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ORIGINAL ARTICLE

Dopamine D3 autoreceptor inhibition enhances cocaine potency at the dopamine transporter

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Abstract

Cocaine is a commonly abused central nervous system stimulant that enhances dopamine (DA) neurotransmission through its ability to block dopamine transporters (DATs). Recent evidence suggests there may be an interaction between DATs and D2/D3 autoreceptors that modulates cocaine's effects. The purpose of this study was to explore how D2/D3 autoreceptors modulate the ability of cocaine to inhibit DA uptake through DATs on pre-synaptic DA terminals. Using fast-scan cyclic voltammetry in brain slices containing the nucleus accumbens core from male and female C57BL/6J mice, we first sought to examine the effects of global autoreceptor blockade using the non-selective D2/D3 autoreceptor antagonist, raclopride. We found that the ability of cocaine to inhibit DA uptake was increased by raclopride and that this effect was consistent across sexes. Furthermore, using D2 (L-741,626) or D3 (SB-277011-A) autoreceptor selective antagonists, we discovered that blockade of D3, but not D2, autoreceptors was responsible for the increased cocaine potency. Alterations in cocaine potency were attributable to alterations in uptake inhibition, rather than cocaine effects on vesicular DA release, suggesting that these results may be a product of a functional D3/DAT interaction apart from the canonical inhibitory actions of D3 autoreceptors on DA release. In addition, application of D2 (sumanirole) and D3 (PD 128907) autoreceptor-specific agonists had inverse effects, whereby D2 autoreceptor activation decreased cocaine potency and D3 autoreceptor activation had no effect. Together, these data show that DA autoreceptors dynamically regulate cocaine potency at the DAT, which is important for understanding cocaine's rewarding and addictive properties.

Keywords: addiction, D2 autoreceptor, mouse, nucleus accumbens, sex differences, voltammetry.

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Cocaine is a commonly abused psychostimulant that produces its reinforcing effects primarily by enhancing dopamine (DA) neurotransmission in the brain, specifically in regions such as the nucleus accumbens (NAc) (Wu *et al.* 2001b). The NAc is critically involved in guiding the selection and execution of motivated behaviors based on previous experience, in which DA plays a key role (Day and Carelli 2007; Humphries and Prescott 2010). Furthermore, DA within the NAc plays a major role in mediating the reinforcing effects of addictive drugs (Di Chiara and Imperato 1988; Koob 1992). Cocaine increases extracellular DA levels by binding to and blocking the function of the dopamine transporter (DAT) in pre-synaptic terminals of DA neurons, thereby inhibiting the reuptake of synaptic DA (Kahlig and Galli 2003; Mortensen and Amara 2003) and leading to a prolonged intensity and duration of DA signaling in the brain.

DAT function as well as DA release and synthesis are modulated by feedback-inhibitory autoreceptors. In addition to serving as classical post-synaptic receptors, D2 and D3 receptors located on DA nerve terminals function as pre-synaptic autoreceptors. As autoreceptors, they regulate extracellular DA levels through the modulation of DA firing rate, DA synthesis, DA uptake rate, and DA release as part of a negative feedback loop (Diaz *et al.* 2000; Chen *et al.* 2009; Beaulieu and Gainetdinov 2011; Rice *et al.* 2011; Ford 2014). Mice deficient in D2 autoreceptors show increased DA release and synthesis (Bello *et al.* 2011). In addition, mice with the selective loss of D2 autoreceptors exhibit increased place preference for cocaine (Bello *et al.* 2011) as well as heightened acquisition of cocaine self-administration

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Abbreviations used: DA, dopamine; DAT, dopamine transporter; ERK1/2, extracellular signal-regulated kinases 1/2; FSCV, fast scan cyclic voltammetry; NAc, nucleus accumbens; PI3K, phosphoinositide 3-kinase.

and intensified reactivity to cocaine-paired cues (Holroyd *et al.* 2015), providing strong evidence implicating D2 autoreceptors in the modulation of cocaine effects. Furthermore, a physical interaction between the N-terminus of the DAT and the third intracellular loop of D2 autoreceptors has been reported, which influences DAT surface localization and activity (Bolan *et al.* 2007; Lee *et al.* 2007; Chen *et al.* 2013). However, the role of D3 autoreceptors in cocaine actions is less well understood.

Previous studies suggest that D3 autoreceptors also regulate DA neurotransmission and interact with the DAT, suggesting that they may play a role in modulation of cocaine potency. For example, DA D3 agonists inhibit DA release in the NAc and caudate-putamen (Maina and Mathews 2010), and a physical interaction between D3 autoreceptors and DATs has been demonstrated following prolonged treatment with a D3 agonist, which results in reduced DA uptake in the striatum (Castro-Hernández *et al.* 2015). Our laboratory and others have previously shown that chronic cocaine exposure reduces DA terminal autoreceptor function (Yi and Johnson 1990; Gifford and Johnson 1992; Jones *et al.* 1996; Mateo *et al.* 2005; Edwards *et al.* 2007; Park *et al.* 2013), but whether autoreceptors in turn may modulate cocaine effects at the DAT remains unknown.

As is the case with several other drugs of abuse, there is evidence suggesting that females may be more vulnerable to some aspects of psychostimulant abuse than males (Becker and Hu 2008). For example, female rats form conditioned place preference for cocaine after a fewer number of pairings and at a lower dose, and acquire cocaine self-administration more rapidly and self-administer greater amounts of cocaine than male rats (Hu *et al.* 2004). For these reasons, it is important to study both sexes. In this study, we used fast-scan cyclic voltammetry (FSCV) in brain slices containing the NAc core to assess the effects of autoreceptor blockade and activation on the ability of cocaine to inhibit the DAT in male and female mice. Given the contribution of D2/D3 autoreceptors to DAT function and cocaine's addictive properties, it is important to understand these functional relationships.

Methods and materials

Animals

Adult male and female C57BL/6J mice aged 9–12 weeks (Jackson Laboratories, Bar Harbor, ME, USA) were maintained on a 12 : 12 h light/dark cycle (6:00 am lights on; 6:00 pm lights off) with food and water *ad libitum*. Female mice were not examined for their estrous cycle phase when voltammetry experiments were conducted in this study. However, previous *in vivo* and *in vitro* analyses of DA release, maximal velocity of DA uptake, and the affinity of the DAT for DA reveal that none of these parameters vary across the female estrous cycle (Walker *et al.* 2000). All animals were maintained in accordance with the National Institutes of Health guidelines in Association for Assessment and Accreditation of Laboratory Animal Care accredited facilities. The experimental protocol was approved by the Institutional Animal Care and Use Committee at Wake Forest School of Medicine.

In vitro voltammetry

FSCV was used to characterize pre-synaptic DA signaling and cocaine potency in the NAc core. A vibrating tissue slicer was used to prepare 400-μm-thick coronal brain sections, as previously described (Siciliano *et al.* 2014). The tissue was immersed in oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl (126), KCl (2.5), NaH_2PO_4 (1.2), $CaCl_2$ (2.4), $MgCl_2$ (1.2), $NaHCO_3$ (25), glucose (11), L-ascorbic acid (0.4) at a pH of 7.4. Once sliced, the tissue was transferred to testing chambers containing bath aCSF (32°C), which flowed at 2 mL/min. A carbon fiber microelectrode (50–150 μM length, 7 μM radius) and bipolar stimulating electrode were placed in close proximity in the NAc core. Extracellular DA was recorded by measuring changes in current at the oxidation and reduction potentials for DA (0.6 and −0.2 V, respectively). A triangular voltage waveform (−0.4 to +1.2 to −0.4 V vs. Ag/AgCl, 400 V/s) was applied to the recording electrode every 100 ms. DA release was evoked by a single electrical pulse (350 μA, 4 ms, monophasic) applied to the tissue every 3 min until a stable baseline was established (three collections within 10% variability in peak height).

To explore the possibility of an interaction between D2/D3 autoreceptors and cocaine-induced inhibition of the DAT, raclopride (100 nM), a D2/D3 receptor antagonist, L-741,626 (30 nM), a D2 receptor-selective antagonist, SB-277011- A (300 nM), a D3 receptor-selective antagonist, sumanirole (300 nM), a D2 receptor-selective agonist, or (+)-PD 128907 (10 nM), a D3 receptor-selective agonist, were bath applied to separate slices after pre-drug baseline measures of peak DA concentration of the electrically evoked signal were stable. Once stability was reestablished following agonist/antagonist application, increasing cocaine concentrations (0.3–30 μM) were cumulatively bath applied to each slice. Stimulations were repeated until evoked DA levels reached stability at each concentration (approximately 30 min). Control slices were stimulated for the same amount of time using the same cocaine concentrations, except no agonist/antagonist was applied.

Data analysis

For analysis of FSCV data, Demon Voltammetry and Analysis software was used (Yorgason *et al.* 2011). Recording electrodes were calibrated by recording responses (in electrical current; nA) to a known concentration of DA (3 μM) using a flow-injection system. Calibrations were used to convert electrical current to peak DA concentration. Michaelis–Menten modeling parameters were used to determine the maximal rate of DA uptake and cocaine-induced uptake inhibition (Wightman *et al.* 1988). Michaelis–Menten modeling provides parameters that describe the amount of DA released following electrical stimulation, the maximal rate of DA uptake (V_{max}), and alterations in the ability of DA to bind to the DAT, or apparent K_m. For pre-drug modeling, we followed standard voltammetric modeling procedures by setting the baseline K_m parameter to 160 nM based on the affinity of DA for the DAT (Wightman *et al.* 1988; Wu *et al.* 2001a), whereas V_{max} values were allowed to vary as the pre-drug measure of the rate of DA uptake. Following drug application, apparent K_m was allowed to vary to account for changes in drug-induced DA uptake inhibition while the respective V_{max} value determined for that subject at baseline was

held constant. The apparent K_m parameter models the amount of DA uptake inhibition following a particular concentration of drug (Ferris *et al.* 2013).

Statistics

Graph Pad Prism (version 5; La Jolla, CA, USA) was used to statistically analyze datasets and create graphs. DA kinetics were compared with a two-tailed paired-samples *t*-test to test for differences between baseline (pre-drug) and antagonist/agonist application. Uptake inhibition and release data were subject to a mixed-model repeated measure two-way ANOVA with cocaine concentration (within) and drug treatment (between) as the factors. When main effects were obtained, differences between groups were tested using Bonferroni's *post hoc* test. Following the raclopride experiments, all subsequent data comparisons were *a priori*. All p values of < 0.05 were considered to be statistically significant.

Results

The ability of cocaine to inhibit DA uptake was increased with autoreceptor blockade

To determine whether D2/D3 autoreceptors interact with cocaine inhibition of the DAT, we used the D2/D3-receptor antagonist, raclopride (100 nM), to block D2 and D3 autoreceptors in the NAc core. In males, there was no change in DA release (Fig. 1a) or uptake (Fig. 1b) following bath application of raclopride, confirming previous findings that there is no constitutive DA release, or DA tone, present in an acute slice preparation (Phillips *et al.* 2002; Wilson *et al.* 2004). Following bath application of cocaine, we found that cocaine-induced increases in apparent K_m for DA uptake were greater in the presence of raclopride than in control slices which were run under raclopride-free bath conditions (Fig. 1c): repeated measures two-way ANOVA (cocaine concentration × raclopride treatment, $n = 5$), cocaine concentration ($F_{4,32} = 172.3$, $p < 0.0001$), interaction ($F_{4,32} = 4.689$, $p = 0.0043$), Bonferroni's post-test: control versus raclopride (cocaine 30 μM) $p < 0.01$. In addition to enhancing DA transmission through inhibition of the DAT, which blocks DA uptake, cocaine can mobilize reserve pools of DA-containing vesicles, resulting in increased exocytotic/stimulated release of DA (Venton *et al.* 2006). We found that cocaine-induced effects on release (Fig. 1d): repeated measures two-way ANOVA (cocaine concentration × raclopride treatment, $n = 5$), cocaine concentration ($F_{4,32} = 56.99$, $p < 0.0001$), interaction ($F_{4,32} = 4.501$, $p = 0.0053$). However, there was no difference in cocaine-induced changes in DA release between raclopride treatment and control recordings. These data demonstrate that autoreceptors act to regulate cocaine potency at the DAT.

Similarly, in females, there was no change in DA release (Fig. 1e) or V_{max} (Fig. 1f) following raclopride bath application. Following bath application of cocaine, we found that cocaine-induced uptake inhibition was augmented in the presence of raclopride (Fig. 1g): repeated measures two-way

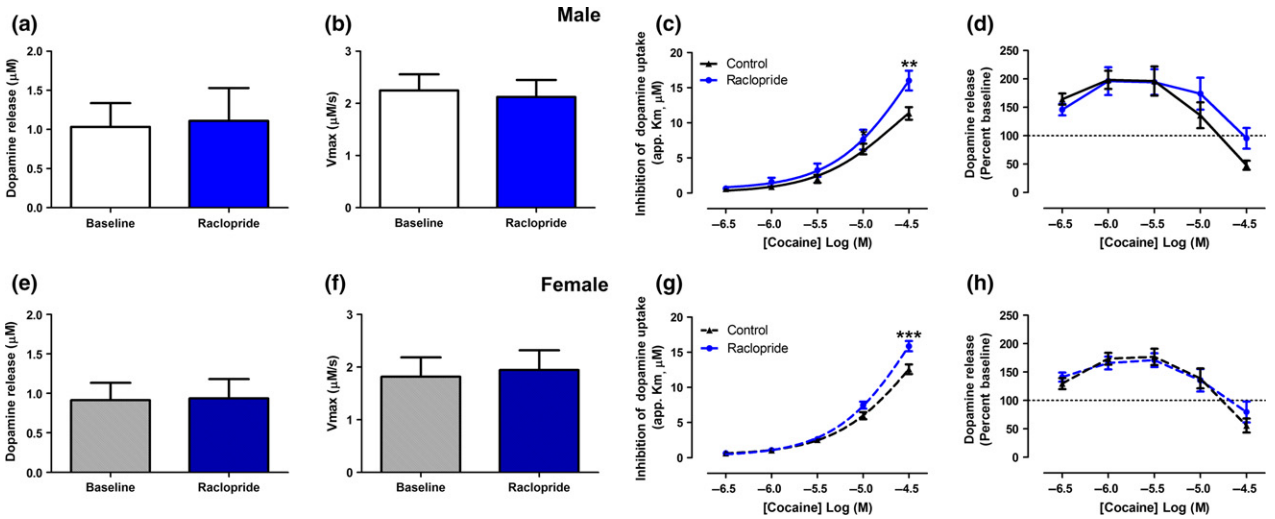

Fig. 1 Global autoreceptor blockade enhances cocaine's ability to inhibit dopamine uptake at the dopamine transporter. Male data (a–d) showing (a) no change in dopamine release or (b) maximal rate of dopamine uptake (V_{max}) between baseline and the D2/D3 antagonist (raclopride, 100 nM), application. (c) Cocaine concentration–response curve (CRC) demonstrating an increase in the inhibition of dopamine uptake at the dopamine transporter (apparent K_m) in the presence of raclopride. (d) Cocaine altered dopamine release over concentrations but there were no differences between control and raclopride groups. The 0 μM concentration (baseline) to which the data is normalized is denoted by a dotted line at 100%. Female data (e–h) showing (e) no change in dopamine release or (f) maximal rate of dopamine uptake between baseline and the D2/D3 antagonist application. (g) Cocaine CRC depicting an increase in the apparent K_m in the presence of raclopride. (h) Dopamine release across cocaine concentrations indicating no change in cocaine-induced dopamine release between groups. $^{**}p < 0.01$; $^{***}p < 0.001$ as compared to control. Male, $n = 5$, female $n = 6$.

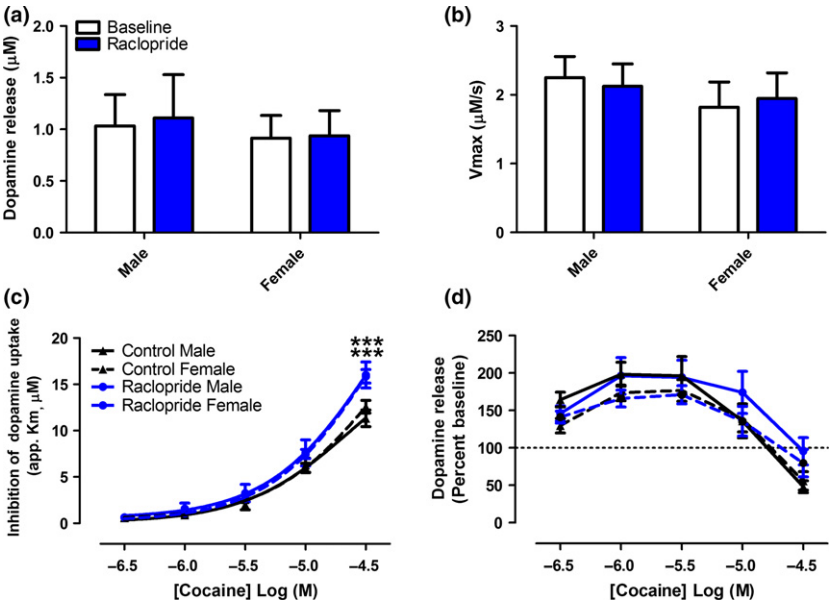

Fig. 2 Sex has no impact on dopamine kinetics and cocaine effects at the dopamine transporter. Data showing no sex differences in (a) dopamine release, (b) maximal rate of dopamine uptake, (c) cocaine-induced inhibition of dopamine uptake (although there is still a difference between raclopride and control of same sex), or (d) cocaine-induced dopamine release, under drug-free bath conditions or in the presence of raclopride. ***$p < 0.001$ versus same sex control group. Male, $n = 5$, female $n = 6$.

ANOVA (cocaine concentration × raclopride treatment, $n = 6$), cocaine concentration ($F_{4,40} = 400.8$, $p < 0.0001$), raclopride treatment ($F_{1,40} = 9.765$, $p = 0.0108$), interaction ($F_{4,40} = 6.465$, $p = 0.0004$), Bonferroni's post-test: control versus raclopride (cocaine 30 μM) $p < 0.001$. Cocaine altered release in both groups but there were no differences in cocaine-induced changes in DA release between raclopride treatment and control recordings (Fig. 1h): repeated measures two-way ANOVA (cocaine concentration × raclopride treatment, $n = 6$), cocaine concentration ($F_{4,40} = 18.80$, $p < 0.0001$). In addition, there were no differences between males and females in any of the parameters measured (Fig. 2a–d). The effects of increased cocaine-induced uptake inhibition in the presence of raclopride remained for raclopride versus control of the same sex (Fig. 2c): repeated measures two-way ANOVA (cocaine concentration × raclopride treatment, $n = 5$–6), cocaine concentration ($F_{4,72} = 518.7$, $p < 0.0001$), interaction ($F_{12,72} = 3.800$, $p = 0.0002$), Bonferroni's post-test: control male versus raclopride male (cocaine 30 μM) $p < 0.001$, control female versus raclopride female (cocaine 30 μM) $p < 0.001$. Similarly, the effect of cocaine on DA release was consistent across group or sex (Fig. 2d): repeated measures two-way ANOVA (cocaine concentration × raclopride treatment, $n = 5$–6), cocaine concentration ($F_{4,72} = 66.72$, $p < 0.0001$). Since there were no sex differences in any of the parameters measured, we chose to complete the remainder of the study in male subjects.

D3, but not D2, autoreceptor inhibition increases the ability of cocaine to inhibit DA uptake

Using the D2-receptor selective antagonist, L-741,626 (30 nM), and the D3-receptor selective antagonist, SB-277011-A (300 nM), we sought to determine whether the increase in cocaine potency that was observed in the presence of raclopride was mediated through DAT functional interactions with the D2 or D3 autoreceptor. In line with what we found upon raclopride application, neither L-741,626 nor SB-277011-A bath application resulted in a change in DA release (Fig. 3a and c) or V_{max} (Fig. 3b and d) between baseline and antagonist application. However, we found that cocaine-induced uptake inhibition was amplified in the presence of the D3-receptor selective antagonist, SB-277011-A (Fig. 3e): repeated measures two-way ANOVA (cocaine concentration × antagonist treatment, $n = 6$), cocaine concentration ($F_{4,56} = 273.1$, $p < 0.0001$), Bonferroni's post-test: control versus SB-277011-A (cocaine 30 μM) $p < 0.05$. Interestingly, there was no difference in cocaine effects in the presence of the D2-receptor selective antagonist, L-741,626, as compared to controls. This demonstrates that autoreceptor modulation of cocaine potency was mediated through DA D3 receptors. Consistent with the raclopride experiment, there were no differences in the observed cocaine-induced increases in DA release between D2 or D3 antagonist treatment and control (Fig. 3f): repeated measures two-way ANOVA (cocaine concentration × antagonist treatment, $n = 5$–6), cocaine concentration ($F_{4,56} = 64.26$, $p < 0.0001$).

D2, but not D3, autoreceptor activation decreases the ability of cocaine to inhibit DA uptake

Having demonstrated that D3, but not D2, autoreceptor blockade augmented cocaine potency at the DAT, we hypothesized that activation of D3, but not D2 autoreceptors, would have the opposite effect of the antagonist and result in a decrease in the ability of cocaine to inhibit DA uptake. To test this hypothesis, we used the D2-receptor selective

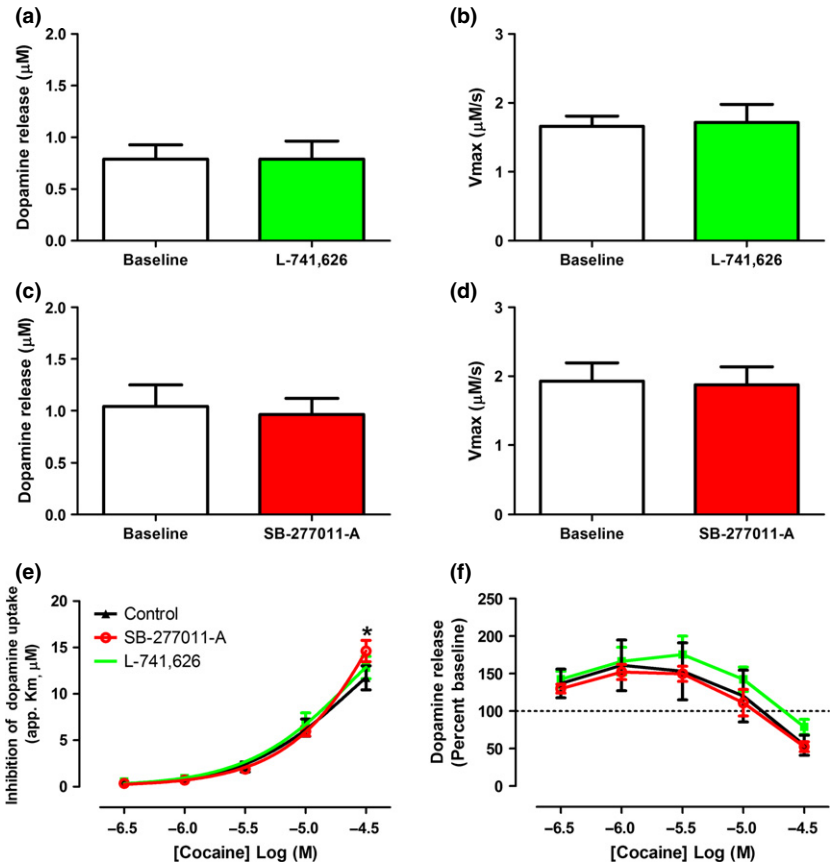

Fig. 3 D3, but not D2, autoreceptor blockade increases the ability of cocaine to inhibit dopamine uptake. (a) No effect of the D2 selective antagonist L-741,626 (30 nM) on dopamine release or (b) maximal rate of dopamine uptake as compared to baseline. (c) No effect of the D3 selective antagonist SB-277011-A (300 nM) on dopamine release or (d) maximal rate of dopamine uptake. (e) D3 autoreceptor antagonist augments cocaine-induced increases in K_m but the D2 antagonist has no effect. (f) No effect of D2 or D3 autoreceptor blockade on cocaine-induced dopamine release. *$p < 0.05$ as compared to control. Male, $n = 6$.

agonist, sumanirole (300 nM), and the D3-receptor selective agonist, (+)-PD 128907 (10 nM). Drug concentrations were selected based on pilot data demonstrating that the IC_{50} for DA release was 300 and 10 nM for sumanirole and (+)-PD 128907, respectively. As expected, following bath application of 300 nM sumanirole, we saw a 53% decrease in DA release as compared to baseline (Fig. 4a): paired-samples *t*-test, $t\ (5) = 5.862$, $p = 0.002$. Similarly, following bath application of (+)-PD 128907, we saw a 41% decrease in DA release as compared to baseline (Fig. 4c): paired-samples *t*-test, $t\ (4) = 5.259$, $p = 0.0063$. There was no change in V_{max} with either agonist (Fig. 4b and d).

Contrary to our hypothesis, we found that following bath application of cocaine, there was no change in the inhibition of DA uptake in the presence of (+)-PD 128907, and a decrease in uptake inhibition in the presence of sumanirole (Fig. 4e): repeated measures two-way ANOVA (cocaine concentration × agonist treatment, $n = 5–6$), cocaine concentration ($F_{4,52} = 293.1$, $p < 0.0001$), agonist treatment ($F_{2,52} = 4.146$, $p = 0.0405$), interaction ($F_{8,52} = 2.891$, $p = 0.0096$), Bonferroni's post-test: control versus sumanirole (30 μM) $p < 0.01$, (+)-PD 128907 versus sumanirole (30 μM) $p < 0.001$. In addition, cocaine altered DA release but there was no difference in cocaine-induced increases in DA release between D2 or D3 agonist treatment and control recordings (Fig. 4f): repeated measures two-way ANOVA (cocaine concentration × agonist treatment, $n = 5–6$), cocaine concentration ($F_{4,52} = 41.64$, $p < 0.0001$), interaction ($F_{8,32} = 2.230$, $p = 0.0398$). Taken together, these data suggest that there is modulation of cocaine potency at the DAT by both D3 and D2 autoreceptors, such that when D3 autoreceptors are blocked, there is an increase in cocaine-induced inhibition of DA uptake and when D2 autoreceptors are activated, there is a decrease in the inhibition of DA uptake.

Discussion

In this study, we show that in the presence of the D2/D3 autoreceptor antagonist, raclopride, the ability of cocaine to inhibit the DAT was increased in the NAc core of both male and female mice. We did not observe any changes in DA release or the rate of DA reuptake (V_{max}) between baseline and raclopride application, and these effects were consistent across sex. Furthermore, we found that raclopride-induced augmentation of cocaine potency was mediated by blockade of D3 type autoreceptors. Finally, we show that D2 and D3 type autoreceptors dynamically modulate cocaine potency, with D2 activation and D3 blockade producing attenuated and augmented cocaine potency, respectively. These data are consistent with previous results showing a synergistic increase in extracellular DA concentration when cocaine is

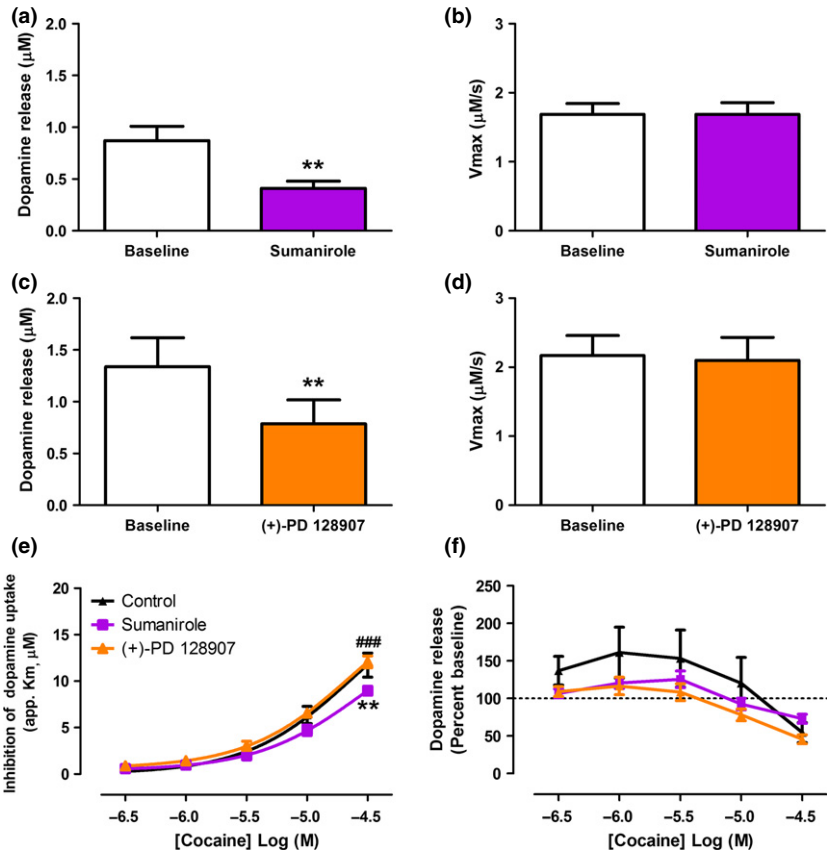

Fig. 4 D2, but not D3, autoreceptor activation decreases the ability of cocaine to inhibit dopamine uptake. (a) ~ 50% decrease in dopamine release following application of the D2 receptor agonist sumanirole (300 nM) as compared to baseline. (b) No effect of sumanirole on maximal rate of dopamine uptake. (c) ~ 40% decrease in dopamine release following application of the D3 receptor agonist (+)-PD 128907 (10 nM). (d) No effect of (+)-PD 128907 on maximal rate of dopamine uptake. (e) Cocaine's ability to inhibit dopamine uptake at the dopamine transporter is decreased in the presence of sumanirole, with no effect in the presence of (+)-PD 128907. (f) No change in cocaine effects on dopamine release with sumanirole or (+)-PD 128907. $^{**}p < 0.01$ as compared to control; $^{###}p < 0.001$ sumanirole versus (+)-PD 128907. Male, $n = 6$.

administered following non-selective autoreceptor blockade with raclopride (Aragona *et al.* 2008). These findings suggest that D2/D3 autoreceptors function to negatively modulate cocaine potency; however, if autoreceptors are 'offline' then cocaine can maximally exert its effects.

When D3 autoreceptors were blocked using the selective antagonist, SB-277011-A, the ability of cocaine to inhibit DA reuptake at the DAT was increased. However, it does not appear that this effect was occurring through antagonist-inhibition of canonical autoreceptor actions on DA release. This is evidenced by our data indicating there was no change in DA release upon antagonist application, which coincides with other studies that note that there is minimal DA tone, and thus no tonic agonist activation of autoreceptors, in an acute slice preparation (Phillips *et al.* 2002; Wilson *et al.* 2004). In addition, we found no change in cocaine-induced increases in DA release in the presence of raclopride or SB-277011-A, again indicating that the D3 receptor-mediated effect on cocaine potency was not through loss of feedback inhibition on DA release. Thus, it is more likely through an allosteric mechanism which may involve direct interactions between D3 autoreceptors and DAT. This idea is supported by a recent study which reported a physical interaction between D3 receptors and DAT, as assessed by co-immunoprecipitation and *in situ* proximity ligation assay (Castro-Hernández *et al.* 2015). It is therefore possible that a D3 receptor antagonist may disrupt this interaction to produce effects on cocaine potency. Similar physical interactions have been seen between D2 receptors and DAT (Bolan *et al.* 2007; Lee *et al.* 2007; Sullivan *et al.* 2013), as well as between D3 and D2 receptors (Maggio and Millan 2010), suggesting that a disruption of the physical interaction between D3 autoreceptors and DATs is a potential explanation for our findings.

In addition to physical interactions, it is also possible that intracellular mechanical and/or signaling mechanisms are involved in the functional D3-DAT interactions. Members of the D2-like DA receptor family, including D2 and D3 receptors, couple to the inhibitory Gαi/o family of heterotrimeric G-proteins for signal transduction. This family is commonly characterized by inhibition of adenylyl cyclase signaling and inactivation by pertussis toxin treatment. Specifically, the D2 receptor can couple to both Gαo and Gαi (Lledo *et al.* 1992; Gazi *et al.* 2003). Interestingly, in addition to Gαo and Gαi subtypes, the D3 receptor can also couple to Gαz and Gαq, which activate phospholipase C (Sidhu and Niznik 2000; Lane *et al.* 2008). Previous studies indicate that various agonists may induce selectivity for one G-protein subtype over another (Cordeaux *et al.* 2001) thereby leading to differential downstream effects, which could differentially affect the DAT. In addition, both D2 and D3 receptors are involved in the regulation of protein kinase

B, also known as Akt, signaling; however, DA inhibition of Akt is dependent on D2 receptor activation but only modulated by D3 receptors (Beaulieu *et al.* 2007), providing an example of differential regulation produced by these receptors. Previous studies have also found that D2 and D3 autoreceptors regulate DAT function through different mechanisms such that both phosphoinositide 3-kinase and extracellular signal-regulated kinases inhibitors abolish the effect of D3 receptor activation on DAT function (Zapata *et al.* 2007), whereas there is a role of extracellular signal-regulated kinases 1/2, but not phosphoinositide 3-kinase, in meditating D2 receptor regulation of DAT (Bolan *et al.* 2007). Taken together, these data offer another explanation for our results such that differential coupling of D2 versus D3 autoreceptors with downstream intracellular signaling pathways could directly or indirectly affect DAT activity.

Furthermore, DA activates different DA receptors with various affinities (Beaulieu and Gainetdinov 2011). Specifically, DA itself has more than 20 times higher affinity at the D3 than at the D2 receptor (Sokoloff *et al.* 1990). This is another possible mechanism that could account for the differential effects of D2 versus D3 autoreceptors on cocaine-regulation of DAT activity. In particular, the D3 antagonist may be eliciting an effect because the D3 receptors have such a high affinity for DA that even though there is very low DA tone in a slice, these low concentrations may still be sufficient to activate D3, but not the D2 autoreceptors. On the other hand, activation of D2 receptors would require much higher levels of DA in the slice, which may explain why we see no effect of the D2 antagonist.

Initially our findings may appear contradictory to those that report deleting or blocking D2 autoreceptors alters cocaine actions (Bello *et al.* 2011; Holroyd *et al.* 2015). However, these discrepancies are likely because of the differences in animal/tissue preparation, and lack of agonist activity at autoreceptors in an acute slice preparation, as discussed above. In both of the studies cited above, the authors used AutoDrd2KO mice, which are generated by crossing *Drd2loxP/loxP* mice with *Dat+/IRES-cre* mice. The resulting mice have a selective loss of D2 autoreceptors only in neurons that also express the DAT in the midbrain. Bello *et al.* (2011) reported that AutoDrd2KO mice displayed elevated DA synthesis and release, hyperlocomotion and supersensitivity to the psychomotor effects of cocaine. These effects are likely because of the reduced D2 autoreceptor inhibition, as in wild-type animals D2 receptors provide strong inhibition of DA cell firing and DA release during times of elevated DA levels, such as following cocaine administration. In addition, Holroyd *et al.* (2015) found that the selective loss of D2 autoreceptors impairs the feedback inhibition of DA release, which allows cocaine to induce an amplified effect on DA transmission in the NAc. In both of these studies, deletion of D2 receptors alters cocaine effects primarily *in vivo*, where there is constant DA activity at the receptor. We show that an agonist at D2 receptors decreases cocaine potency; therefore an antagonist in the presence of an agonist would increase cocaine potency. However, we saw no change in potency with the antagonist because there was no DA tone in our slice preparation. Although the studies above report some alterations *in vitro*, for example, amplification of the effect of cocaine on DA transmission, these effects may be a consequence of compensatory changes in DA neurons because of the long-term nature of genetic deletion. For example, there is elevated DA synthesis and release in the D2 KO mice as compared to their controls, which is likely responsible for the increased cocaine effect *in vitro*. Importantly, to our knowledge this is the first study to use D2 and D3 selective drugs to examine the role of autoreceptors on cocaine's effects, as previous studies have used non-selective D2/D3 antagonists (Aragona *et al.* 2008; Bello *et al.* 2011; Holroyd *et al.* 2015).

Together, we propose a model whereby pre-synaptic DA autoreceptors dynamically modulate cocaine potency through two separate mechanisms. D2 receptors, as noted by Bello *et al.* (2011), Holroyd *et al.* (2015), and in this study, bi-directionally modulate cocaine potency with receptor activation decreasing and receptor inhibition (in the presence of an agonist) augmenting cocaine potency, respectively. On the other hand, D3 receptors most likely modulate cocaine potency through an allosteric mechanism which can be disrupted by an antagonist or through differential coupling with downstream signaling pathways that have differential effects on cocaine regulation of DAT activity. This is the first investigation to show that D3 autoreceptors play a role in cocaine potency. Future studies are needed to examine the exact mechanism in which D3 is modulating DAT to determine if it is a physical interaction or if it is operating through a separate mechanism such as intracellular signaling.

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

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