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Enhance Dopamine Autoreceptor Functioning During Blockade of Dopamine Transporters

Rachel Laura Pace

ENHANCED DOPAMINE AUTORECEPTOR FUNCTIONING DURING BLOCKADE OF DOPAMINE TRANSPORTERS

by

Rachel Laura Pace

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Abstract

Dopamine autoreceptors (DARs) and dopamine transporters (DATs) influence dopamine transmission in the brain's mesolimbic pathway. Prior studies have focused on how activation of DARs influences trafficking of DATs, but how DATs influence DARs remains unclear. Male and female C57BL/6J mice received daily injections of either cocaine (DAT blocker) or saline for seven days then underwent stereotaxic surgery to obtain fixed potential amperometric recordings of DAR-mediated dopamine release. All mice received a mid-surgery injection of cocaine, and DAR-mediated dopamine release was assessed once more during maximal DAT blockade. The current study found that DAR functionality was increased during peak cocaine effects, suggesting DAR work harder to maintain homeostasis. Additionally, DAT function was increased following chronic cocaine exposure, but DAR functioning was unchanged, indicating DAR may be resistant to drug-induced alterations. Understanding the neurochemical mechanisms that control dopamine signaling is critical for informing treatment efforts for addiction, ADHD, and depression.

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The Mesolimbic Dopamine System

The mesolimbic dopamine system—dubbed the "reward" pathway—is a collection of dopamine neurons with somas contained in the ventral tegmental area (VTA) and axons extending to and terminating in the nucleus accumbens (NAc). Although the term "reward" has been habitually linked to dopamine both scientifically and colloquially, dopamine may be better characterized as a neural representation of incentive salience, or the level of motivation for a given reward (see Berridge & Robinson, 1998; Chow et al., 2016). Mesolimbic dopamine has accordingly been shown to be involved in instrumental behavior, anticipation of reinforcement, behavioral activation, and effort-related processes (Salamone et al., 2006; Salamone & Correa, 2012). Given that substance use disorder is characterized by compulsive and impulsive drug-seeking and consumption (Koob & Volkow, 2010), it is logical that the mesolimbic pathway is involved in the formation and maintenance of these addiction-related behaviors, primarily via neurochemical substrates impact on reward-motivated actions brought on by attentionally salient environmental cues. Drugs of abuse, especially psychostimulants, result in increased levels of extracellular dopamine in this pathway (Nestler, 2005; Luscher & Malenka, 2011), which is thought to be responsible for the drug's reinforcing effects.

Moreover, the mesolimbic dopamine system is implicated in the attentional, motivational, and energy-related dysfunctions seen in attention-deficit/hyperactivity disorder (ADHD; Volkow et al., 2007; Dalley et al., 2007; Volkow et al., 2009; Volkow et al., 2011) and depression (Nestler & Carlezon, 2006; Salamone et al., 2006; Dunlop & Nemeroff, 2007; Treadway & Zald,

2011). For example, in individuals with ADHD, inattentiveness appears to stem from disrupted dopamine neurotransmission (Volkow et al., 2007), and reward-motivation deficits seem related to decreased availability of D_2/D_3 receptors and dopamine transporters in the NAc and midbrain regions, including the VTA (Volkow et al., 2009; Volkow et al., 2011). Consistently, selective inhibition or activation of VTA dopamine neurons correspondingly induces or relieves multiple distinct depression-like behaviors, such as anhedonia and decreased motivation (Tye et al., 2013). Given that low levels of D_2/D_3 receptors in the NAc is not only associated with reward-motivation deficits in ADHD but is also correlated with greater risk for substance abuse (Volkow et al., 2009; Dalley et al., 2007) and that dysregulation of the VTA is implicated in both depression and addiction (Nestler & Carlezon, 2006; Koob & Volkow, 2010), mesolimbic dopamine dysfunctionality is a feasible explanation for the increased vulnerability for substance use problems seen in those with ADHD and depression (Elkins, McGue & Iacono, 2007; Polter & Kauer, 2014). Thus, increased understanding of the neural mechanisms that regulate both tonic and phasic dopamine release in the mesolimbic pathway is critical for informing prevention and treatment efforts for ADHD, depression, and substance use disorder, in addition to their comorbid presentation.

Presynaptic Control of Extracellular Dopamine Concentrations

Dopamine transporters (DATs): DATs and dopamine autoreceptors are neural mechanisms that control dopamine signaling and synaptic homeostasis. DAT are specialized surface proteins that operate at the presynaptic membrane of dopamine-synthesizing neurons and are primarily responsible for rapidly clearing extracellular dopamine from the synapse via uptake following exocytotic events (Giros, et al., 1996; Schmitz et al., 2003; Ford et al., 2010). DAT is

the primary determinant of terminating dopamine diffusion to remote receptor cites, as well as the duration of extracellular dopamine, as evidenced by the 300-fold increase in extracellular dopamine levels for mice lacking DAT (Giros et al., 1996; Jones et al., 1998). The findings that DAT knockdown mice show an enhanced tendency to work for a food reward (Cagniard et al., 2006) and that greater DAT surface expression appears to facilitate attribution of incentive salience to discrete reward cues (Singer et al., 2016) are both consistent with the aforementioned role of mesolimbic dopamine in instrumental behavior and motivational processes. Additionally, low availability DAT in the NAc seems to contribute to ADHD's reward-motivation dysfunction (Volkow et al., 2009), and DAT knockdown in the NAc reduces depression-related behaviors (Bahi & Dreyer, 2019). Expectedly, pharmacological treatment options for ADHD and depression, such as stimulants and certain tricyclic antidepressants, block DAT.

DAT are also the primary site of action for the psychostimulant cocaine, which produces its effects by blocking uptake and increasing synaptic concentrations of DA. Consistently, the dopamine system plays an essential role in the pathology of cocaine addiction, and cocaine exposure reciprocally produces alterations in neural mechanism underlying dopamine signaling. For example, cocaine produced elevations in DAT functioning in both human and animal studies and has been shown to increase surface expression of DAT (Mash et al., 2002; Daws et al., 2002). Studies of genetic mutations targeting DAT have shown significant effects on cocaine self-administration and conditioned place preference for cocaine (see Chen et al, 2006; Thomsen et al., 2009). Moreover, cocaine potency at DAT correlates with reinforcing efficacy and corresponds to differences in perseverative responding for sub-threshold doses of cocaine (Siciliano & Jones, 2017), and similarly, a positive correlation has been observed between DAT occupancy levels and the subjective "high" produced by cocaine (Volkow et al., 1997).

Dopamine autoreceptors (DARs): DARs are part of the D2-class of dopamine receptors that are present on the somas, dendrites, and axon terminals of midbrain dopamine neurons in the mesolimbic dopamine system (Beaulieu & Gainetdinov, 2011). DARs are critical to dopamine regulation by delivering inhibitory feedback that controls both neuronal firing and the release, synthesis, and uptake of dopamine (Ford, 2014). Somatodendritic DARs modulate dopamine efflux directly through activation of G-protein gated inwardly rectifying potassium (GIRK) channels, that allow outward potassium conductance that results in hyperpolarization and decreased neuronal excitability (Beckstead et al., 2004; Courtney, Mamaligas & Ford, 2012). Presynaptic DARs decrease vesicular dopamine release (Benoit-Marand, Borrelli & Gonon, 2001; Phillips, Hancock & Stamford, 2002), possibly via modulation of both voltage-dependent potassium channels that induce hyperpolarization (Fulton et al., 2011; Martel et al., 2011) and P/Q- and N-type calcium channels responsible for the calcium entry that triggers dopamine release (Cardozo & Bean, 1995; Phillips & Stamford, 2000). Presynaptic DARs also reduce dopamine synthesis by inhibiting tyrosine hydroxylase (Wolf and Roth, 1990), which alters the distribution and expression of vesicular monoamine transporters (VMAT; Truong et al., 2004) and reduces the filling of presynaptic dopamine vesicles (Pothos et al., 1998).

Given the aforementioned role of dopamine in motivation, it is unsurprising that previous studies have demonstrated that manipulation of D_2 receptors or DARs produces changes in motivation for rewards and drugs of abuse. For example, overexpression of D_2 receptors in the NAc of adult mice increased motivation, as evidenced by higher levels of operant responding as work requirements increased, even in the presence of a less desirable but freely available reinforcer (Trifilieff et al., 2013). Similarly, downregulation of D_2 receptors in the VTA of adult rats appears to increase incentive motivation, as shown by increased responding for both sucrose

and cocaine under a progressive ratio schedule of reinforcement (De Jong et al., 2015). While the findings from these two studies appear to support DARs' role in motivation, the results may not be specific to DARs since all D_2 receptors (auto- and heteroreceptors) were affected. However, a study by Holroyd et al. (2015) found that mice lacking D_2 autoreceptors acquired a cued-operant self-administration task for cocaine faster that littermate controls and had cue reactivity for cocaine—but not sucrose—during extinction. Additionally, mice lacking D_2 autoreceptors displayed not only expected elevations in dopamine synthesis and release, but also enhanced place preference for cocaine and heightened motivation for food reward (Bello et al., 2011). These results help solidify the role of DAR in anticipation of reinforcement, effort-related processes, and motivation, especially related to drugs of abuse.

Interaction of DATs and DARs

DAT and DAR have been shown to interact, but the relationship between these two presynaptic proteins is intricate and still encompasses some ambiguity. As mentioned by Chen et al. (2020), most prior studies have focused on DAR regulation of DAT trafficking and reuptake function. For instance, studies show that DAR impact dopamine transmission by increasing DAT activity, partially via downstream increases in DAT expression on the plasma membrane (Cass and Gerhardt, 1994; Mayfield & Zahnieser, 2001; Wu et al., 2002; Truong et al., 2004), but this may only occur during excessive DAR activation or prolonged stimulation (Benoit-Marand et al., 2011). Additionally, the D_2 agonist sumanirole has been shown to decrease cocaine potency at DAT (McGinnis, Siciliano & Jones, 2016). Still, a few studies have assessed DAT influence on DAR. For example, DAT knockout mice exhibited nearly complete loss of DAR functions (Jones et al., 1999), and two early studies with rats found that repeated cocaine administration—

a inhibitor of DAT—attenuates the ability of DAR stimulation to inhibit dopamine release (Pierce, Duffy, & Kalivas, 1995; Jones et al, 1996) although both studies applied the D2-agonist quinpirole to induce DAR inhibition, which lacks the specificity to distinguish between auto- and heteroreceptors.

Current Study

The purpose of the present study is to expand current comprehension of the relationship between DAT and DAR functioning and their impact the dynamics of dopamine release events in the mesolimbic pathway. This study will evaluate the effect of chronic DAT blockade on DAR functionality. Chronic DAT blockade will be achieved via cocaine administration, and the ability of DAR to inhibit dopamine release will be measured using fixed potential amperometric recordings in the NAc of anesthetized mice. Based on previous studies, we hypothesize that chronic DAT blockade would attenuate the ability of DAR to inhibit synaptic dopamine release and that acute DAT blockade would not alter DAR functionality. Further elucidating the details of the complex interaction between DAT and DAR and their impact on dopamine transmission in the mesolimbic pathway is critical to informing clinical treatment efforts for addiction, ADHD, and depression, as these neural mechanisms are potential targets for therapeutic manipulation of dopamine availability.

Methods

All experiments included in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Memphis and conducted in accordance with

the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Efforts were made to reduce the number of animals used and to minimize pain and discomfort.

Animals

Sixteen male and sixteen female C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed 2-4 per cage in a temperature-controlled environment (21± 1° C) on a 12-hour light/dark cycle (with lights on a 0600) and with food and water available ad libitum. All mice weighed between 15-35 grams at the time of surgery. Mice were assigned to either the experimental condition where they received one injection of cocaine (20mg/kg, i.p.) per day for seven days or the control group where they received one injection of saline (0.9% NaCl) per day for seven days. All mice where given one injection of cocaine (20mg/kg, i.p.) mid-surgery prior to both measurement of stimulation-evoked dopamine efflux and a second assessment of DAR functionality during cocaine's peak effects (20 minutes post i.p. injection). Efforts were made to reduce the number of animals used and to minimize pain and discomfort.

Surgery

All mice were anesthetized with urethane (1.5 g/kg, i.p.), assessed for pain response by foot and tail pinch, and mounted in a stereotaxic frame (David Kopf Instruments, Tujundga, CA) to ensure the skull was flat. Body temperature was maintained at 36± 0.5° C with a temperature-regulated heating pad (TC-1000; CWE, NY). Stereotaxic coordinates are in mm from bregma, midline, and dura according to the mouse atlas of Paxinos and Franklin (2001). First, three 1 mm diameter trephine holes were drilled in the skull for electrode placements. Then a concentric bipolar stimulating electrode was inserted in to the left VTA (coordinates: AP -3.3 mm from bregma, ML +0.3 mm from midline, and DV 4.0 mm from dura); a stainless-steel auxiliary and

Ag/AgCl reference electrode combination was placed on the surface of contralateral cortical tissue -2.0 mm from bregma; and a carbon fiber recording electrode (active recording surface of 250 μm length by 7 μm o.d.) was positioned in the left NAc (coordinates: AP +1.5 mm from bregma, ML +1.0 mm from midline, and DV −4.0 mm from dura). Finally, a fixed current of +0.8 V was applied to the recording electrode with dopamine oxidation currents being continuously monitored (10 K samples/sec) by an electrometer (ED401 e-corder 401 and EA162 Picostat, eDAQ Inc., Colorado Springs, CO) filtered at 50 Hz. All amperometric recordings were made within a Faraday cage to increase the signal to noise ratio.

Electrical Stimulation and Drug Administration

Fixed potential amperometry (FPA) paired with carbon fiber recording microelectrodes has been confirmed as a valid technique for monitoring real-time, stimulation-evoked dopamine release in the NAc (Forster & Blaha, 2003; Lester et al., 2010) and boasts a high temporal resolution (10 K samples/sec) for assessing this dopamine release in subcortical brain regions (Holloway et al., 2019; Estes et al., 2019). Following surgical setup, DAR sensitivity was assessed by applying a pair of test stimuli (T1 and T2, each 10 pulses at 50 Hz with 10 s between T1 and T2) to the VTA every 30 s to evoke dopamine efflux (Fielding et al., 2013; Mittleman et al., 2011). Six sets of prepulses (1, 5, 10, 20, 40, and 80; 0.5-ms pulse duration at 15 Hz) were delivered prior to T2 such that there was 0.3 s between the end of the prepulse train and initiation of T2, as autoinhibition of dopamine release is maximal between 150 and 300 msec after the end of prepulse stimulation (Benoit-Marand, Borrelli & Gonon, 2001). DAR mediated inhibition of evoked dopamine efflux is expressed in percent inhibition, i.e. the change in the amplitude of T2 with respect to T1 (T2/T1*100) for each set of prepulses. Thus, low-to-high DAR sensitivity is represented as a low-to-high percent inhibition of evoked dopamine efflux (i.e., high sensitivity

resulted in a lower amplitude of T2 relative to T1). The DAR sensitivity test took approximately 20 min to complete.

To assess pre-drug dopamine efflux, a series of cathodal monophasic pulses (20 pulses at 50 Hz) were applied every 30 s. After a 5 min baseline recording of VTA stimulation evoked dopamine release and half-life, mice received an injection of cocaine (20 mg/kg, i.p.), and recordings continued for 20 min. At 20 min post cocaine injection, the DAR sensitivity test was repeated to assess DAR functionality during cocaine's peak effects (Lester et al., 2010; Holloway et al., 2019). Then assessment of post-drug dopamine efflux (as outlined previously) continued for approximately 40 minutes. Phasic dopamine release was quantified as the magnitude of the stimulation-evoked response, and dopamine uptake, an indication of DAT functioning, was measured by dopamine half-life decay (the time for 50% decrease from the maximum evoked increase to the prestimulus baseline level). Finally, a 3-minute continuous stimulation was delivered (9,000 pulses at 50 Hz) to quantify the neuronal supply of DA.

At the completion of each amperometric recording session, a direct anodic current (400 Amps for 10 s) was applied to the simulating electrode for placement confirmation. Once complete, recording electrodes were removed from the brain for in vitro calibration using know concentrations of dopamine solutions (0.2–1.2 μM) administered with a flow injection system (Michael & Wightman, 1999; Prater, Swamy, Beane, & Lester, 2018), and change in dopamine oxidation current (μAmp) was converted to dopamine concentration (μM). Measured current changes correspond to dopamine efflux, as pharmacologically confirmed by previous studies using the same electrical stimulation technique (Holloway et al., 2019).

Statistical Analyses

Establishing an index of autoreceptor functioning: To confirm that the prepulse conditions in the autoreceptor test altered dopamine release as in our previous publications (Freels et al., 2020; Holloway et al., 2019), a one-way repeated measures analyses of variance (ANOVA) will be used to examine the effect of the number of prepulses on autoreceptor-mediated dopamine release (T2/T1). If a significant effect is found (as indicated by $p < .05$), pairwise comparisons will be used to determine which prepulse conditions (number of prepulses: 1, 5, 20, 40, 80) decreased autoreceptor-mediated dopamine release from the control condition (0 prepulses). Autoreceptor-mediated dopamine release will then be averaged across the sets of prepulses conditions that differ significantly from the control condition. This process will be done for both autoreceptor tests, resulting in an average autoreceptor-mediated dopamine release value for both before and after the mid-surgery cocaine challenge.

Comparing differences in autoreceptor functioning: A two-way between-subjects ANOVA will be used to assess the effect of sex and drug pretreatment (cocaine/saline) on baseline autoreceptor-mediated dopamine release (the autoreceptor inhibition index calculated from the initial autoreceptor test, prior to the in-test cocaine injection). In order to determine the interactive effects of the in-test cocaine injection, a three-way mixed ANOVA will be used to assess the effect of sex and drug pretreatment (both between-subject factors) on autoreceptor-mediated dopamine release before and after the in-test cocaine challenge (within-subjects factor), and significant findings will be evaluated via pairwise comparisons. We tested for a main effect of DAR function pre- and post-injection (pre-post) and 3 interactions (pre-post x sex, pre-post x drug pretreatment, and pre-post x sex x drug pretreatment).

Comparing differences in dopamine release and synaptic half-life: Dopamine release and half-life following the cocaine injection were expressed as percent change of baseline release and half-life (with baseline representing 100%). To assess the timing of the cocaine-induced changes in dopamine efflux, three-way mixed ANOVAs were used to determine the effects of sex and drug pretreatment (between-subjects factors) on percent change of dopamine release and half-life following cocaine administration over the 1-hour recording period in 10 min intervals (within-subject factor). We tested for a main effect of time post-injection and 3 interactions (time post-injection x sex, time post-injection x drug pretreatment, and time post-injection x sex x drug pretreatment).

To assess differences at the peak effect time of cocaine, two-way between-subjects ANOVAs were used to determine the effect of sex and drug pretreatment on percent change in dopamine release and half-life at 20 min post-injection. We tested for a main effect of sex, a main effect of drug pretreatment, and an interaction between sex and drug pretreatment.

Histology

After amperometric recordings were complete, an iron deposit was made in brain sites by passing direct anodic current (100 μAmps for 10 s) through the stimulating electrode. Mice were then euthanized with a 0.25-ml intracardial injection of urethane (0.345 g/ml). Brains were extracted and immersed in 10% buffered formalin containing 0.1% potassium ferricyanide to cause a redox reaction at the stimulation site resulting in a Prussian blue spot. Brains were then stored in 30% sucrose/10% formalin solution for a least 1 week prior to sectioning. Coronal sections (30 μm) were sliced using a cryostat at -20° C and electrode placement were determined under a light microscope and recorded on representative coronal diagrams (Paxinos & Franklin, 2001).

Chemicals

Urethane and cocaine hydrochloride were obtained from Sigma-Aldrich Chemical (St Louis, MO). Urethane was used at a dose of 1.5 g/kg, i.p. for anesthesia prior to surgeries. Cocaine hydrochloride was used at a dose of 20 mg/kg, i.p. once per day for seven days for the mice assigned to the experimental condition and once mid-surgery for all mice included in the study. Urethane was dissolved in distilled water and cocaine hydrochloride was dissolved in sterile PBS (pH 7.4).

Results

Establishing an Index of Dopamine Autoreceptor Functionality

Autoreceptor-mediated inhibition of evoked dopamine release was expressed in terms of the percentage change between test stimulation (T1 and T2) for each set of prepulses, with greater percent change of T2 relative to T1 indicating more efficient autoreceptor function. A one-way repeated measure ANOVA was conducted to compare autoreceptor-mediated dopamine release across all prepulse conditions for both the initial autoreceptor test and the second mid-drug autoreceptor test. Mauchly's test denoted that the assumption of sphericity had been violated for both autoreceptor tests [initial: $\chi^2(20) = 127.01$, $p < .001$; mid-drug: $\chi^2(20) = 145.70$, $p < .001$], so the Greenhouse–Geisser correction was applied when evaluating main effects. As expected per previous studies (Freels et al., 2020; Holloway et al., 2019), a robust significant main effect for the number of prepulses was found for both the initial and mid-drug autoreceptor test [initial: $F(2.11, 65.39) = 84.41$, $p < .001$, $\eta_p^2 = .73$; mid-drug: $F(2.10, 56.66) = 154.23$, $p < .001$, $\eta_p^2 = .85$] (see Figure 2A and Figure 2B). Post hoc analyses with a Bonferroni adjustment revealed that all prepulse conditions for both autoreceptor tests differed significantly from the

control condition of 0 prepulse stimulations ($p < .001$ for all comparisons, see Table 2). Therefore, the autoreceptor-mediated dopamine efflux for all prepulse conditions were averaged for each test of autoreceptor functionality to create two inhibition indices: one for the initial autoreceptor test (pretest inhibition index) and one for the mid-drug autoreceptor test (posttest inhibition index).

Comparing Differences in Dopamine Autoreceptor Functioning

First, a two-way between-subjects ANOVA was conducted to analyze the impact of pretreatment condition (cocaine/saline) and sex (male/female) on DAR function during the initial autoreceptor test, as represented by the pretest inhibition index. Sex of the mice did not significantly impact DAR-mediated dopamine efflux for the initial autoreceptor test, [$F(1, 28) = 1.20$, $p = .28$]. Similarly, whether the mice received daily cocaine injections or daily saline injections (pretreatment) did not significantly change DAR functionality as shown by the pretest inhibition index, [$F(1, 28) = .232$, $p = .63$] (see Figure 2C). Additionally, the interaction between sex and pretreatment did not significantly influence DAR capability to inhibit dopamine release, [$F(1, 28) = 2.84$, $p = .10$].

A three-way mixed ANOVA was then used to assess the effect of sex and pretreatment on DAR-mediated dopamine release for both the initial and mid-drug autoreceptor tests by comparing the pretest and posttest inhibition indices (pre-post). There was a substantial main effect for alterations in DAR-mediated dopamine efflux following the mid-surgery DAT blockade via cocaine, as indicated by a substantial change in between the inhibition indices from pretest to posttest [$F(1, 24) = 90.41$, $p < .001$, $\eta_p^2 = .79$]. Post hoc analysis with a Bonferroni adjustment revealed that DAR functionality was enhanced during the mid-drug autoreceptor test, as indicated by lower DAR-mediated dopamine release compared to the initial autoreceptor test

[19.00 (95% CI, 15.35 to 23.85), p < .001] (see Figure 2C). However, there were no significant interactions between the pretest/posttest inhibition indices and sex [$F(1, 24) = .50$, $p = .49$] or between the pretest/posttest inhibition indices and pretreatment [$F(1, 24) = .13$, $p = .72$]. Likewise, the interaction between sex, pretreatment, and the pretest/posttest inhibition indices was also nonsignificant [$F(1, 24) = 1.80$, $p = .19$].

Comparing Differences in Dopamine Release and Half-Life

Changes in dopamine release: A three-way mixed ANOVA was used to evaluate the effect of sex and pretreatment (cocaine/saline) on percent change of baseline dopamine release (with baseline representing 100%) following mid-surgery cocaine administration over the duration of the recording period (in 10-minute intervals, within-subjects factor). Mauchly's test showed that the assumption of sphericity had been violated [$\chi^2(20) = 82.22$, $p < .001$], so the Greenhouse–Geisser correction was applied when appraising main effects and interactions. As expected per previous studies' results (Lester et al., 2010; Holloway et al., 2019), there was a significant main effect of time following cocaine administration on percent change in dopamine release [$F(2.76, 57.86) = 48.98$, $p < .001$, $\eta_p^2 = .70$], with pairwise comparisons showing all 10-minute interval timepoints differed significantly from the baseline dopamine release ($p < .001$ for all conditions). Yet, the interactions of time by sex and of time by pretreatment were nonsignificant [time x sex: $F(2.76, 57.86) = .626$, $p = .59$; time x pretreatment: $F(2.76, 57.86) = 1.08$, $p = .36$]. Additionally, the interaction of time, sex, and pretreatment condition did not significantly contribute to percent changes in dopamine release following cocaine administration [time x sex x pretreatment: $F(2.76, 57.86) = .888$, $p = .45$].

Changes in dopamine half-life: Another three-way mixed ANOVA was then used to evaluate the effect of sex and pretreatment (cocaine/saline) on percent change of baseline

dopamine half-life (with baseline representing 100%) following mid-surgery cocaine administration over the duration of the recording period (in 10-minute intervals, within-subjects factor). The Greenhouse–Geisser correction was applied when appraising main effects and interactions because Mauchly's test revealed that the assumption of sphericity had been violated [$\chi^2(20) = 107.04$, $p < .001$]. Similar to outcomes concerning dopamine release, there was a significant main effect of time on changes in dopamine half-life [$F(2.30, 48.27) = 28.23$, $p < .001$, $\eta_p^2 = .57$]. Given that dopamine transporters are the primary determinant of synaptic dopamine half-life (Jones et al., 1998), this finding that half-life was markedly longer following the mid-surgery drug administration (pairwise comparisons indicate $p < .001$ for all conditions) supports that cocaine had the intended effect of dopamine transporter blockade. However, there were no significant interactions between time x sex nor between time x pretreatment [time x sex: $F(2.30, 48.27) = .212$, $p = .84$; time x pretreatment: $F(2.30, 48.27) = 2.13$, $p = .12$]. The interaction of time x sex x pretreatment condition was also nonsignificant [$F(2.30, 48.27) = .141$, $p = .89$].

Peak dopamine release: Next, a two-way between-subjects ANOVA was used to assess differences based on sex and pretreatment on dopamine release during cocaine's peak effects (20 minutes post-injection). Sex did not significantly influence peak dopamine release following cocaine administration [$F(1, 26) = .551$, $p = .47$]. Similarly, pretreatment had a non-significant effect on dopamine release 20 minutes post-injection [$F(1, 26) = .747$, $p = .40$]. Likewise, the interaction of pretreatment by sex was not significant [$F(1, 26) = .375$, $p = .55$].

Peak dopamine half-life: Another two-way between-subjects ANOVA was utilized to evaluate the influence of sex and pretreatment on dopamine half-life during peak cocaine effects at the 20 minutes post-injection timepoint. As it was with dopamine release, the impact of sex on

dopamine half-life during peak cocaine effects was not significant [F (1, 26) = .150, p = .70]. However, pretreatment condition significantly altered dopamine half-life at 20 minutes post-injection [F(1, 26) = 6.89, p = .01, η_p^2 = .21]. Then a post-hoc independent samples t-test was used to determine how pretreatment impacted dopamine synaptic half-life during peak cocaine effects. The cocaine pretreated mice (M = 300.49; SD = 87.54) had significantly lower half-lives compared to the saline pretreated mice (M = 399.16; SD = 108.23) [t (28) = -2.75, p = .01, two-tailed] (see Figure 3C). However, the interaction between sex and pretreatment was not significant [F(1, 26) = .328, p = .57].

Discussion

Mesolimbic dopamine (Berridge & Robinson, 1998; Salamone et al., 2006; Salamone & Correa, 2012; Chow et al., 2016) and its regulatory surface proteins DAT and DAR (Cagniard et al., 2006; Bello et al., 2011; Holroyd et al., 2015; Singer et al., 2016) are centrally involved in motivational states and accompanying behavioral activation. DATs control the duration of extracellular dopamine by modulating clearance from the synapse via uptake, while DARs deliver inhibitory feedback to reduce neuronal excitability and the release, synthesis and uptake of dopamine. These two fundamental influencers of mesolimbic dopamine also underlie symptomologies related to depression (Bahi & Dreyer, 2019), ADHD (Volkow et al., 2009), and problematic substance use (Chen et al, 2006; Thomsen et al., 2009; Bello et al., 2011). There is ample literature on the individual impact of DAT and DAR, and several studies have focused on DAR regulation of DAT trafficking (Cass and Gerhardt, 1994; Mayfield & Zahnieser, 2001; Wu et al., 2002; Truong et al., 2004; Chen et al., 2020). Yet, how changes at DAT impact DAR functionality is unclear. Therefore, this study used fixed-potential amperometry to quantify

DAR-mediated dopamine release both before and during DAT blockade for both mice that had experienced chronic exposure to a DAT-blocker (cocaine pretreatment) and mice receiving their initial exposure to DAT inhibition (saline pretreatment). Results indicate discrete DAR activity in the presence and absence of DAT blockade, as well as expected changes in DAT function following cocaine exposure.

Dopamine Release and Synaptic Half-Life following Cocaine Administration

Cocaine precipitates increases in extracellular dopamine concentrations not only by blocking DAT reuptake capability (Giros et al., 1996; Schmitz et al., 2003; Ford et al., 2010), but also by mobilizing synapsin-dependent vesicles filled with dopamine for exocytosis (Venton et al., 2006). Our findings that dopamine synaptic half-life and release increased significantly in all mice following the mid-surgery cocaine injection is consistent with this prior research. Our findings also indicate that dopaminergic responses to cocaine are altered by previous exposure to cocaine. Specifically, the mice exposed to daily cocaine injections displayed an attenuated dopaminergic response to cocaine, specifically a lower percent change in the synaptic half-life of evoked dopamine, compared to saline pretreated mice. DAT functionality remained more intact following the in-test cocaine injection in the cocaine-exposed mice. Given that the dopamine reuptake rate is determined by the number of functional DAT on the cell surface (Daws et al., 2002), the cocaine-exposed mice likely had more DAT left uninhibited following the in-test cocaine injection. These results fit with studies showing that chronic cocaine exposure leads to an upregulation in DAT expression on the plasma membrane (Fang & Rønnekleiv, 1999; Mash et al., 2002). However, prior cocaine exposure had no effect on cocaine-induced changes in dopamine release (indicated by response amplitudes) in the NAc, which is consistent with the prominent notion that DAT (which controls extracellular dopamine duration)—not exocytosis of

synapsin-dependent vesicles—is the primary mechanism by which cocaine elevates dopamine concentrations (Giros et al., 1996; Jones et al., 1998). Finally, although a previous study indicates cocaine produced greater dopamine efflux in the striatum of females (Walker, Ray & Kuhn, 2006), the similarities found in cocaine-induced changes in dopamine release and half-life between male and female mice regardless of prior drug exposure in our study may be due to the aforementioned differences only being present at higher stimulation frequencies (30 pulses at 50 and 60 Hz), or because those sex differences are mediated by estradiol fluctuations within the female estrous cycle (Calipari et al., 2017), which we did not control for.

DAR Functionality following Cocaine Administration

Benoit-Marand et al. (2001) conducted an in vivo electrochemical study that showed electrically stimulated dopamine release activates DAR, and the subsequent inhibition of further dopamine release is maximal between 150-300 ms after the end of that stimulation. The current study examined DAR functionality in the NAc of anesthetized mice by using sets of prepulses (1, 5, 10, 20, 40, and 80) delivered 300 ms prior to the second test stimulation (T2) that was compared to a matching stimulation in the absence of prepulse stimulation (T1), so that higher percent inhibition of dopamine efflux would results in lower amplitude of T2 in relation to T1, indicating greater DAR function. The control condition was T1 and T2 applied with no prepulses before either stimulation (0 prepulses), resulting in nearly equivalent amplitudes and percent change approximately 100%. All prepulse conditions (1, 5, 10, 20, 40, and 80) produced changes in DAR-mediated dopamine release that were significantly different from that produced by the control conditions (0 prepulses) for both tests of DAR functionality. This finding is consistent with those from prior studies assessing autoreceptor functioning (Freels et al., 2020; Holloway et al., 2019) and helps confirm fixed potential amperometry as a valid technique for measuring

autoinhibition of dopamine, especially given the need for a high temporal resolution to capture this transient event. An index of DAR functionality was then calculated for each autoreceptor test by averaging the DAR-mediated dopamine efflux across prepulse conditions. These indices allowed us to compare changes in DAR function across conditions. We found no sex differences in DAR functionality, which fits with findings from a similar study using pharmaceutical blockade of D2/3 receptors and electrochemical measures of dopamine release (Pitts et al., 2020).

The in-test injection of cocaine did alter DAR functioning. Specifically, DAR-mediated dopamine release was lower following mid-surgery cocaine administration, suggesting DAR functioning is elevated during DAT blockade. Given the paucity of prior research on DAR activity during peak cocaine effects, these are novel findings. However, more research is needed to determine the mechanistic cause. Cocaine-induced increases in DAR functioning could indicate that DAT and DAR directly communicate via the mutual downstream signaling molecule PKCβ (Foster et al., 2002; Namkung & Sibley, 2004), with DAT blockade signaling increases in DAR functioning, or another explanation could be that DAR work harder during DAT blockade because of increased activation via greater extracellular dopamine concentrations. A recent study by Robinson et al. (2017b) indicated that there may be drug-induced plasticity differences in certain splice variants of DAR, with the D2L (long) showing reduced desensitization (i.e., higher functioning) following acute cocaine exposure compared to D2S (short). Thus, it is not implausible that D2L may be contributing to this increase in DAR functionality immediately following cocaine exposure, but studies utilizing splice-specific genetic knockout mice paired with amperometric assessments of DAR function are needed further evaluate this possibility.

Contrary to expectations, DAR functionality was not altered following chronic DAT blockade via cocaine administration when compared to saline-treated controls. This contradicts previous findings that DAR ability to inhibit dopamine release is diminished following chronic cocaine administration (Pierce, Duffy, & Kalivas, 1995; Jones et al, 1996). As stated previously, both of these studies applied the D2-agonist quinpirole to induce DAR inhibition, which lacks the specificity to distinguish between auto- and heteroreceptors, and may account for different findings from our study that utilized an amperometric approach for functional measurements. Furthermore, the results indicating attenuated DAR function following cocaine administration must be considered within the limitations of their methodological approaches: one study used microdialysis which has low temporal and spatial resolution, and the other used fast scan cyclic voltammetry in brain slices which partially limits the generalizability of the results. The study by Jones et al. (1996) also indicated that subcutaneous daily injections of cocaine induce sensitization while a continuous infusion regimen of cocaine lead to tolerance, and these were correspondingly associated with autoreceptor sub- and super-sensitivity. The possibility that route and schedule of administration affects whether tolerance or sensitization occurs has already been studied, with high intake resulting in tolerance and intermittent temporal patterns of intake leading to sensitization (Calipari et al., 2013). Thus, it is possible that our results would have been different if utilizing a different dosing regimen.

Although there is a lack of prior studies on i.p. injection regimens that produce tolerance and it was not directly evaluated here, it is still arguable that the cocaine regimen used in our study produced tolerance given that cocaine increases DAT surface expression (Fang & Rønnekleiv, 1999; Mash et al., 2002; Daws et al., 2002), and that cocaine-induced changes in dopamine half-lives were shorter for the cocaine-exposed group in our study. However, the

question of whether tolerance leads to DAR super-sensitivity (Jones et al., 1996) remains unanswered. Our study shows potential markers of tolerance but no differences in the cocaine and saline pretreatment groups in DAR function, suggesting DAR are resistant to changes following chronic cocaine exposure despite changes in DAT. Similarly, a study by Robinson et al. (2017a) showed that DAR are resistant to agonist-induced internalization. Given that receptor trafficking is related to tolerance and sensitization and our findings that DAR function is unchanged following chronic cocaine exposure, DAR stability may serve a preservative function to alterations in neuroplasticity.

Conclusions

The results of this study helped further elucidate the reciprocal relationship between DAT and DAR functionality, and their impact on phasic dopamine transmission in the brain's mesolimbic circuit. Specifically, this study provided the novel finding that DAR activity is enhanced during peak cocaine effects. Additionally, it was shown that chronic cocaine exposure produces changes in DAT but not DAR activity. Taken together, these findings highlight the potential that DAR may offer compensatory action to changes in neurocircuitry following drug exposure. Still, the complexity of the DAR interactome offers many intricacies that need further evaluation. Future studies should seek to clarify how drug-induced states of tolerance and sensitization influence DAR action in the mesolimbic system, and, more broadly, how DAR or the molecules in its signaling cascade could be targets for pharmacological restoration of disrupted dopamine homeostasis brought on by drugs of abuse.

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Appendix

Table 1. Experimental Groups and Sample Sizes

Strain	*N*	Sex	*N*	Treatment	Drug Challenge	*N*
C57/BL6J	32	Female	16	Cocaine	Cocaine	8
				Saline	Cocaine	8
		Male	16	Cocaine	Cocaine	8
				Saline	Cocaine	8

Table 2. Descriptive Statistics for DAR-Mediated Dopamine Release for Different Prepulse Conditions During Tests of DAR Functionality and Calculated Inhibition Index

Test	Prepulses	*N*	*M*	*SD*
Initial	0	32	97.17	6.80
	1	32	89.93*	9.17
	5	32	77.40*	9.54
	10	32	72.31*	12.17
	20	32	69.71*	17.15
	40	32	59.07*	17.83
	80	32	53.29*	18.55
During DAT Blockade	0	28	91.22	6.00
	1	28	82.73*	10.23
	5	28	57.84*	13.12
	10	28	51.99*	14.02
	20	28	44.64*	17.54
	40	28	38.33*	17.22
	80	28	36.86*	18.01

Means are expressed as percent change in amplitude of the second stimulation (T2) compared to initial stimulation lacking any prepulses (T1) so that DAR-mediated dopamine release is T2/T1*100 * differed significantly from control (0 prepulse), $p < .001$

Table 3. Dopamine Autoreceptor Inhibition Indices for the Cocaine and Saline Pretreatment Conditions Before and After Dopamine Transporter Blockade

	Saline			Cocaine		
Time period	*N*	*M*	*SD*	*N*	*M*	*SD*
Pre-DAT blockade	14	70.56	11.98	14	73.19	12.16
Post-DAT blockade	14	50.40	12.38	14	53.73	14.31

Table 4. Dopamine Autoreceptor Inhibition Indices for Males and Females Before and After Dopamine Transporter Blockade

	Females			Males		
Time period	*N*	*M*	*SD*	*N*	*M*	*SD*
Pre-DAT blockade	12	70.97	13.39	16	72.55	11.10
Post-DAT blockade	12	52.82	14.17	16	51.50	12.94

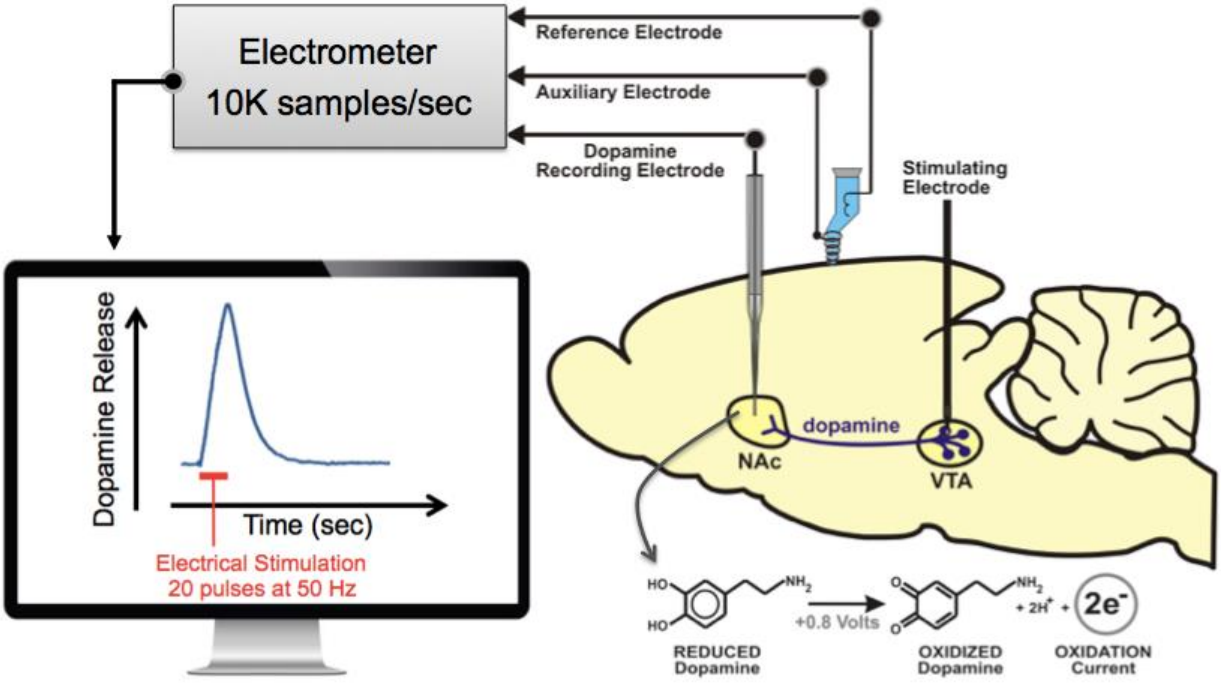

Figure 1. Amperometric Set-up. In vivo fixed potential amperometry was used to quantify nucleus accumbens (NAc) dopamine release evoked by stimulation of dopamine cell bodies within the ventral tegmental area (VTA).

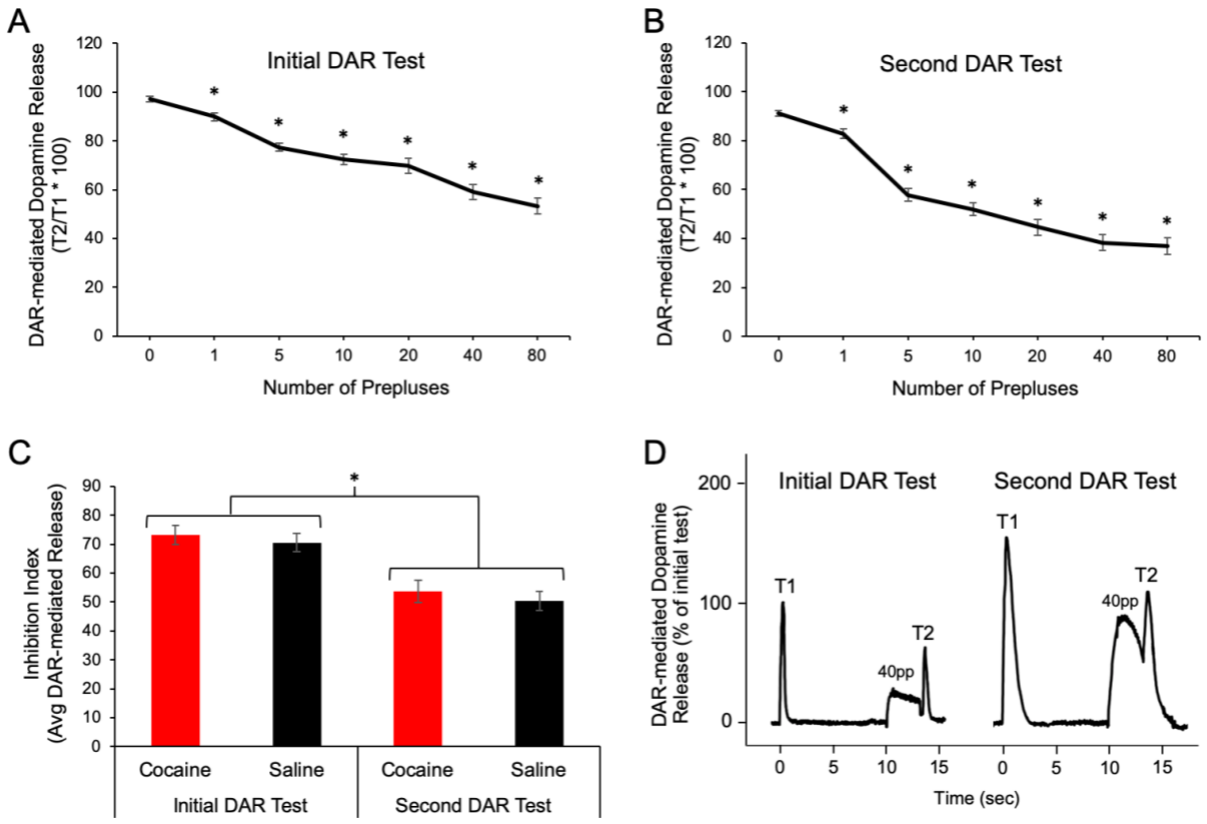

Figure 2. Autoreceptor-mediated inhibtion of dopamine release. (A, B) All prepulse conditions resulted in dopamine autoreceptor (DAR)-mediated dopamine release (% of T2/T1) that was significantly different from the control condition of 0 prepulses for each autoreceptor test. (C) Mean (±SEM) differences in inhibition indices between the initial DAR test and second DAR test (which took place 20 min after the in-test cocaine injection). * indicates $p < .05$. (D) Profiles indicate example responses from each DAR test.

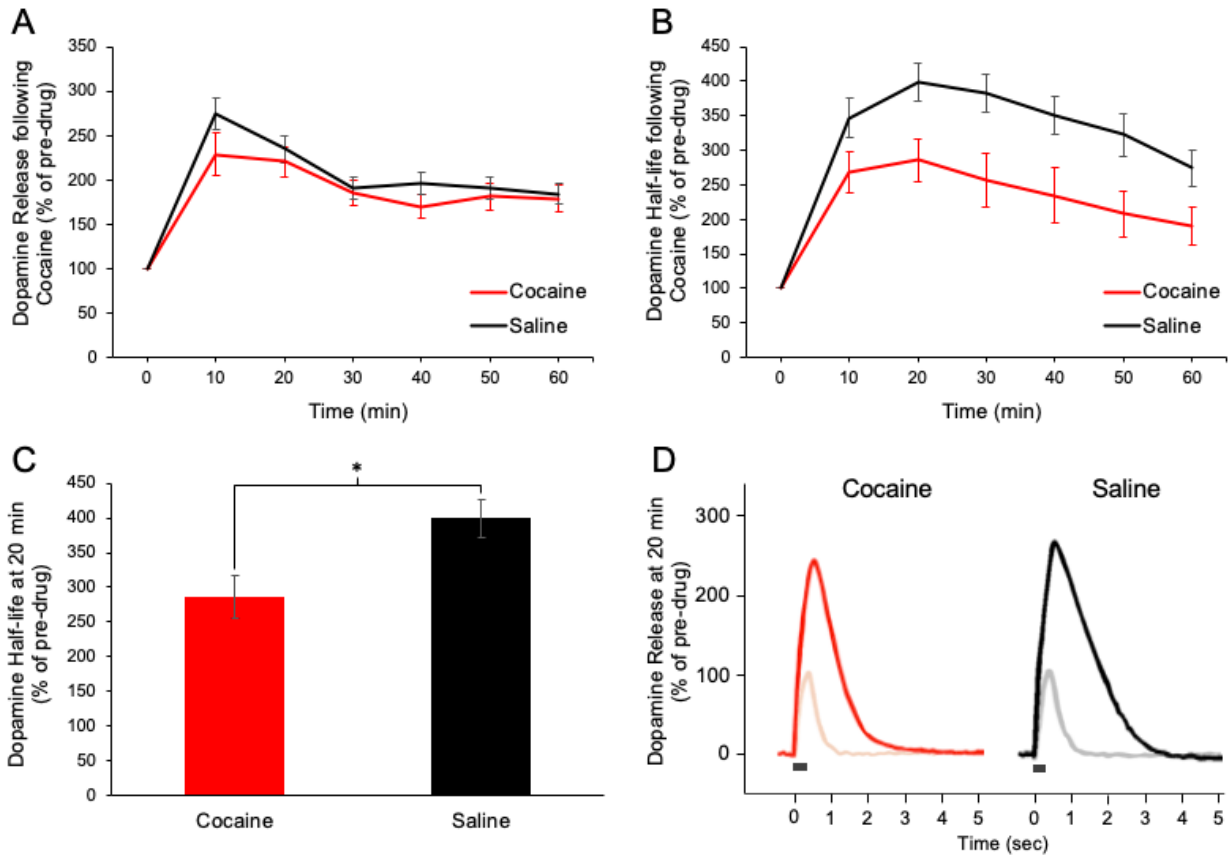

Figure 3. Stimulation evoked dopamine release and half-life following mid-surgery drug challenge of cocaine (DAT-blocker). (A) Mean (±SEM) dopamine release over the recording session following cocaine administration. (B) Mean (±SEM) dopamine half-life over the recording session following cocaine administration. (C) The synaptic half-life of evoked dopamine at the 20-minute time point was different between mice chronically exposed to cocaine and saline. (D) Profiles indicate example responses at the 20-minute time point (i.e., cocaine's peak effect time) in both pretreatment groups. The lighter lines represent pre-drug responses.