrane fractions. Neither the D₂ nor the D₅ receptor was present in any significant amount in buoyant membranes where flotillin, caveolin and D₁ receptors were found (Fig 4B, 4C).

FIGURE 4 HERE

2.4 Cocaine induces cellular subdomain redistribution of D₁ receptors in the frontal cortex

Following a schedule of daily cocaine administrations over seven days, the distribution of D₁ receptors was significantly shifted out of the DRM into the DSM and cytoplasmic fractions (Fig 5A). In contrast, cocaine administration did not alter the intercompartmental distribution of D₅ or D₂ receptors (Fig 5B, 5C). To control for equivalent sample loading, blots were reprobed with β 3-tubulin (Fig 5D), α -synuclein (Fig 5E) and PSD-95 (Fig, 5F). While β 3-tubulin and α -

synuclein were abundant in cytoplasmic and DSM membranes, and PSD-95 was observed solely in the DRM fraction, chronic cocaine administration did not alter the content or localization of any of these control proteins.

FIGURE 5 HERE

Striatal tissue from these same animals also was fractionated and assessed for changes in D₁, D₅, and D₂ receptor distribution (Fig 6). Basal distribution of the receptors and subcellular markers in the saline-treated animals was similar to the distribution in naïve cortical tissue. Contrary to the observations in the frontal cortex, however, cocaine treatment failed to induce any significant redistribution of the dopamine receptor subtypes in the striatum.

FIGURE 6 HERE

3.0 Discussion

We observed that dopamine receptor subtypes are differentially distributed among subcellular compartments, with the D₁ subtype localized almost exclusively to detergent-resistant membranes where raft and caveolar proteins are found, while the D₅ subtype was largely localized to a cytoplasmic milieu that resisted sedimentation upon centrifugation up to 300,000g. The D₂ receptor subtype showed variable distribution between cytoplasmic, detergent-soluble and detergent-resistant membranes. Subacute cocaine treatment caused D₁ receptors to redistribute from detergent-resistant to detergent-soluble membranes, thus increasing the proportion of cellular D₁ receptors that are juxtaposed with D₂ receptors. A differential subcellular distribution of dopamine receptor subtypes in the brain, and the susceptibility of such distribution to pharmacological modulation, probably bears important implications for clarifying the mechanisms of functional alterations in dopamine signal transduction.

3.1 Localization of dopamine receptors in cortex

D₁ receptors were preferentially localized to detergent-resistant brain membranes. Such membrane subdomains that are resistant to solubilization in cold Triton X-100 include postsynaptic densities, lipid rafts and caveolae. Thus, the present observations correlate with published reports of direct D₁ receptor interactions with NMDA receptors, PSD-95 or caveolin-1 – proteins that are known to reside in detergent-resistant subdomains or lipid rafts (Fiorentini et al., 2003; Kong et al., 2007; Lee et al., 2002; Pei et al., 2004; Zhang et al., 2007). Consistent with previous reports that D₁ receptors resist solubilization in the absence of a reducing agent (Bergson et al., 1995; Sidhu, 1990), we found that disulfide bond disruption with β -mercaptoethanol or DTT was essential to visualize D₁ receptor monomers and dimers by Western analysis. This was intriguing as it implies that D₁ receptors form covalently associated multimers (or the multimers may be covalently associated with some other membrane proteins). This inference is supported by the frequent observation of residual high molecular weight material on PAGE blots that reacts strongly with D₁ receptor antibodies but fails to dislodge or migrate through the gel. The size of this residual complex was reduced in samples treated with reducing agent (data not shown). It was, however, unperturbed by treatment with strong denaturants such as urea or potassium iodide which effectively disrupt hydrophobic protein-protein interactions.

Inclusion of the cytoplasm for analysis was originally intended only as a control, as it was assumed that G protein-coupled receptors would ordinarily not reside to any appreciable extent in a cytoplasmic fraction. Nevertheless, D₅ receptors were reproducibly and predominantly detected in the cytoplasm (200,000- 300,000g supernatant), an observation that was further confirmed with a monoclonal D₅ receptor antibody. This characteristic distribution distinguishes the D₅ from D₁ receptors (Bergson et al., 1995). D₅ receptors were typically detected as a

predominant band of approximately 50kD, with a second band at approximately 45kD. Previously published reports demonstrated that treatment of membrane extracts with deglycosylating enzymes caused the approximately 50kD D₅ receptor band to migrate with the 45kD marker (Bergson et al., 1995; Karpa et al., 1999), thus we postulate that the 45kD protein represents the deglycosylated form of the receptor. The nature of the approximately 66kD cytoplasmic protein detected with mouse anti-D₅ receptor antibody has not been determined (Figure 1G). Previously published immunohistochemical analyses of D₅ receptors in brain indicated that this receptor was present primarily in the somal cytoplasm in tissues where the receptor was expressed (Ariano et al., 1997; Bergson et al., 1995; Ciliax et al., 2000; Khan et al., 2000; Oda et al.). Similarly, in cultured cells D₁ receptors were localized to the plasma membrane, whereas D₅ receptor immunoreactivity was high in the somal cytoplasm and also present on the cell surface (He et al., 2009). The present determination that D₅ receptors do not sediment at 300,000g suggests that these receptors are associated with a microvesicular membrane of high lipid content, rendering it difficult to sediment. Evidence for such microvesicles exists primarily in plants (Yao et al., 1991), although evidence for phospholipase C/phosphatidic acid-containing vesicles that resist sedimentation also exists in mammalian cells (Olsen et al., 1993). The recent finding that D₅ receptors mediate stimulation of phosphoinositide signaling as well as CDP-diacylglycerol synthesis from phosphatidic acid (Sahu et al., 2009), together with the current finding of cytoplasmic localization of D₅ receptors and PLC β 1, would be consistent with a preferential distribution of the receptors to sedimentation-resistant phospholipase C/phosphatidic acid-containing microvesicles.

D₅ receptor detection by Western blot in previous reports may have been missed or underestimated due to the receptor's failure to sediment at forces reaching 300,000g. This single factor may have contributed to the conclusion that D₅ protein levels are at the lower limit of detection by Western analysis (Rivera et al., 2002). Our enhanced method for separating membranes based on a combination of differential centrifugation and detergent solubility yielded

a broad estimate of receptor localization. Additional studies are required to more specifically address the source and nature of the microvesicular membranes with which D₅ receptors prefer to associate. Finally, the observation that D₅ receptor immunoreactivity was detected in detergent-resistant membranes of striatum (Figures 6 and Supplemental Figure 1C) does suggest that D₅ receptor distribution may be brain-region dependent.

D₂ receptors were observed in all fractions and, compared to D₁ or D₅ subtypes, the D₂ distribution was rather labile as the compartmental proportions varied with conditions such as exposure to reducing agent or whether membranes had been freshly isolated from tissue or from slices pre-equilibrated *ex vivo* for 45 min (Lee et al., 2000). For example, D₂ receptors were more frequently observed in the cytoplasm when extracts were rapidly obtained from acutely isolated cortical tissue, but were almost completely redistributed into the membrane fraction after slices were equilibrated by incubation in oxygenated HEPES-bicarbonate buffer at 37 °C for up to 45 min (Supp Fig 2). Hence, D₂ receptor localization is dynamic, shifting between compartments in response to conditions in the cellular environment. We hypothesize that the shifting of D₂ receptors between compartments represents a biological phenomenon that reflects synaptic levels of neurotransmitter. Interestingly, it is well understood by electrophysiologists using the acute brain slice preparation that slices typically require a period of recovery, generally one hour post-processing. The data we present here may well provide a biological explanation for the need to re-equilibrate slices *ex-vivo* prior to initiation of electrophysiological analyses: the equilibration incubation allows for repositioning of the receptors in appropriate compartments for subsequent action. This is also consistent with existing evidence for alterations in D₂ receptor function as a consequence of tissue handling (Vazquez et al., 2007).

3.2 Membrane subdomains are enriched in specific receptor subtypes

We present here the first demonstration of D₁ receptor subcellular localization into lipid raft-like entities in brain. D₁, but not D₂ or D₅ receptors were observed in sucrose gradient fractions where lipid rafts and caveolae float. While the existence of caveolae in neurons is controversial, physical association of D₁ receptors with caveolin has been demonstrated in HEK-293 cells, COS-7 cells and whole brain extracts (Kong et al., 2007; Yu et al., 2004).

D₁ receptors were also found in the pellet at the bottom of the gradient, where membranes resistant to solubilization with 1% TX-100 are found, and where post-synaptic density proteins are located. D₁ receptor co-localization with synaptosomal proteins was also noted in detergent-resistant synaptosomal membranes (Fig. 4). Distribution of D₁ receptors within these substructures is consistent with studies concluding that D₁ receptor interactions with NMDA receptors or PSD-95 influence D₁ receptor function (Fiorentini et al., 2003; Pei et al., 2004; Zhang et al., 2007). The observation that D₁ receptors were found throughout the sucrose gradients may reflect an ability of the receptors to associate with diverse membranous structures in different cellular compartments (Vickery and von Zastrow, 1999), each perhaps characterized by distinct lipid compositions. The results of differential centrifugation support this, since D₁ receptors were nearly equally distributed in DRM throughout the cell.

3.3 Synaptic dopamine drives receptor localization

Lipid rafts are biochemically distinct membrane subdomains theoretically providing flexibility and efficiency in signal transduction by segregating related signaling mediators. A hallmark of lipid rafts is the physicochemical segregation of cellular mediators that specify the coupling mechanisms for receptor-mediated signaling (Hancock, 2006; Harding and Hancock, 2008; Ostrom and Insel, 2004). For example, translocation of G-proteins and other signaling molecules into and out of rafts aids in directing cellular responses to specific agonist stimulation (Allen et al., 2007; Head et al., 2006; Patel et al., 2008). Since repeated exposure to abused

drugs results in molecular adaptations in neuronal signaling, we questioned whether the associated changes in synaptic dopamine concentrations could induce dopamine receptor subtypes to translocate between subcellular domains in a manner that might predicate changes in signaling function. Seeing that cocaine treatment induced a significant and selective redistribution of D₁ subtype receptors, we may conclude that synaptic dopamine activity can modulate receptor distribution within the plasma membrane and possibly in exchange with other cellular compartments such as endosomes. A recent in vitro experiment with D₁ and D₅ receptor-expressing cells observed a similar behavior of these two receptors in response to agonist: D₁ receptors translocated from the plasma membrane to intracellular sites while D₅ receptors did not undergo a significant shift when exposed to agonist (He et al., 2009). It is generally known that excessive or persistent stimulation can induce internalization of G protein-coupled receptors, leading to lysosomal degradation of the receptors or subsequent recycling of the receptors back to the plasma membrane. In the present study, it is not clear if the redistribution of D₁ receptors was to reposition them for alternative signal transduction coupling, to gain access to compartments where the receptors would be degraded, or if the receptors were being sequestered to be preserved for subsequent recycling and reuse. The observation that total D₁ receptor immunoreactivity across all compartments appeared unchanged between saline and cocaine treatment groups suggests that the receptor redistribution is probably aimed at modulating the type or strength of coupled signaling cascades.

In summary, we have uncovered several features related to differences in the subcellular distribution of D₁, D₂ and D₅ dopamine receptors. These differences are predicted to have significant impact on receptor signaling as well as the potential susceptibility of various receptor subtypes to functional modulation by alterations in synaptic neurotransmission. Additionally, localization of dopamine receptor subtypes within physicochemically distinguishable cellular subdomains in brain tissues could set the stage for diverse cellular-level receptor-receptor interactions (or lack thereof), yielding new insights into the regulation and function of these

receptors. Dopamine receptors are critical components of neuronal circuits involved in memory, motor coordination and reward. Addiction, Parkinson disease and schizophrenia are among the diseases exhibiting altered dopaminergic signaling (Anderson and Pierce, 2005; Berke and Hyman, 2000; Graybiel et al., 1994). The present finding of differential but dynamic localization of dopamine receptor subtypes suggests a potentially novel mechanism for the regulation of dopamine neurotransmission under physiologic conditions or as part of the pathophysiology of various dopamine-associated disorders.

4.0 Experimental methods

4.1 Animals

Male Sprague-Dawley rats weighing 250-275g were purchased from Zivic Laboratories, Pittsburgh, PA. The animals were caged in groups of three and housed in climate-controlled facilities with a 12-h light/dark cycle and free access to food and water. Protocols for the care and use of the experimental animals were approved by the Institutional Animal Care and Use Committee and conformed to the principles set forth in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

4.2 Materials

Cocaine hydrochloride was obtained from the National Institute on Drug Abuse, Bethesda, MD. All other reagents, including a protein inhibitor cocktail comprising 4-(2-aminoethyl) benzenesulfonyl fluoride, E-64, bestatin, leupeptin and aprotinin, were obtained from Sigma-Aldrich (St. Louis, MO).

4.3 Cocaine administration

Rats were administered intraperitoneal (i.p.) injections of either 0.9% sterile saline as control or 20 mg/kg cocaine dissolved in 0.9% sterile saline. Injections were administered to the animals in their home cage, and repeated once each day for seven days. Animals were euthanized 24 h after the last injection. Brains were quickly removed and frontal cortical tissues were dissected out as previously described (Undie and Friedman, 1990).

4.4 Preparation of tissue slices

Dissected cortices were chopped into 350 x 350 μm slices using a McIlwain tissue chopper. Slices were washed three times in 20 volumes of HEPES-bicarbonate buffer containing, in mM: NaCl 122, KCl 4.9, KH_2PO_4 1.2, MgCl_2 1.2, NaHCO_3 36, HEPES 30 and glucose 10. The suspension was aerated with 95% O_2 /5% CO_2 and incubated for 15 min at 37 °C with continuous reciprocal shaking. The buffer was then exchanged by washing three times with HEPES bicarbonate buffer that contained 1.2 mM CaCl and slices were equilibrated in a fresh aliquot of the buffer for an additional 45 min. Following a brief low-speed centrifugation, excess buffer was removed to obtain a slurry of packed slices (Undie and Friedman, 1990).

4.5 Isolation of cytoplasmic and membrane fractions

Tissue from one rat was collected into 1 ml of homogenization buffer comprised of 10 mM HEPES, pH 7.4, 2 mM EDTA, 320 mM sucrose and 2% (v/v) protease inhibitor cocktail. The material was transferred to a glass/Teflon homogenizer and homogenized gently on ice with 18-20 strokes. The homogenate was centrifuged at 1000g for 10 min to pellet nuclei and cellular debris (P1 fraction). The supernatant (S1) was reserved and the P1 pellet was washed with 1 ml of homogenization buffer and centrifuged again at 1000g for 5 min. The pooled supernatants were centrifuged for 5 min at 1000g to remove residual debris. This post-nuclear fraction was then centrifuged for 45 min at 200,000g. The supernatant (S2) corresponding to a crude

cytoplasmic fraction was saved; the crude membrane pellet (P2) was washed in 1 ml of membrane buffer (25 mM HEPES, pH 7.4, 2 mM EDTA and protease inhibitors) and both the cytoplasmic and membrane fractions were centrifuged again at 200,000g for 30 min. The cytoplasmic fraction was saved for further analysis. The P2 pellet was resuspended in membrane buffer and homogenized with 8-10 strokes on ice. To solubilize membranes, Triton X-100 (or sodium deoxycholate as indicated) was added to a final concentration of 1% and the extracts were incubated overnight at 4°C. Membranes were subsequently centrifuged at 200,000g for 45 min. The supernatant (S3) from this step was retained as the DSM fraction. The pellet (P3) comprised the DRM fraction. The DRM pellet was washed with membrane buffer, recovered by centrifugation at 200,000g for 30 min, and then resuspended in membrane buffer containing 1% Triton X-100. The DSM fraction was further cleared by centrifugation at 200,000 g for 30 min, retaining the supernatant for subsequent analyses.

In some experiments, differential centrifugation was used to obtain a mitochondrial fraction (12,500g, 15 min), plasma membrane (23,000g, 30 min), microsomal membrane (100,000g, 1.5h), and ribosomal membrane fractions (300,000g, 2h). Synaptosomes were enriched from the mitochondrial fraction by separation over a discontinuous sucrose gradient. The gradient was formed by layering 2.4ml of 1.2M sucrose in the bottom of a 13 ml tube, followed by a layer of 7.2ml of 0.8M sucrose. The mitochondrial/synaptosomal pellet was resuspended in 2.4ml of 0.3M sucrose and layered on top of the 0.8M sucrose layer. Gradients were centrifuged at 54,000g for 2h. The synaptosomal layer was collected from the interface between 1.2 and 0.8M sucrose and centrifuged at 100,000g for 1 h in order to pellet synaptosomal membranes. Triton X-100-soluble and resistant synaptosomal fractions were isolated as described above by pretreating the sample with 1% Triton X-100 followed by centrifugation at 200,000g for 30 min.

4.6 Sucrose gradient ultra-centrifugation and TCA precipitation

All manipulations were carried out on ice or at 4°C. Cortical tissues were isolated from three animals and the tissues pooled for processing in order to recover sufficient amounts of DRM material for analysis. The cytoplasmic and P2 membrane fractions were isolated as described above. The P2 membrane pellet was resuspended in 0.9 ml of MES buffered saline (MBS; comprised of 40 mM MES, pH 6.5, 150 mM NaCl, 2% protease inhibitors) and homogenized 8-10 strokes on ice. Triton X-100 was added to a final concentration of 1%. Membranes were mixed 1:1 with 85% sucrose in MBS, placed in the bottom of a 13 ml polyallomer tube and carefully overlaid with 6 ml of cold 35% sucrose/MBS followed by 4 ml of 5% sucrose/MBS using a 22 G syringe. The gradients were centrifuged for 19 h at 182,000g and 4°C in an SW40Ti rotor (Beckman-Coulter). Fractions (1ml) were gently collected from the top of the gradient (from Fraction #1 at the top to Fraction #12 at the bottom). Each pellet was resuspended in 0.5 ml MBS and mixed with an equal volume of 85% sucrose.

Cold 100% trichloroacetic acid (TCA) was added to each fraction such that the final concentration was 20%. Samples were kept on ice for 15 min and then centrifuged at 22,500g and 4°C for 15 min. Samples were immediately placed on ice, and the clear upper layer was removed leaving the cloudy lower layer. To each sample, 700µl of acetone was added and tubes were centrifuged for 10 min at 22,500g. Acetone was removed and samples were dried overnight in a fume hood. To resuspend, 250µl of 20 mM Tris, pH 8.0 was added to each sample, followed by trituration with 8 strokes of a glass/Teflon homogenizer. Triton X-100 was added to a final concentration of 1%, and fractions were subsequently analyzed by Western blotting.

4.7 Dopamine receptor antibody specificity

Cell lysates from Sf9 cells were a generous gift from R.R. Luedtke. Sf9 cells were either untransfected, transfected with D_{2L} receptor, or transfected with D₃ receptor. Prior to western

blotting, these extracts were tested for D_{2L} receptor binding activity by the Luedtke laboratory. Only Sf9 cells transfected with D_{2L} receptor demonstrated activity in a D₂ receptor radioligand binding assay. Protein content was quantified and 1µg of each lysate was loaded per lane. HEK293 cells were stably transfected with a triple HA-tagged D_{2L} dopamine receptor, and selective pressure was maintained with 100µg/ml G418. Cell growth media was DMEM with 10% fetal bovine serum, penicillin/streptomycin and glutamine. HEK293 cells (no D₂ receptor) and HEK293-D_{2L} receptor-expressing cells were collected and lysed with 10mM Tris, Ph 7.4, 150mM NaCl and 1% Triton X-100, and the resultant membranes used for downstream analyses.

4.7 Protein quantitation and Western blot analysis

Sample aliquots were taken for quantitation of protein using the Pierce BCA protein assay kit (Rockford, IL). Equivalent amounts (micrograms) of each sample were separated on Invitrogen (Carlsbad, CA) NuPage 4-12% Bis-Tris gels with MES running buffer. Gels were transferred to nitrocellulose membranes at 30V for 16h at 4°C. Membranes were stained post-transfer with 2% Ponceau S to identify and rerun blots with uneven lane loading. Membranes were blocked with 10% milk/Tris-buffered saline, 1% Tween 20 (TBST) and probed with primary antibody in 5% milk/TBST. Horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-mouse, 1/5000; KPL, Gaithersburg, MD); donkey anti-rabbit, 1/10,000 Chemicon) were used in conjunction with Pierce West Pico chemiluminescent reagents to visualize immunoreactivity. For visualization of dopamine receptors, Pierce Femto reagent was added to Pico at a 20:1 (Pico:Femto) ratio. Rabbit polyclonal antibody against D₁ dopamine receptor (D₁DR) or D₅ dopamine receptor (D₅DR) was used at a 1/1000 dilution for Western blot analysis; mouse anti-D₂ dopamine receptor (D₂DR) was used at a 1/500 dilution. Primary antibodies used in this study included the following. D₁ receptor (rabbit polyclonal, a.a. hum 338-446 (H-109), sc-

14001), D₂ receptor (mouse monoclonal, a.a. hum 1-50, clone (B-10), sc-5303), D₅ receptor (rabbit polyclonal, a.a. hum 371-477, (H-107), sc-25650), a-synuclein (rabbit polyclonal, (C-20) 7011-R), G α_{olf} (rabbit polyclonal sc-385) G α_i -1 (rabbit polyclonal sc-28586), Gq/11 (rabbit polyclonal sc-392) and PLC β 1 (rabbit polyclonal sc-205) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The D_{1b} antibody (mouse monoclonal, specific for the D_{1b}/D₅ receptor) was a generous gift from R. Luedtke (University of North Texas Health Science Center, Fort Worth, TX). β 3-tubulin (mouse clone 3D10, T-8660), β -actin (A-5316) and caveolin-1 (rabbit c-3237) were obtained from Sigma-Aldrich (St. Louis, MO). PSD95 (mouse clone 6G6-1c9, MA1-045) was purchased from Affinity Bioreagents (Golden, CO) and Rap-1 (rabbit, KAP-GP120) was from StressGen (Ann Arbor, MI). Rabbit anti-mGluR5 was purchased from Upstate Biotechnology Inc. (Waltham, MA). Src (mouse clone 327) was a gift from Jo Bolen (Millenium Pharmaceuticals), Fyn (rabbit BL90) was a gift from Mike Tomlinson (University of Birmingham, UK), and calnexin (rabbit, Sigma) was a gift from Peter Swaan (University of Maryland). The Santa Cruz anti-D5 receptor antibody was used in all experiments except for that shown in Figure 1G. Selectivity of the D5 and D2 receptor antibodies are demonstrated in Supplemental Figure 1. Data reported in the graphs have not been normalized to loading controls, because the distribution of loading controls was not equivalent across the three compartments examined (cytoplasmic, DSM, DRM). This critical issue has been addressed recently (Aldridge et al., 2008). Chemiluminescence images were collected onto Kodak Blue XB-1 film, and subsequently scanned and quantitated by densitometry using ImageQuant (v. 1.2, Molecular Dynamics).

4.8 Data analysis

Quantitative data are expressed as mean \pm SEM. Statistical analysis was conducted using one-way analysis of variance (ANOVA), followed by a post-hoc Bonferroni-Dunnett test (Statview).

5.0 Conflict of interest statement

The authors declare no conflict of interest.

6.0 References

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7.0 Figure legends

Fig. 1. D₁, D₅ and D₂ dopamine receptors are differentially distributed among subcellular fractions in rat cortex. Samples of cytoplasm (Cy), detergent-soluble membranes (Ds), and detergent-resistant membranes (Dr) were electrophoresed under either reducing (A, C, E, G) or non-reducing (B, D, F) conditions. A, Under reducing conditions D₁ receptors are visualized primarily in the Dr membrane fraction. B, D₁ receptors are undetectable in the blots under non-reducing conditions. C, D, D₅ receptor monomers and dimers are most abundant in the cytoplasmic fraction, but also present in Ds membranes (rabbit polyclonal antibody). E, D₂ receptor immunoreactivity is present mainly in Ds and Dr fractions. F, Under non-reducing conditions D₂ receptors migrate as high molecular weight, multimeric forms, most predominantly in the cytoplasm, but monomers and multimers are also present in Ds and Dr fractions. G, distribution of D₅ receptor immunoreactivity is similar when detected with a mouse monoclonal antibody. Each extract was loaded with 20 µg protein except the Dr analysis for D₁ receptor detection (#) where only 4 µg protein was loaded. Blots were reprobed with β-actin to confirm equal loading of protein. The data in this figure are representative of 4 separate experiments.

Fig. 2. Distribution of signaling molecules into cytoplasmic, detergent soluble and detergent resistant membrane fractions in cortical tissue. A, Components of dopamine-sensitive signaling pathways are primarily localized to Ds and Dr membranes; only PLCβ1 was detected in the cytoplasmic fraction. B, The tyrosine kinases Src and Fyn were associated with Ds and Dr membranes; Fyn, but not Src could be detected in the cytoplasm. C, Another G protein coupled receptor, metabotropic glutamate receptor 5 (mGluR5) distributed into both the Ds and Dr membrane fractions. Twenty micrograms of protein was loaded per lane, except for Dr in panel B, where only 4µg total protein needed to be loaded.

Fig. 3. Differential centrifugation of cortical membrane fractions reveals dopamine receptor distribution across distinct cellular subdomains. Fractions are designated as: S, synaptosomal fraction (12,500g pellet); P, plasma membrane fraction (23,000g pellet); µ, microsomal membrane fraction (100,000g sediment); R, ribosomal fraction (300,000g sediment); and Cy,

cytoplasmic fraction (300,000g supernatant). A, D₁ receptors are restricted to Dr membranes of various buoyancies throughout the cell. B, D₅ receptors are visualized primarily in the 300,000g cytoplasmic fraction. C, D₂ receptors are observed in every fraction examined, with varying percentages in the Ds versus Dr fractions. Blots were reprobed for PSD-95 and calnexin to validate the efficiency of fractionation.

Fig. 4. Cortical D₁ receptors, but not D₅ or D₂ receptors float on sucrose density gradients. Western blot analysis was conducted using discontinuous sucrose gradient fractions, and shown in the figure are representative gradient profiles. A, D₁ receptor-containing membranes localize to fractions where the lipid raft protein flotillin (D) and the caveolar protein caveolin (E) are found. D₁ receptors are also present in diverse detergent-soluble membranes and in the detergent-resistant pellet, where PSD-95 (F) is located. B, D₅ receptors are only detectable in the 200,000g supernatant (cytoplasmic) fraction. C, D₂ receptors are distributed between the cytoplasmic, detergent-resistant pellet and detergent-soluble fractions, but are clearly absent from the raft fraction. All panels were derived from the same gradient. Graph inserts on the right represent densitometric quantification of the immunoreactivity in each fraction (compiled from two independent experiments) as a percentage of summed immunoreactivity in all fractions, for each individual protein.

Fig. 5. Cocaine treatment causes subcellular redistribution of cortical D₁ receptors, but not D₂ or D₅ receptors. Twenty micrograms of each extract was loaded per lane, except for detection of D₁ receptors in Dr (#, 4μg). A, In saline-treated rats, D₁ receptors are localized to the Dr fraction, while cocaine treatment shifts D₁ receptors from Dr into Ds and Cy. B, Cocaine administration does not alter D₅ receptor location. C, Following repeated saline or cocaine injection, D₂ receptors were preferentially found in Dr membranes. D, E, F, Blots were reprobed with β3-tubulin, α-synuclein or PSD-95 to demonstrate equal loading, and the lack of a generalized cocaine effect on cellular distribution of these proteins. For each condition, immunoreactivity was quantified in each fraction for each protein and expressed as a percentage of summed immunoreactivity in all fractions of the protein for that condition. Data are expressed as mean ± SEM (n = 5). *p < 0.05.

Fig. 6. Repeated cocaine administration does not elicit changes in dopamine receptor localization in the dorsal striatum. Striatal tissue from the same animals analyzed in Figure 4 was fractionated and assessed for changes in dopamine D₁, D₅ and D₂ receptor content

following repeated dosing with cocaine. Twenty micrograms of each extract was loaded per lane, except for detection of D₁ receptors in Dr (#, 4µg). A, striatal D₁ receptors are enriched in the Dr fraction. B, striatal D₅ receptors are detected primarily in the cytoplasmic fraction and to some extent in the Dr membranes. C, striatal D₂ receptors are found mainly in the Dr membrane fraction. Blots were reprobed with β3-tubulin, α-synuclein or PSD-95 to demonstrate equal loading, and check for any generalized effect of cocaine on subdomain distribution of cellular proteins. For each condition, immunoreactivity was quantified in each fraction for each protein and expressed as a percentage of summed immunoreactivity in all fractions of the protein for that condition. Data are expressed as mean ± SEM (n = 5) *p< 0.05.

Supplemental Figure 1. Selectivity of dopamine receptor antibodies. A, D₂ receptor immunoreactivity was detected only in lysates from HEK 293 cells that expressed D2L receptors; 20µg of lysate was loaded per lane. B, D₂ receptor immunoreactivity was observed in lysates from Sf9 cells that overexpressed D2L, but not D3 receptors; 1µg of lysate was loaded per lane. C, D₅ receptor immunoreactivity was observed in hippocampal, but not liver extracts. Twenty micrograms of cytoplasmic, DSM or DRM was loaded per lane. β-actin was used to demonstrate presence of protein in the liver extracts.

Supplemental Figure 2. Detection of D₂ receptors in cortical cytoplasmic fractions is dependent on preparation. A, D₂ receptors were observed in cytoplasm of acutely isolated tissue extracts, whereas further processing (acute slices for *ex-vivo* stimulation) rendered the D₂ receptors undetectable in cytoplasmic extracts for up to 60 minutes post-slice preparation. B, C, D₂ receptor immunoreactivity in DSM and DRM was not altered by preparation. All panels, 20µg total extract was loaded per lane.

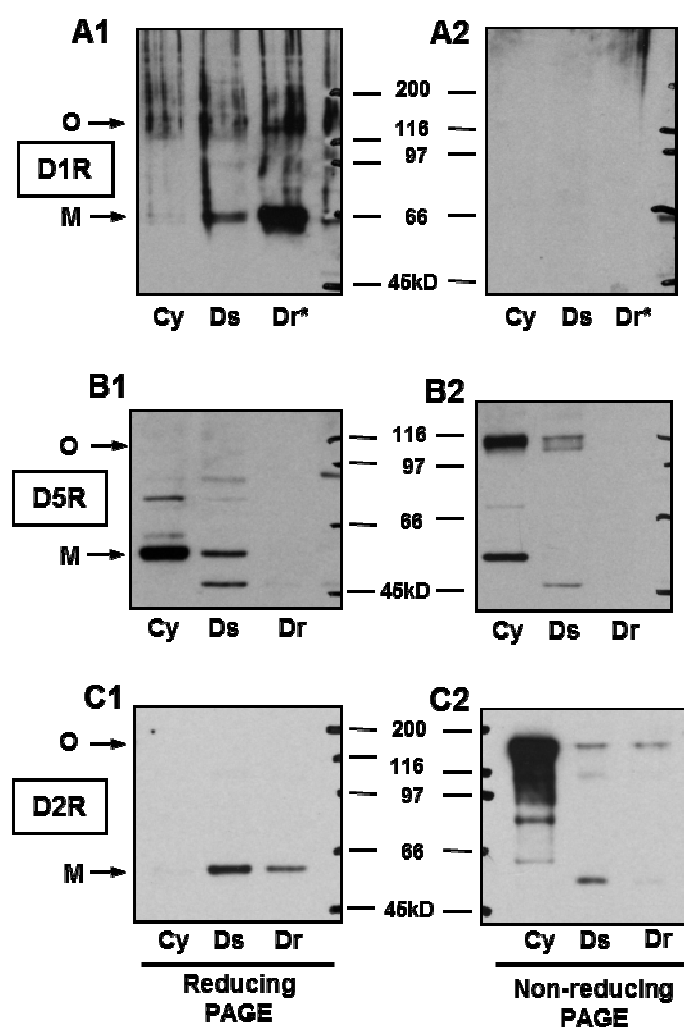


Figure 1

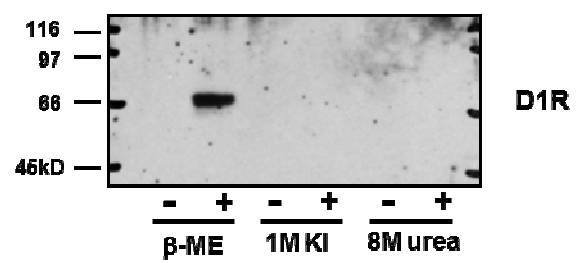


Figure 2

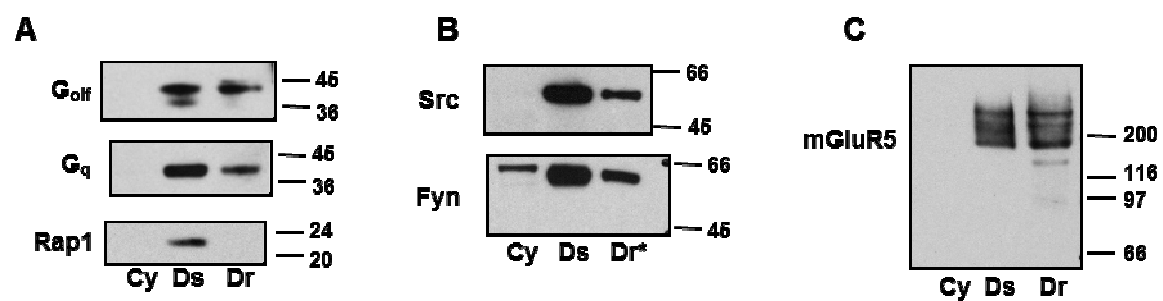


Figure 3

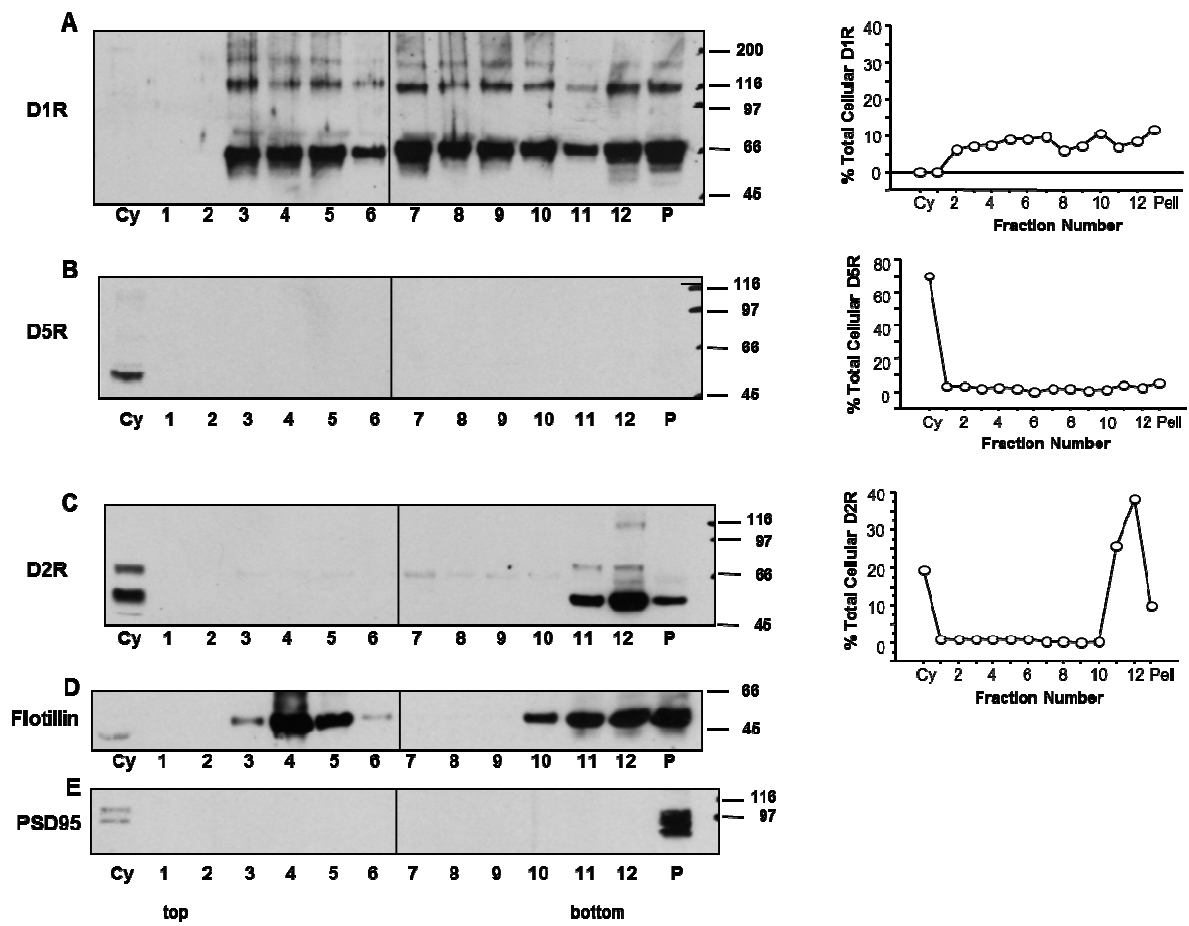


Figure 4

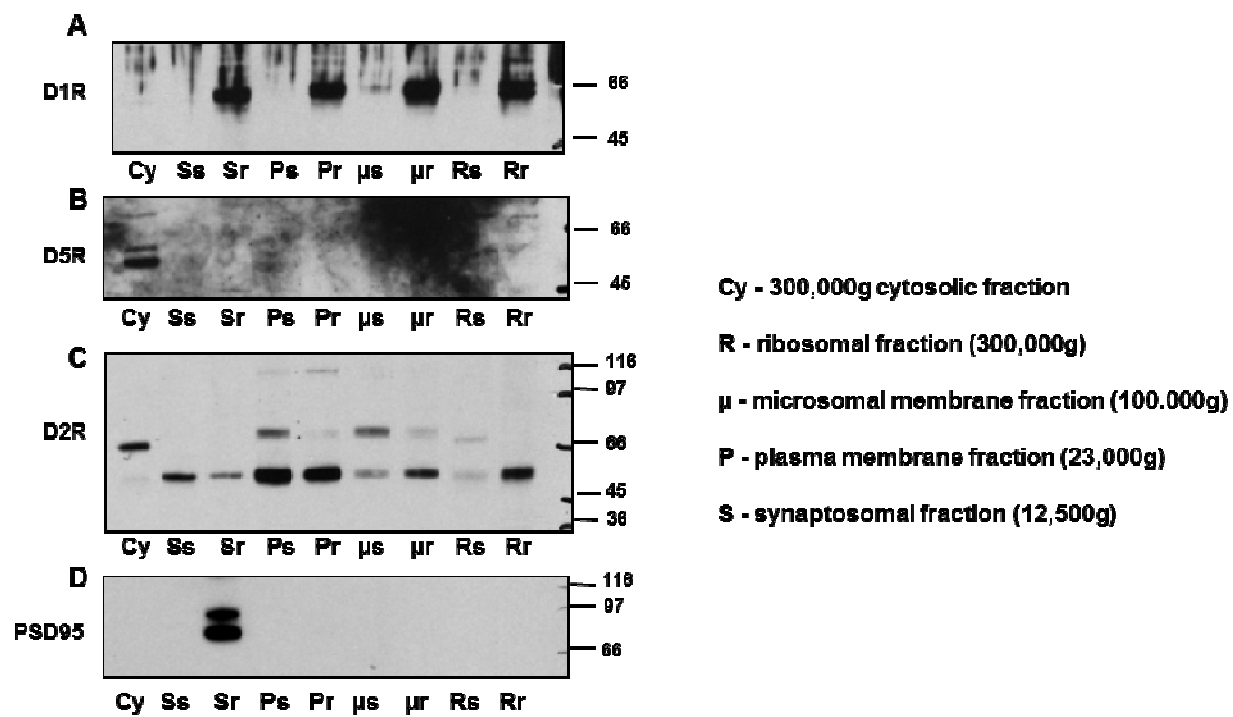


Figure 5

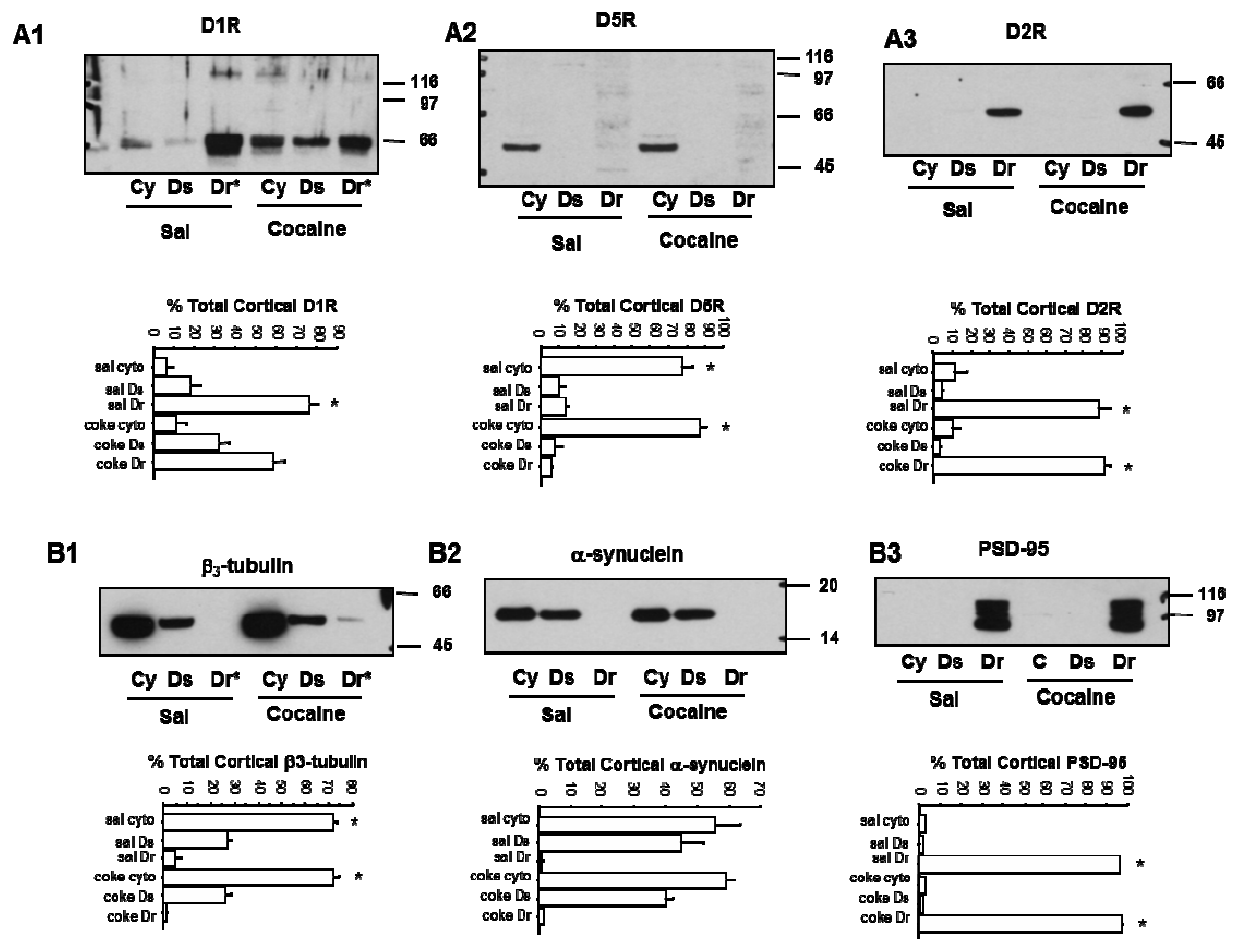


Figure 6