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Dopamine receptor expression and distribution dynamically change in the rat nucleus accumbens after withdrawal from cocaine self-administration

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Abstract

Dopamine receptors (DARs) in the nucleus accumbens (NAc) are critical for cocaine's actions but the nature of adaptations in DAR function after repeated cocaine exposure remains controversial. This may be due in part to the fact that different methods used in previous studies measured different DAR pools. In the present study, we used a protein crosslinking assay to make the first measurements of DAR surface expression in the NAc of cocaine-experienced rats. Intracellular and total receptor levels were also quantified. Rats self-administered saline or cocaine for ten days. The entire NAc, or core and shell subregions, were collected one or 45 days later, when rats are known to exhibit low and high levels of cue-induced drug seeking, respectively. We found increased cell surface D1 DARs in the NAc shell on the first day after discontinuing cocaine self-administration (designated withdrawal day 1, or WD1) but this normalized by WD45. Decreased intracellular and surface D2 DAR levels were observed in the cocaine group. In shell, both measures decreased on WD1 and WD45. In core, decreased D2 DAR surface expression was only observed on WD45. Similarly, WD45 but not WD1 was associated with increased D3 DAR surface expression in the core. Taking into account many other studies, we suggest that decreased D2 DAR and increased D3 DAR surface expression on WD45 may contribute to enhanced cocaine-seeking after prolonged withdrawal, although this is likely to be a modulatory effect, in light of the mediating effect previously demonstrated for AMPA-type glutamate receptors.

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Keywords

cocaine; dopamine receptors; nucleus accumbens; receptor trafficking

Alterations in dopamine (DA) receptor (DAR) signaling are widely believed to contribute to addiction (Volkow et al., 2009). Many studies have therefore examined the effects of cocaine self-administration and withdrawal on the expression of D1-like (D1 and D5) and D2-like (D2, D3, and D4) classes of DARs in the nucleus accumbens (NAc). Studies in humans and non-human primates have used positron emission topography (PET) to provide an indirect measure of available DAR cell surface receptors. In rat studies, binding assays or *in vitro* receptor autoradiography have been utilized; these techniques measure DARs in a number of compartments, including but not limited to the cell surface pool. Particularly in rodent studies, results appear to depend on the drug regimen and timing of the experiment (Anderson and Pierce, 2005). However, another important variable is the use of different methods that measure different DAR pools, combined with recently uncovered complexities regarding DAR aggregation, trafficking, and signaling. All of these factors complicate the measurement of functional DAR species.

It is well established that D1-like DARs and D2-like DARs are positively and negatively coupled, respectively, to adenylyl cyclase, and that each family can also influence other signal transduction cascades (Lachowicz and Sibley, 1997; Neve et al., 2004). More recently, it has been appreciated that D1, D2 and D3 DARs form dimers and higher order complexes (Lee et al., 2000a; George et al., 2002; Javitch, 2004). Oligomerization, which occurs early in the biosynthetic pathway at the level of the endoplasmic reticulum, may be necessary for targeting DARs and other G-protein coupled receptors (GPCRs) to the cell surface (Lee et al., 2000b; Bulenger et al., 2005). DAR oligomers are formed by disulfide bonds but also by hydrophobic transmembrane domain interactions, making them partially resistant to reducing conditions and leading to the observation of monomer, dimer and oligomer bands in Western blotting studies (e.g., Lee et al., 2003). DARs also contain a variable number of N-linked glycosylation sites (Missale et al., 1998) that may be required, for the D2 DAR, for cell surface trafficking (Free et al., 2007). Glycosylation of the D2 DAR contributes to an additional ~70-75kDa band commonly observed in Western blots (David et al., 1993; Fishburn et al., 1995; Lee et al., 2000b). Intriguingly, DARs have been shown to form hetero-oligomers between different DAR subtypes and with other GPCRs and non-GPCRs; by activating DARs within these multimeric complexes, DA agonists may activate signaling pathways distinct or altered in magnitude from those linked to the individual DARs (e.g., Rocheville et al., 2000; Ginés et al., 2000; Scarselli et al., 2001; Lee et al., 2004; Fiorentini et al., 2003; 2008; Marcellino et al., 2008; So et al., 2009).

In abstinent human cocaine users, vulnerability to relapse often increases after the acute drug withdrawal stage (Gawin and Kleber, 1986; Kosten et al., 2005). An analogous phenomenon has been observed after withdrawal from extended access cocaine self-administration in rats (Neisewander et al., 2000; Grimm et al., 2001; Lu et al., 2004a, b; Conrad et al., 2008). These studies have shown that cue-induced drug seeking increases between day one and day 90 of drug withdrawal, and then returns towards baseline by 6 months. The rising phase is termed “incubation”. The goal of the present study was to determine if incubation of cue-induced cocaine craving is accompanied by alterations in D1, D2, or D3 DAR levels in the NAc. In order to selectively measure changes in the functional DAR pool expressed on the cell surface, we adapted a protein crosslinking assay used previously by our laboratories to measure glutamate receptor cell surface expression after *in vivo* treatments (Boudreau and Wolf, 2005; Boudreau et al., 2007; 2009; Conrad et al., 2008; Nelson et al., 2009; Ferrario et al., 2010). Using this assay, surface, intracellular and

total DAR levels were determined in aliquots of NAc tissue obtained from rats either 1 day or 45 days after discontinuing extended access cocaine or saline self-administration.

EXPERIMENTAL PROCEDURES

Animals and behavioral procedures

Experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23; revised 1996) and were approved by our Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering. The present study analyzed DAR distribution in aliquots of NAc tissue obtained from the same rats used previously to demonstrate incubation of cocaine craving and associated changes in expression of α -amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor subunits after 45 days of withdrawal from cocaine self-administration (Conrad et al., 2008). Tissue was not available for all rats used in our prior study, accounting for some differences in N values. Two cohorts of rats were used. The entire NAc (core + shell) was dissected in the first, whereas core and shell were dissected separately in the second. These studies employed male Sprague Dawley rats (Harlan, Indianapolis, IN) weighing 250-275g upon arrival and housed individually on a reverse 12h/12h light-dark cycle (lights out at 0900 hours). Procedures for surgery and self-administration training were described previously (Conrad et al., 2008). Briefly, rats were allowed to nose-poke to self-administer cocaine or saline for 10 days (6h/day) in self-administration chambers (MED Associates, St. Albans, VT) in sound-attenuating cabinets. Nose-poking in the active hole delivered an infusion of saline or cocaine (0.5 mg/kg/100 μ L over 3s), paired with a 30-s discrete light cue inside the nose-poke hole. Nose-poking in the inactive hole had no consequences. A time-out period of 10s was used during the first hour or for the first 10 infusions (whichever occurred first) and extended to 30s for the remaining time to prevent cocaine overdose. Rats that self-administered cocaine averaged 120 infusions each day (~60mg/kg/day), whereas rats that self-administered saline averaged 20 infusions each day (data not shown). Food and water were present at all times. After discontinuing saline or cocaine self-administration, rats were returned to their home cages for 1 or 45 days before NAc tissue was obtained for protein crosslinking studies (see next section). Thus, four experimental groups were formed: saline rats killed on withdrawal day 1 (WD1-Sal), cocaine rats killed on WD1 (WD1-Coc), saline rats killed on WD45 (WD45-Sal) and cocaine rats killed on WD45 (WD45-Coc). The term "WD" refers simply to the number of days that drug was not available, and does not imply a set of physiological symptoms resulting from the cessation of chronic drug taking.

Protein crosslinking

This method has been described in detail previously (Boudreau and Wolf, 2005; Ferrario et al., 2010). The rats were decapitated, their brains were rapidly removed, and the entire NAc (or core and shell subregions) was dissected on ice from a 2mm coronal section obtained using a brain matrix. Whole NAc tissue was immediately chopped into 400 μ m slices using a McIlwain tissue chopper (Vibratome, St. Louis, MO), whereas the smaller core and shell subregions were minced by hand using a scalpel. Tissue was then added to Eppendorf tubes containing ice-cold artificial CSF spiked with 2 mM bis(sulfosuccinimidyl)suberate (BS³; Pierce Biotechnology, Rockford, IL). The crosslinking reaction was allowed to proceed for 30 min at 4°C with gentle agitation, and was then terminated by addition of 100mM glycine (10 min at 4°C). Tissue was pelleted by brief centrifugation, re-suspended in ice-cold lysis buffer containing protease and phosphatase inhibitors, sonicated for 5 sec, and centrifuged again. Aliquots of the supernatant were stored at -80°C until analyzed by Western blotting.

Western blot analysis of DARs in crosslinked tissue

Samples (20-30µg total protein/lysate) were electrophoresed on 4-15% Tris-HCl gels (Biorad, Hercules, CA). Proteins were transferred onto polyvinylidene fluoride membranes for immunoblotting using constant current (1.15mA) for 1.5 h. A cooling coil was used to prevent excessive heating. Complete transfer of high molecular weight aggregates was confirmed by staining gels after transfer with Coomassie blue. Furthermore, we verified that crosslinked DAR proteins were not detected in the stacking gel (data not shown). After transfer, membranes were washed in ddH₂O, air-dried for 1 hr at room temperature (RT), re-hydrated with 100% MeOH, washed in 1x Tris buffered saline (TBS) and immersed in 0.1M NaOH, pH 10 for 15 min at RT. Then, they were washed in TBS, blocked with 3% Bovine Serum Albumin (Sigma-Aldrich, St. Louis, MO) in TBS-Tween-20 (TBS-T), pH 7.4, for 1 hr at RT, and incubated overnight at 4°C with antibodies recognizing the D1 DAR (1:1000; Millipore; Cat # AB1765P), D2 DAR (1:1000; Millipore, Billerica, CA; Cat # AB5084P), and D3 DAR (1:1000; Millipore; Cat # AB1786P). D4 and D5 DARs were not analyzed due to lack of antibodies recognizing both crosslinked and intracellular receptors. It should be noted that the DAR antibody lots used in these experiments were purchased in 2005-06; current lots of these antibodies (2009-10) show different banding patterns that are not altered in tissue from DAR knockout mice (unpublished observations). After the primary antibody incubation, membranes were washed with TBS-T solution, incubated for 60 min with HRP-conjugated anti-rabbit IgG or anti-mouse IgG (1:10,000; Upstate Biotechnology, Lake Placid, NY), washed with TBS-T, rinsed with ddH₂O, and immersed in chemiluminescence detecting substrate (Amersham GE, Piscataway, NJ). After blots were developed, images were captured with Versa Doc Imaging Software (Bio-Rad). Diffuse densities of surface and intracellular bands were determined using Quantity One software (Bio-Rad). Values for surface, intracellular and total (surface + intracellular) protein levels were normalized to total protein in the lane determined using Ponceau S (Sigma-Aldrich) and analyzed with TotalLab (Nonlinear Dynamics, Newcastle, UK). The surface/intracellular ratio did not require normalization, because both values are determined in the same lane. To examine antibody specificity, preabsorption studies were conducted for the DAR antibodies with the peptide used to generate each antibody. D1, D2, or D3 DAR antibody was combined with a 10-fold excess concentration of peptide in 500µl of TBS, mixed for 4 hrs at 4°C, diluted to a final volume of 20ml, added to the membrane, and incubated overnight at 4°C.

Data analysis

Data were analyzed using SPSS with ANOVA using Drug exposure (Saline vs. Cocaine) and Withdrawal Day (WD1 vs. WD45) as the between-subjects factors, followed by a post hoc Tukey test. Significance was set at $p < 0.05$.

RESULTS

DAR analysis with the BS³ crosslinking assay

The purpose of this study was to analyze cell surface and total expression of D1, D2 and D3 DARs in aliquots of NAc tissue obtained after discontinuing cocaine self-administration (6 h/day for 10 days). As described in Methods, groups are designed WD1 or WD45 to indicate the number of days spent in home cages without access to cocaine prior to DAR analysis. NAc tissue from the same rats was previously used to demonstrate that the formation of GluR2-lacking AMPA receptors underlies the expression of incubated cue-induced cocaine craving in cocaine-exposed rats on WD45 (Conrad et al., 2008). To assess DAR distribution, we used the same BS³ crosslinking assay used previously to study AMPA receptor distribution. BS³ is a membrane impermeant protein crosslinking agent and therefore selectively crosslinks cell surface proteins, forming high molecular weight aggregates. Intracellular proteins are not modified. Thus, surface and intracellular pools of a particular

protein can be distinguished by SDS-polyacrylamide gel electrophoresis and Western blotting (Boudreau and Wolf, 2005; Boudreau et al., 2007; 2009; Conrad et al., 2008; Nelson et al., 2009; Ferrario et al., 2010). In addition to quantifying surface and intracellular protein levels, we used the sum of surface + intracellular levels as a measure of total receptor protein and the surface/intracellular ratio as a measure of receptor distribution.

Fig. 1 illustrates the method by comparing crosslinked (X) and non-crosslinked (Non) tissue probed for each DAR. Surface bands are present only after crosslinking. Intracellular bands are diminished in crosslinked tissue compared with an equal amount of non-crosslinked tissue because, in the former, the surface-expressed portion of the total receptor pool is now present in the surface band. Accordingly, total DAR protein levels in the non-crosslinked lanes are approximately equal to the sum of S and I values in the crosslinked lanes (see legend to Fig. 1; the same equivalence was observed in all other experiments). It should be noted that although BS³ provides an accurate measure of relative differences in S/I ratios between experimental groups, the absolute level of S/I that is measured depends on the experimental conditions and the antibody. For example, consider two proteins, A and B, that are similarly distributed between S and I compartments. If antibody to A recognizes its crosslinked form less avidly than the unmodified (intracellular) form, whereas antibody to B recognizes both forms equally well, the measured S/I ratio will be lower for A than B, even though the proportion of each protein on the surface is actually the same.

For D1 and D3 DARs, we quantified a single intracellular and single surface band (Fig. 1a, c). For the D2 DAR, three intracellular bands were detected. Consistent with other studies (e.g., Fishburn et al., 1995; Kim et al., 2008), we identified these bands as monomeric (~55kDa), glycosylated (~75kDa) and dimeric (~100kDa) D2 DARs (Fig. 1b). A surface band was also detected. All three of the intracellular species contributed to the surface-expressed D2 DAR pool, based on decreased intensity of all three intracellular bands in crosslinked tissue relative to non-crosslinked controls. All three of the D2 DAR intracellular bands were summed to generate the intracellular value used to determine total D2 DAR levels (surface + intracellular) and the D2 DAR surface/intracellular ratio. A faint band was also detected at ~200kDa, but its immunoreactivity was too low to quantify (Fig. 1b). Preabsorption studies, conducted with peptides used to generate each antibody, demonstrated immunospecificity of all bands quantified in our experiments, including surface bands (Fig. 1d, e, f). Furthermore, the banding patterns we observed were similar to those found in previous immunoblotting studies using the same antibodies (e.g., Huang et al., 1992 – D1 DAR; Boundy et al., 1993a – D2 DAR; Boundy et al., 1993b – D3 DAR), and immunohistochemical studies with these antibodies revealed the expected anatomical distribution for D1 DARs (Huang et al., 1992) and D2 DARs (Boundy et al., 1993a; Wang and Pickel, 2002; Paspalas and Goldman-Rakic, 2004; Pinto and Sesack, 2008).

D1 DARs

No significant differences between cocaine and saline groups were found on WD45. However, effects of cocaine self-administration were evident on WD1. Analysis of the entire NAc indicated a significantly higher D1 DAR surface/intracellular ratio in the WD1-Coc group compared with groups that self-administered saline (Fig. 2a). This was attributable to a modest increase in surface D1 DARs combined with a modest decrease in intracellular D1 DARs (neither of these latter two effects were statistically significant), in the absence of any change in total D1 DAR levels (surface + intracellular) (Fig. 2a). Within the NAc core, no significant effect was found for any D1 DAR measure (Fig. 2b). However, the NAc shell displayed changes that were similar to those observed in the entire NAc but slightly more robust (Fig. 2c). The surface/intracellular D1 DAR ratio was increased in the WD1-Coc group due to a significant increase in surface D1 DAR expression. Intracellular levels were unchanged, but there was a trend towards increased total D1 DAR levels. In summary, a

greater portion of D1 DAR protein was surface-expressed in the NAc shell of WD1-Coc rats compared with WD1-Sal rats. D1 DAR distribution returned to the control state after 45 days of withdrawal from cocaine self-administration.

D2 DARs

In the entire NAc, the major effect observed was decreased D2 DAR expression in rats that self-administered cocaine compared with saline controls (Fig. 3a). This was most pronounced on WD45, when decreases were observed in the surface band, all three intracellular bands (~55, 75 and 100kDa), and in total D2 DAR levels compared with saline controls. The surface/intracellular D2 DAR ratio was slightly but significantly increased in the WD45-Coc group, due to a greater decrease in intracellular than surface D2 DARs, perhaps indicating that the cells are compensating for decreased D2 DAR expression by distributing a greater portion of available D2 DARs to the surface. It is important to keep in mind that the elevated surface/intracellular ratio does not suggest increased D2 DAR transmission in this particular case, because the absolute level of surface-expressed D2 DARs was decreased. In the WD1-Coc group, the only significant effect was a decrease in intracellular levels of the D2 DAR monomer (~55kDa), compared to both WD45-Sal and WD1-Sal groups, although several other measures also tended to decrease (Fig. 3a).

An overall decrease in D2 DAR expression was also evident in core and shell subregions of the NAc (Fig. 3b and 3c, respectively), although effects tended to be more pronounced in the shell. Thus, surface D2 DAR levels decreased in cocaine rats only on WD45 in core, but on WD1 and WD45 in shell. Total D2 DARs decreased significantly only in shell. Decreases in intracellular D2 DAR bands occurred on both withdrawal days in both core and shell, although there were withdrawal- and region-specific differences in terms of which intracellular band showed a statistically significant effect. In summary, D2 DAR surface and intracellular protein levels were decreased in the NAc after cocaine self-administration. Some decreases were already evident by WD1.

D3 DARs

Significant changes in D3 DAR distribution were not observed on WD1 after cocaine self-administration, but developed by WD45. Within the entire NAc, the WD45-Coc group had a higher D3 DAR surface/intracellular ratio than all other groups, attributable to the combination of a modest increase in surface levels and a modest decrease in intracellular levels (neither effect was significant); total D3 DAR levels were unchanged (Fig. 4a).

The NAc core showed similar but more pronounced changes. Thus, the WD45-Coc group had higher surface D3 DAR levels compared to all other groups, resulting in a higher surface/intracellular ratio (Fig. 4b). In the NAc shell, the only significant change relative to saline controls was an increase in the D3 DAR surface/intracellular ratio (Fig. 4c). In both core and shell, D3 DAR total protein levels were higher in WD45-Coc compared with WD1-Coc rats (Fig. 4b, c). Functionally, the most important change is probably the increased D3 DAR surface expression in the NAc on WD45, an effect that was most evident in the core subregion.

DISCUSSION

We analyzed D1, D2 and D3 DAR surface and intracellular levels in the NAc of rats on WD1 or WD45 after discontinuing extended access cocaine self-administration. Although behavioral results are not presented here, we showed previously that rats exposed to this cocaine regimen exhibit incubation of cue-induced cocaine craving on WD45 (Conrad et al., 2008). Furthermore, the same cocaine-exposed rats used to obtain the NAc tissue analyzed

herein were previously shown to exhibit elevated cell surface GluR1 levels on WD45, indicative of the formation of GluR2-lacking AMPA receptors that accompanies the incubation of cue-induced cocaine craving (Conrad et al., 2008). The role of DARs in incubation has not been studied previously. Furthermore, our study is the first to measure surface-expressed DARs in any animal model of addiction. As described below, although all three DARs studied showed time-dependent changes after discontinuing cocaine self-administration, we speculate that time-dependent decreases in D2 DAR surface expression and increases in D3 DAR surface expression in the NAc core are most likely to contribute to incubation of cue-induced cocaine seeking.

In addition to observing time-dependent changes, we observed different DAR changes in core and shell subregions. The core is implicated in motor responding to conditioned reinforcers, whereas the shell is more involved in processing information related to the reinforcing effects of psychostimulants (Ito et al., 2000; 2004; Rodd-Henricks et al., 2002; Ikemoto, 2003; Fuchs et al., 2004; Ikemoto et al., 2005). Consistent with this, the core is an important part of the neural circuitry that underlies incubation of cue-induced cocaine seeking (Conrad et al., 2008). This suggests that DAR adaptations in the core are more likely to be related to incubation. However, it must be kept in mind that core and shell cannot be considered in isolation, because they interact as part of spiraling anatomical loops linking cortical, limbic and basal ganglia regions (Haber, 2003). Furthermore, these loops rely on many transmitters in addition to DA, such as glutamate. Keeping in mind core-shell interactions and the role of multiple transmitter systems may help explain some apparent discrepancies in the core-shell literature. For example, functional inactivation studies implicate core but not shell in cocaine-primed and cue-induced reinstatement (McFarland and Kalivas, 2001; Fuchs et al., 2004). Yet, as will be discussed in more detail below, both shell and medial core (but not lateral core) are implicated in DAR regulation of cocaine-primed reinstatement (Anderson et al., 2003; 2008; Bachtell et al., 2005; Schmidt and Pierce, 2006; Schmidt et al., 2006).

We have limited the scope of our literature review by focusing on DAR adaptations after cocaine self-administration rather than non-contingent cocaine treatment (for reviews of the latter topic, see Pierce and Kalivas, 1997; Anderson and Pierce, 2005). Likewise, we have focused on studies utilizing intra-NAc injection of DAR subtype-selective drugs rather than systemic drug administration (e.g., Self et al., 1996; De Vries et al., 1999). However, it is interesting to note that time-dependent changes in responding to systemic DA agonists have been found after discontinuing cocaine self-administration (De Vries et al., 2002; Edwards et al., 2007). These changes could be related to the DAR expression changes reported herein, or they could reflect changes in DAR function in other brain regions.

D1 DAR surface expression transiently increases in the NAc shell after discontinuing cocaine self-administration

After cocaine self-administration, D1 DAR surface expression was increased in the NAc shell on WD1 but normalized by WD45, whereas no changes were observed in the core, indicating a transient increase confined to shell. Similar results have been obtained in previous studies using receptor autoradiography. Ben-Shahar et al. (2007) found increased D1 DAR density in the NAc shell of rats 20 min (but not 14 or 60 days) after discontinuing extended access (6 hr/day) cocaine self-administration, whereas no changes were observed in the core or after short access cocaine self-administration (2 hr/day). Nader et al. (2002) observed a small increase in D1 DAR density in shell but not core of rhesus monkeys killed after the last of 100 cocaine self-administration sessions. Monkeys evaluated 30 days after discontinuing the same regimen showed increased D1 DAR density in rostral NAc and in both core and shell at more caudal levels, but D1 DAR density had normalized by 90 days (Beveridge et al., 2009). All of these results, like ours, indicate a transient increase in D1

DAR levels, particularly in shell, after discontinuing cocaine self-administration. However, an earlier study by this group indicated a decrease in D1 DAR density in the NAc (most robust in the shell) of rhesus monkeys that had self-administered cocaine for a much longer period of time (18 months; Moore et al., 1998a). Decreased D1 DAR binding in the NAc was also found 18 hr after discontinuing an extended access regimen in rats, although total cocaine intake in this study was higher than in rat studies discussed above (De Montis et al., 1998). These results indicate that D1 DAR adaptations depend on many aspects of cocaine exposure. Another consideration is that receptor autoradiography measures total cellular receptors, whereas our protein crosslinking experiments can distinguish between surface and intracellular receptors. Interestingly, an immunoblotting study found a trend towards increased D1 DAR levels in the NAc of human cocaine users (Worsley et al., 2000).

Is the transient increase in D1 DAR surface expression that we observed in the NAc shell important for the incubation of cue-induced cocaine craving? This is difficult to assess, because no studies have tested the effect of intra-NAc injection of D1 DAR agonists or antagonists on cue-induced cocaine seeking after home-cage withdrawal (or cue-induced reinstatement of cocaine seeking after extinction training). However, D1 receptors in the medial NAc (shell and medial core) are implicated in cocaine-primed reinstatement of cocaine seeking after extinction, apparently through a mechanism requiring cooperative activation of D1 and D2 DARs (Anderson et al., 2003; 2008; Bachtell et al., 2005; Schmidt and Pierce, 2006; Schmidt et al., 2006). Together with our results, this could suggest that neurons in the NAc shell are more responsive to D1 DAR-mediated cocaine seeking in early withdrawal due to transient D1R upregulation. However caution must be used in extrapolating from reinstatement to incubation studies, because extinction training and home-cage withdrawal are associated with different neuroadaptations in the NAc (Sutton et al., 2003; Ghasemzadeh et al., 2009; Wolf and Ferrario, 2010). It is important to note that D1 DARs in the basolateral amygdala and prefrontal cortex are also important for cue-induced reinstatement of cocaine seeking (e.g., Ciccocioppo et al., 2001; Alleweireldt et al., 2006; Berglind et al., 2006).

On a cellular level, both presynaptic and postsynaptic DARs can modulate the excitability of medium spiny neurons, the predominant cell type and output neuron of the NAc (Nicola et al., 2000; O'Donnell, 2003). Repeated non-contingent cocaine administration is known to enhance some effects of D1 DAR activation in the NAc. Thus, one day to one month after discontinuing cocaine treatment, enhanced ability of D1 DAR agonists to inhibit the activity of medium spiny neurons (driven by iontophoretic glutamate) was observed throughout the NAc (Henry and White, 1991; 1995). However, the increased D1 DAR surface expression reported here is unlikely to explain these prior results, because it is restricted to the shell and has only been demonstrated on WD1. One day after a cocaine challenge administered 10-14 days after discontinuing repeated cocaine injections, Beurrier and Malenka (2002) observed an enhancement of DA-mediated inhibition of excitatory synaptic responses in NAc medium spiny neurons that was apparently mediated by presynaptic D1-like DARs on glutamate nerve terminals. However, possible effects of the challenge injection (for example, see Boudreau et al., 2007 and Kourrich et al., 2007), combined with species differences and lack of recordings in core, make it difficult to compare their findings to our own. It should also be noted that the DAR agonists and antagonists used by Henry and White (1991; 1995) and Beurrier and Malenka (2002) did not distinguish between D1 and D5 DARs.

D2 DAR levels decrease in the NAc after discontinuing cocaine self-administration

The main effect observed in our study was a decrease in D2 DAR protein in both NAc core and shell after discontinuing cocaine self-administration, relative to saline controls. This was more pronounced in the shell, where intracellular, surface and total bands were decreased on both WD1 and WD45. In core, D2 DAR surface expression was decreased only on WD45

and total D2 DAR levels did not decrease significantly. Several other studies have similarly found decreased D2 DAR expression after discontinuing cocaine self-administration. In rhesus monkeys with extensive cocaine self-administration experience, D2 DAR density, measured with receptor autoradiography, was decreased in many striatal regions including NAc core and shell when tissue was obtained immediately after the last session (Moore et al., 1998b; Nader et al., 2002). Using PET, this effect in the basal ganglia was detected within 1 week of initiating cocaine self-administration (Nader et al., 2006). The rate at which D2 DAR levels recover during withdrawal may depend on total cocaine intake. In an autoradiography study, D2 DAR levels in the NAc recovered to control values after 30 or 90 days of withdrawal from 100 sessions of cocaine self-administration (Beveridge et al., 2009). However, in a PET study of monkeys with longer exposure (1 year) and thus higher total cocaine intake, 3 of 5 monkeys showed recovery of D2 DAR levels after 90 days while 2 monkeys showed no recovery even after 12 months (Nader et al., 2006). Overall, these results correspond well with our findings of decreased D2 DAR levels throughout withdrawal.

PET studies of human cocaine addicts have also found reduced D2 DAR levels in many striatal regions, including ventral striatum, that were evident in early withdrawal as well as after 3-4 months of detoxification (Volkow et al., 1990, 1993, 1997). However, the significance for behavior is unclear, as D2 DAR availability did not correlate with positive subjective effects of cocaine or the decision to take more cocaine after a priming dose (Martinez et al., 2004). It is important to note that while cue-induced cocaine craving shows a time-dependent increase during withdrawal (“incubation”), this does not occur for cocaine-primed cocaine seeking (Lu et al., 2004a). Therefore, the results of Martinez et al. (2004) leave open the possibility that D2 DAR availability might correlate with cue-induced cocaine seeking, the focus of the incubation model studied herein. Low D2 DAR availability in human cocaine users does correlate with decreased frontal cortical metabolism (Volkow et al., 1993). Along with other changes, this may contribute to the loss of control that occurs when addicts are exposed to drugs or drug-paired cues, and to greater salience of drugs compared to non-drug rewards (Volkow et al., 2007; Volkow et al., 2009). It should be noted that decreased D2 DAR levels in a PET study can indicate elevated DA release rather than decreased D2 DAR levels, but recent results argue against this explanation in the case of cocaine dependent patients (Martinez et al., 2009). Furthermore, a postmortem study of human cocaine users found a trend towards decreased D2 DAR levels in the NAc using immunoblotting (Worsley et al., 2000).

Studies in human cocaine addicts cannot determine whether decreased D2 DAR availability is a predisposing trait or a result of cocaine exposure, but other results indicate that both are true. On the one hand, experiments in non drug-abusing humans have found an inverse correlation between D2 DAR availability and reports of “drug liking” when administered methylphenidate (Volkow et al., 1999; 2002). These findings suggest that low D2 DAR availability may increase vulnerability to addiction. A similar conclusion is supported by studies in rhesus monkeys. In socially housed monkeys, the achievement of social dominance increases D2 DAR availability in the striatum and this is associated with lower sensitivity to the reinforcing effects of cocaine compared to subordinate monkeys (Morgan et al., 2002). Social status is also correlated with striatal D2 DAR availability in drug free human volunteers (Martinez et al., 2010). On the other hand, both PET and receptor autoradiography studies show that long term cocaine self-administration decreases striatal D2 DAR receptor availability in individually housed monkeys, as discussed above (Moore et al., 1998b; Nader et al., 2002; Nader et al., 2006). Chronic cocaine self-administration also appears to decrease D2 DAR availability in dominant socially housed monkeys (Czoty et al., 2004). Thus, after long-term cocaine self-administration, there were no longer significant differences in D2 receptor availability or reinforcing effects of cocaine between dominant

and subordinate monkeys (Czoty et al., 2004). However, elevated D2 DAR levels remerged in the dominant monkeys during abstinence and this was correlated with longer latency in reaction to novelty, a trait predictive of decreased sensitivity to cocaine's reinforcing effects (Czoty et al., 2010).

As in humans and monkeys, rat studies indicate that low D2 DAR availability is a risk factor for cocaine vulnerability. Thus, PET studies in rats with high impulsivity (a trait associated with increased cocaine self-administration) show reduced D2/D3 DAR availability in the ventral striatum (Dalley et al., 2007). D2 DAR levels in the NAc are also reduced in rats that show a high locomotor response to novelty, another trait associated with addiction vulnerability (Hooks et al., 1994). Our results in rats indicate that decreased D2 DAR levels in the NAc can also be a consequence of repeated cocaine exposure, consistent with studies in monkeys and humans (above). However, two receptor autoradiography studies in rats found results that differ from ours. Ben-Shahar et al. (2007) did not observe decreased D2 DAR levels in the NAc after withdrawal (20 min, 14 days of 60 days) from an extended access cocaine self-administration regimen similar to our own (6 hr/day), although decreases were observed in the NAc shell after a limited access regimen (2 hr/day) and 14 days of withdrawal (Ben-Shahar et al., 2007). Stéfanski et al. (2007) found no changes in D2 DAR levels in core or shell 24 h after discontinuing limited access cocaine self-administration (2 hr/day), although D2 DAR levels did decrease in yoked cocaine controls. As noted above, receptor autoradiography measures total cellular receptors, while PET and protein crosslinking studies measure cell surface receptors.

Overall, studies on the relationship between D2 DAR levels and cocaine self-administration support a model in which D2 DARs normally limit cocaine self-administration. Therefore, we suggest that the decreased D2 DAR levels observed in our experiments may contribute to cue-induced cocaine seeking after cocaine withdrawal. In particular, the fact that D2 DAR surface expression in the NAc core was decreased on WD45 but not WD1, combined with a key role for NAc core in cue-induced cocaine seeking, suggests that time-dependent D2 DAR downregulation in the NAc core may contribute to the time-dependent intensification of cue-induced cocaine seeking. This would predict that intra-NAc infusion of a D2 agonist during withdrawal would reduce cue-induced cocaine seeking. Unfortunately, no studies have examined effects of intra-NAc D2 DAR drugs in the incubation model. On the other hand, studies of cocaine-primed reinstatement indicate that D1 and D2 DARs in the shell and medial core work cooperatively to promote cocaine seeking (Anderson et al., 2003; Bachtell et al., 2005; Schmidt and Pierce, 2006; Schmidt et al., 2006). Based on these findings, the decreased D2 DAR expression observed in our experiments might be predicted to reduce cocaine seeking, that is, produce an effect opposite to the withdrawal-dependent intensification that is actually observed. The discrepancy may reflect problems introduced by generalizing from cocaine-primed reinstatement after extinction training to cue-induced cocaine seeking after withdrawal.

A time-dependent increase in D3 DAR surface expression occurs in the NAc core after discontinuing cocaine self-administration

Studies of D3 DAR-preferring drugs in cocaine self-administration and reinstatement paradigms suggest that D3 DAR antagonists may be useful in treating cocaine addiction and, in particular, in reducing reactivity to cocaine-associated cues (Heidbreder et al., 2005; 2008; Le Foll et al., 2005; Xi and Gardner, 2007). These results imply that activation of D3 DAR by endogenous DA may be involved in mediating cue-induced cocaine seeking. Our results show that D3 DAR surface expression in the NAc core is unchanged on WD1 from extended access cocaine self-administration but increased on WD45 in association with the incubation of cocaine craving. D3 DAR surface expression did not increase significantly in the shell, although there was a small but significant increase in the surface/intracellular ratio.

Given the role of D3 DAR transmission in responding to cocaine-associated cues and the importance of core for cue-induced cocaine seeking, it is tempting to speculate that increased D3 DAR surface expression in the NAc core contributed to the incubation of cue-induced cocaine craving that is observed on WD45. However, the neural site at which D3 DAR antagonists act to reduce cocaine seeking has not been established. Specifically, no studies have examined the effect of intra-NAc injection of D3 DAR preferring drugs on cue-induced cocaine seeking. In a different model, Schmidt et al. (2006) found that injection of the D3-preferring agonist PD 128,907 into core or shell did not produce reinstatement of cocaine seeking after extinction training.

Our results are generally consistent with receptor autoradiography studies that measured total D3 DAR levels in the NAc after cocaine exposure. Staley and Mash (1996) reported that D3 DAR binding was higher in the NAc of cocaine overdose victims compared with age-matched controls. After exposure to cocaine in a conditioned place preference paradigm and three days of withdrawal, mice exhibited increased D3 DAR binding in the NAc core and shell (Le Foll et al., 2002). Neisewander et al. (2004) measured D3 DAR binding in rats with extensive cocaine self-administration experience that were tested for cocaine-primed reinstatement after various withdrawal periods and then killed 24 h later. D3 DAR binding in the NAc was unchanged on WD1 but increased after a longer time (WD31-32), consistent with our observation of a time-dependent increase. Furthermore, a drug treatment during withdrawal that reduced cocaine seeking also attenuated the increase in D3 DAR binding, suggesting that D3 DAR upregulation is functionally linked to cocaine seeking. It should be noted that D3 DAR increases in Neisewander et al. (2004) were significant in the core whereas only trends were observed in shell, but the subregions were analyzed in a rostral portion of the NAc where core and shell are less distinct. Our analysis was performed on core and shell from rostral and caudal portions of the NAc.

Contrasting changes in D1, D2, and D3 DARs after cocaine self-administration

Important differences in trafficking and intracellular sorting of different DAR subtypes may help explain our observation that D2 DAR levels are decreased on WD45 after cocaine self-administration whereas D1 DAR levels are unchanged. After acute exposure to a DA agonist, all DARs internalize, but D1 DARs rapidly recycle to the surface while D2 DARs are targeted for degradation (Bartlett et al., 2005). If the same occurs after prolonged exposure to elevated DA levels during cocaine self-administration, it could help explain our results of a transient increase in D1 DAR expression but a more persistent decrease in D2 DAR expression. The accumulation of D3 DARs may be related to less agonist-induced internalization compared with D2 DARs (Kim et al., 2001). Caution is necessary, of course, in extrapolating from DAR trafficking responses in expression systems after short-term agonist treatment to their responses in adult neurons following long-term cocaine treatment and withdrawal.

Conclusions

We conducted the first study of DAR surface expression after withdrawal from repeated cocaine exposure, using a cocaine self-administration paradigm that leads to incubation of cocaine craving. D1 DAR surface expression increased in the NAc shell on WD1 but normalized by WD45. Intracellular D2 DAR levels decreased in NAc core and shell at both withdrawal times. However, while D2 DAR surface expression was also decreased in the shell at both withdrawal times, the core showed decreased D2 DAR surface expression on WD45 but not WD1. Cocaine-induced changes in D3 DAR surface and total expression in the core were also time-dependent; both measures were increased on WD45 but not WD1. The functional implications of these changes are complex to predict. However, based on the literature discussed above, including results demonstrating a more important role for core

than shell in cue-induced cocaine seeking, we suggest that the time-dependent decrease in cell surface D2 DAR and increase in cell surface D3 DAR in the NAc core may contribute to the incubation of cue-induced cocaine seeking. However, these effects are likely to be modulatory in light of the “mediating” role of NAc GluR2-lacking AMPA receptors for the expression of incubated cue-induced cocaine craving (Conrad et al., 2008).

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ABBREVIATIONS

AMPA	α -amino-3-hydroxy-5-methylisoxazole-4-propionate
BS³	bis(sulfosuccinimidyl)suberate
DAR	Dopamine receptor
Coc	Cocaine
GPCR	G-protein coupled receptor
NAc	Nucleus accumbens
PET	positron emission topography
RT	room temperature
Sal	Saline
SDS	Sodium dodecyl sulfate
TBS	Tris buffered saline (TBS)
TBS-T	TBS-Tween-20
WD1	Withdrawal day 1
WD45	Withdrawal day 45

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Fig. 1. Measurement of DAR surface expression using a protein crosslinking assay and demonstration of immunospecificity by preabsorbing DAR antibodies with peptides used to raise each antibody

The membrane impermeant protein crosslinking reagent BS3 crosslinks surface-expressed proteins, forming high molecular weight aggregates, but does not modify intracellular proteins. Surface and intracellular DAR can therefore be distinguished by molecular weight. (A-C) Crosslinked (X) and non-crosslinked (Non) NAc tissue (20-30 μ g protein/lane) was probed with antibodies to the D1, D2, and D3 DARs, respectively, in the presence of the vehicle (TBS) used to dissolve the peptides (DAR ab + Veh groups). A surface band (>250 kDa) was evident only in the crosslinked tissue. A single intracellular band was observed for the D1 DAR (A) and D3 DAR (C). For the D2 DAR (B), three intracellular bands were observed, presumably corresponding to the D2 DAR monomer (~55kDa), glycosylated monomer (~75kDa), and dimer (~100kDa). (D-F) Preabsorption of the D1, D2, or D3 DAR antibodies, respectively, with the peptide used to raise each antibody nearly eliminated all surface and intracellular bands (DAR ab + peptide groups). For each of the DAR, we quantified diffuse density of bands in arbitrary units. As expected from prior studies on

AMPA (Boudreau and Wolf, 2005), we found that the sum of surface (S) and intracellular (I) bands in the crosslinked lanes approximately equaled the total (T) DAR protein in the non-crosslinked lanes (i.e., S + I summed to 92.47% of T for the D1 DAR, 109.15% for the D2 DAR, and 98.9% for the D3 DAR).

D1 Dopamine Receptor

Fig. 2. D1 DAR surface expression was increased in the NAc shell after 1 day of withdrawal from cocaine self-administration

Surface (I), intracellular (I), and total (S+I) D1 DAR levels, and the surface/intracellular ratio (S/I), were determined in the entire NAc (A), the core subregion (B), and the shell subregion (C). Data (mean $\pm$ S.E.M, NAc: $n = 7-12$ per group; NAc core and shell: $n = 5-7$ per group) are expressed as a percentage of the WD1-Sal group. S, I and S+I values were normalized to total protein in the lane; the S/I ratio is independent of total protein loaded onto the gel. ¹ $p < 0.05$ compared with WD1-Sal; ² $p < 0.05$ compared with WD1-Coc; ³ $p < 0.05$ compared with WD45-Sal; ⁴ $p < 0.05$ compared with WD45-Coc.

D2 Dopamine Receptor

Fig. 3. Intracellular and surface D2 DAR levels in the NAc were decreased after 45 days of withdrawal from cocaine self-administration

Surface (I), intracellular (I), and total (S+I) D2 DAR levels, and the surface/intracellular ratio (S/I), were determined in the entire NAc (A), the core subregion (B), and the shell subregion (C). For calculations of S+I and S/I, all three intracellular bands (~55, 75 and 100 kDa; see Figure 1) were summed to generate I. Data (mean $\pm$ S.E.M, NAc: $n = 7-12$ per group; NAc core and shell: $n = 5-7$ per group) are expressed as a percentage of the WD1-Sal group. S, I and S+I values were normalized to total protein in the lane; S/I is independent of total protein loaded onto the gel. ¹ $p < 0.05$ compared with WD1-Sal; ² $p < 0.05$ compared with WD1-Coc; ³ $p < 0.05$ compared with WD45-Sal; ⁴ $p < 0.05$ compared with WD45-Coc.

D3 Dopamine Receptor

Fig. 4. D3 DAR surface expression was increased in the NAc after 45 days of withdrawal from cocaine self-administration

Surface (I), intracellular (I), and total (S+I) D3 DAR levels, and the surface/intracellular ratio (S/I), were determined in the entire NAc (A), the core subregion (B), and the shell subregion (C). Data (mean $\pm$ S.E.M, NAc: $n = 7-12$ per group; NAc core and shell: $n = 5-7$ per group) are expressed as a percentage of the WD1-Sal group. S, I and S+I values were normalized to total protein in the lane; S/I is independent of total protein loaded onto the gel. ¹ $p < 0.05$ compared with WD1-Sal; ² $p < 0.05$ compared with WD1-Coc; ³ $p < 0.05$ compared with WD45-Sal; ⁴ $p < 0.05$ compared with WD45-Coc.