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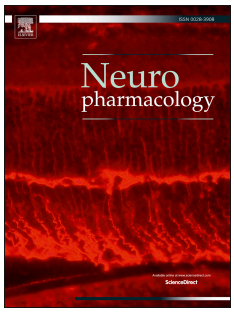

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Abstract

Social isolation rearing (SI) is a model of early life stress that results in neurobiological alterations leading to increased anxiety-like behaviors. These animals also exhibit an increased propensity to administer psychostimulants, such as cocaine; however, the mechanisms governing this increased addiction vulnerability remains to be elucidated. Long-term stressors have been shown to produce important alterations in nucleus accumbens core (NAc) function. The NAc regulates motivated and goal-directed behaviors, and individual differences in NAc function have been shown to be predictive of addiction vulnerability. Rats were reared in group (GH; 4/cage) or SI (1/cage) conditions from weaning (PD 28) into early adulthood (PD 77) and dopamine release was assessed using voltammetry in brain slices containing the NAc and dorsomedial striatum. SI rats exhibited enhanced dopamine release and uptake in both regions compared to GH rats. In regard to psychostimulant effects directly at the dopamine transporter (DAT), methylphenidate and amphetamine, but not cocaine, inhibited uptake more in SI than GH rats. The increased potencies were positively correlated with uptake rates, suggesting that increased potencies of amphetamine-like compounds are due to changes in DAT function. Cocaine's effects on uptake were similar between rearing conditions, however, cocaine enhanced evoked dopamine release greater in SI than GH rats, suggesting that the enhanced cocaine reinforcement in SI animals involves a DAT independent mechanism. Together, the results provide the first evidence that greater psychostimulant effects in SI compared to GH rats are due to effects on dopamine terminals related to uptake dependent and independent mechanisms.

1. Introduction

Early life stress in humans is associated with increased risk for developing severe psychological disorders including schizophrenia, depression, and anxiety disorders, as well as risk for abusing and becoming addicted to drugs such as psychostimulants (Scheller-Gilkey et al., 2004; Nugent et al., 2011; Enoch, 2012). Adolescent social isolation rearing (SI) in rats is a translational model that produces early life stress and results in behavioral changes that model many of the deficits observed in humans exposed to early life stress. Accordingly, our laboratory, as well as others, has reported increased anxiety-like behaviors under SI conditions as compared to group housed (GH) counterparts (Da Silva et al., 1996; McCool and Chappell, 2009; Chappell et al., 2013; Yorgason et al., 2013). SI also increases voluntary consumption and preference for abused drugs, and reduces the latency to acquire cocaine self-administration (Schenk et al., 1987; Bozarth et al., 1989; Yajie et al., 2005; McCool and Chappell, 2009; Chappell et al., 2013; Whitaker et al., 2013), demonstrating that SI increases the reinforcing properties of these drugs. However, while increased reinforcement is well documented, the mechanism by which early life stress increases psychostimulant abuse vulnerability is unclear.

Dopaminergic activity in the striatum is involved in reward learning, drug self-administration and reinforcement behaviors (Pierce and Kumaresan, 2006; Sora et al., 2009). The striatum is divided into multiple subregions, which have been shown to have different functions. The ventral region of the striatum, including the nucleus accumbens core (NAc), is integral in reward-prediction error encoding, and is involved in the acute reinforcing effects of abused compounds as well as motivational and incentive processes (Graybiel, 1995; Di Chiara, 2002; Porrino et al., 2004; Graybiel, 2008; Saddoris et al., 2013). The dorsal regions of the striatum are thought to regulate goal-directed and habitual drug seeking behaviors. For instance, dopamine activity in the dorsomedial striatum (DMS) is required for initial drug seeking behavior, whereas dopamine activity in the dorsolateral striatum is more important for maintaining habitual drug seeking (Murray et al., 2012). Microdialysis studies have established that SI rats have greater dopamine elevations in the NAc after systemic cocaine and amphetamine injections than

GH counterparts (Jones et al., 1992; Hall et al., 1998; Howes et al., 2000; Lapiz et al., 2001; Lapiz et al., 2003), which may help explain increased preference in SI rats. Currently, the direct mechanisms underlying the enhanced ability of stimulants to increase dopamine after SI is unknown. Furthermore, it is unclear if psychostimulant effects on dopamine are similarly enhanced in the DMS of SI rats.

The aim of the present study was to investigate the mechanisms involved in increased psychostimulant effects in SI rats. *Ex vivo* voltammetric methods were used to assess dopamine release and dopamine transporter (DAT) function in the NAc and DMS of SI and GH rats. These measures were examined to determine if changes in dopamine release/uptake kinetics could contribute to the divergent responses to psychostimulants in SI and GH rats. Next, amphetamine, methylphenidate and cocaine's effects on dopamine release and uptake were examined across multiple concentrations in the NAc and DMS of SI and GH rats. Lastly, pre-drug uptake rates and drug effects at maximally tested psychostimulant concentrations were examined for relationships that might help explain increased psychostimulant sensitivity in SI rats. Understanding the early life stress induced adaptations that underlie alterations in psychostimulant potency are particularly important as they may allow for the identification of individuals that are "at risk" or allow for treatments that reverse these adaptations and minimize risk.

2. Materials and Methods

2.1. Animal Housing

SI procedures were performed as previously described (Yorgason et al., 2013). Briefly, male Long-Evans rats (Harlan Laboratories, Indianapolis, IN) were procured on post-natal day (PD) 21 and housed for one week under standard conditions (4 rats/cage, food/water *ad libitum*, 12/12 hr light/dark). On PD 28, rats were randomly assigned to two groups, SI (1 rat/cage; 20x27 cm cages; Allentown Inc, Allentown, NJ) and GH (4 rats/cage; 33x60 cm cages; Ancare, Bellmore, NY) for six weeks. We have previously reported increases in anxiety-like behavior in SI rats (Chappell et al., 2013; Yorgason et al., 2013; McCool and Chappell, 2009). As in these previous studies, preceding the end of the initial housing period (PD 93), SI and GH rats were tested for anxiety-like behavior on the elevated plus maze (PD 74). *Ex vivo* voltammetry experiments were performed from PD 93-116. Multiple brain slices were obtained from the same rat to reduce the amount of animals used in the present study. Brain slices were from 14 GH and 16 SI rats that were spread across two cohorts, and randomly selected on experimental days, with experimenters blinded to rearing conditions. Experimental protocols adhered to the National Institutes of Health guide for the care and use of laboratory animals and were approved by the Wake Forest University Institutional Animal Care and Use Committee.

2.2. Ex Vivo Slice Preparation

Rats were euthanized and their brains rapidly removed and prepared as described previously (Yorgason et al., 2013). Coronal slices (400 µM) of the striatum were maintained at 32° C in oxygen perfused (95% O2-5% CO_2) artificial cerebrospinal fluid which consisted of (in mM): NaCl (126), $NaHCO_3$ (25), D-glucose (11), KCl (2.5), $CaCl_2$ (2.4), $MgCl_2$ (1.2), NaH_2PO_4 (1.2),

L-ascorbic acid (0.4), pH adjusted to 7.4. A capillary glass-based carbon-fiber electrode was positioned ~175 µm below the surface of the slice in the NAc or DMS as outlined in Figure 1. Dopamine release was evoked every 5 min by a 4 ms, single-pulse stimulation (monophasic, 350 µA) from a bipolar stimulating electrode (Plastics One, Roanoke, VA) placed 100-200 µm from the carbon-fiber electrode.

2.3. Fast Scan Cyclic Voltammetry

Fast scan cyclic voltammetry recordings were performed and analyzed using Demon Voltammetry and Analysis software (Yorgason et al., 2011). Carbon fiber electrodes used in voltammetry experiments were made in-house. Briefly, a carbon fiber (~7 µm diameter, Thornel T-650, Cytec, Woodland Park, NJ) was aspirated into a borosilicate glass capillary tube (A-M Systems, Sequim, WA). Subsequently, electrodes were pulled on a PE-22 vertical pipette puller (Narshige International USA, Long Island, NY) and cut so that ~100-200 µm of carbon fiber protruded from the tip of the glass. The electrode potential was linearly scanned as a triangular waveform from -0.4 to 1.2 V and back to -0.4 V (Ag vs AgCl) using a scan rate of 400 V/s. Cyclic voltammograms were recorded at the carbon fiber electrode every 100 ms by means of a potentiostat (Dagan Corporation, Minneapolis, MN). Once the stimulated dopamine response was stable for three successive collections, baseline measurements were taken and evaluated using a Michaelis-Menten based kinetic model (Wightman, 1988; Yorgason et al., 2011). Michaelis-Menten based changes in release and uptake were obtained by setting baseline apparent affinity (K_m) values to 0.16 µM, the affinity of dopamine for the DAT, and establishing a baseline maximal uptake rate (V_{max}) and stimulated dopamine release individually for each subject (Wightman, 1988; Wightman and Zimmerman, 1990; Wu et al., 2001). Extracellular

concentrations of dopamine were assessed by comparing the current at the peak oxidation potential for dopamine with electrode calibration solutions containing 1 μM dopamine (MEAN+-SEM of cal factors).

For psychostimulant studies, DAT inhibitors amphetamine (100 nM-10 μM; Sigma-Aldrich, St. Louis, MO), methylphenidate (300 nM-30 μM; NIDA, Bethesda, MD) and cocaine (300 nM-30 μM; NIDA) were used to examine changes in psychostimulant potency. Concentrations for these psychostimulants were selected based on concentration response curves from previous studies (Calipari et al., 2013). Psychostimulant-induced decreases in uptake rates were determined by measuring changes in apparent K_m of dopamine for the DAT using Michaelis-Menten based kinetic modeling (Wightman, 1988; Wu et al., 2001) . Psychostimulant-induced increases in electrically stimulated dopamine release were compared with pre-drug values (each animal served as its own control) to obtain a percent change in stimulated dopamine release. The concentration–response curve was then plotted as log concentration (M) of amphetamine, methylphenidate and cocaine or versus percent of control dopamine response, and the data were fit using a nonlinear regression curve fit (sigmoidal concentration response curve).

2.4. Western blot hybridization

In a separate group of animals, NAc and Caudate/Putamen (CPu) tissue samples were extracted from GH and SI animals at the end of the six week housing paradigm (NAc, n = 7 in both groups; CPu, n = 7 in both groups). DAT protein levels in the both areas were quantified using Western blot hybridization. SI and GH rats were euthanized and brains were removed and placed into ice-cold oxygenated artificial cerebrospinal fluid (aCSF) before slicing on a vibratome. The NAc and CPu were free-hand dissected from 400μm-thick slices. Tissue samples were placed in

individual eppendorf tubes, flash-frozen in isopentane (2-methylbutane, Fisher Scientific, Waltham, MA), and stored at -80°C prior to use. All tissue was homogenized in RIPA buffer (150 mM NaCl, 1.0% Triton-X-100, 0.5% Sodium Deoxycholate, 0.1% Sodium Dodecyl Sulfate (SDS), 50mM Trizma Base, pH 8.0) and centrifuged at 12,000xg for 30 min. Protein concentrations were determined with a commercially-available BCA protein assay kit (ThermoScientific, Rockford, IL) and Molecular Devices Spectra Max 384 Plus spectrophotometer (Sunnyvale, CA) utilizing the SoftMax Pro software.

Twenty µg of protein was loaded onto 4 – 12% NuPAGE Bis-Tris10-well precast gels (NP0321BOX; ThermoFisher Scientific, Grand Island, NY) along with a MagicMark™ XP molecular weight ladder (LC5602; ThermoFisher Scientific, Grand Island, NY). The membranes were blocked with a 1X solution of Tris Buffered Saline (TBS-T 20X concentrate; J640; Amresco, Solon, OH) and 0.05% Tween-20 (BP337; Fisherbrand, Loughborough, UK) containing 5% Carnation powdered nonfat dry milk (NFM) for 1 hour at room temperature. Subsequently, blots were incubated with agitation for two hours at room temperature in TBS-T/5% bovine serum albumin (05470; Sigma-Aldrich, St. Louis, MO) solution containing the following primary antibody concentrations: DAT (1:5000) (2231; EMD Millipore, Billerica, MA). Following extensive washing with TBS-T, the blots were exposed to a goat anti-rabbit secondary antibody (1:6000) (4914; Sigma, St. Louis, MO) in 5% NFM dissolved in TBS-T for two hours at room temperature with agitation. Detection of bound secondary antibody was performed using enhanced chemiluminescence using Pierce ECL western blotting substrate (32106; ThermoFisher Scientific, Grand Island, NY). Band intensity was quantified with ImageJ densitometry software (National Institutes of Health, Bethesda, MD). Samples from GH and SI animals were always run on the same gel to facilitate direct comparisons.

2.5. Statistical Analyses

All dopamine release voltammetric assessments are reported as μM concentration, or percent of baseline recordings. Our previous study demonstrated increases in dopamine release and uptake in SI reared rats (Yorgason et al., 2013). Therefore, given this prior knowledge on directionality one-tailed t-tests were used in the current study examining release and uptake differences between SI and GH rats. For pharmacological studies, two-way repeated measures mixed analysis of variance (ANOVA) examining the effects of psychostimulants on dopamine uptake and release across increasing concentrations in SI and GH rats, as well as comparing regional differences. Bonferroni post hoc analysis was used to test for differences between groups at each concentration when significant main effects were obtained ($P < 0.05$). Pearson correlation coefficients were calculated in all correlations presented herein. Statistical analyses were performed using GraphPad Prism 5 (IBM, New York NY) and NCSS 8 (NCSS LLC, Kaysville UT).

3. Results

3.1. Increased Dopamine Terminal Function in Socially Isolated Rats

In order to determine if evoked dopamine terminal release and reuptake are different between SI and GH conditions, electrically-evoked dopamine release was measured in the NAc (Figure 2) and DMS (Figure 3). Similar to previous studies describing regional differences (Cragg et al., 2000; Calipari et al., 2012; Siciliano et al., 2014a), dopamine release and uptake were greater in the DMS than in the NAc (Release: $t_{68} = 3.679$, $p = 0.0002$; Uptake: $t_{69} = 6.194$, $p < 0.0001$). We have recently demonstrated that in the NAc, dopamine release and uptake rates are greater in SI than GH rats (Yorgason et al., 2013). Consistent with this previous report, in the present study utilizing a separate cohort of animals, a one-tailed student's t-test revealed that dopamine release was greater in SI compared to GH reared rats in both the NAc ($t_{32} = 1.89$, $p = 0.034$; Figure 2A,B) and the DMS ($t_{34} = 1.997$, $p = 0.0269$; Figure 3A,B). Michaelis-Menten based kinetic modeling revealed higher uptake rates (V_{max}) in the NAc ($t_{34} = 4.303$, $p < 0.0001$; Figure 2C) and DMS ($t_{33} = 5.65$, $p < 0.0001$; Figure 3C) of SI than GH rats.

Western blot analysis was used to compare DAT expression levels between SI and GH animals in the NAc (Figure 2D,E) and the CPu (Figure 3D,E). In the NAc, NAc DAT expression levels in SI animals were significantly greater than those in the GH animals ($t_{12} = 3.58$, $p < 0.01$; Figure 2E). CPu DAT expression was also greater in SI compared to GH rats ($t_{12} = 2.23$, $p < 0.05$; Figure 3E).

3.2. Uptake Inhibition Effects of Psychostimulants are Greater in Socially Isolated Animals

Genetic and behavioral manipulations that increase DAT levels, and dopamine uptake, also enhance psychostimulant potency for uptake inhibition with 'releasers' such as amphetamine, and the structurally similar blocker methylphenidate (Calipari et al., 2013). However, increases in uptake rates do not strongly correlate with DAT inhibition potency for the prototypical DAT blocker cocaine (Calipari et al., 2013). Since DAT uptake rates are increased in SI rats, the concentration dependent effects of these drugs on uptake inhibition were examined after SI. Psychostimulant application produced marked dopamine uptake inhibition (Figure 4). Two-way ANOVA revealed a main effect of amphetamine on

inhibiting dopamine uptake (NAc: $F_{4,32} = 55.48$, $p < 0.0001$; DMS: $F_{4,36} = 106.7$, $p < 0.0001$; Figure 4A,D). Amphetamine-mediated uptake inhibition was greater in SI rats compared to GH rats in the NAc (Housing, $F_{1,8} = 3.702$, $p = 0.0419$; Housing X Concentration, $F_{4,32} = 5.241$, $p = 0.0023$; Figure 4A) and DMS (Housing, $F_{1,9} = 12.02$, $p = 0.0071$; Housing X Concentration, $F_{4,36} = 10.29$, $p < 0.0001$; Figure 4D).

Two-way ANOVA revealed a main effect of methylphenidate on dopamine uptake (NAc: $F_{4,40} = 113.5$, $p < 0.0001$; DMS: $F_{4,40} = 110.8$, $p < 0.0001$; Figure 4B,E). Additionally, methylphenidate-induced uptake inhibition was greater in SI than GH rats in the NAc (Housing, $F_{1,10} = 20.22$, $p = 0.0011$; Housing X Concentration, $F_{4,40} = 7.140$, $p = 0.0002$; Figure 4B) and DMS (Housing, $F_{1,10} = 12.10$, $p = 0.0059$; Housing X Concentration, $F_{4,40} = 7.502$, $p = 0.0001$; Figure 4E).

Cocaine inhibited dopamine uptake in both the NAc and the DMS (NAc, $F_{4,46} = 167.3$, $p < 0.0001$; DMS, $F_{4,41} = 131.3$, $p < 0.0001$). However, there was no significant difference in cocaine-mediated DAT inhibition between SI and GH rats in either region ($p > 0.05$; Figure 4C,F).

3.3. Uptake Inhibition Effects are Greater in the Dorsal Striatum than the Accumbens Core for Amphetamine and Methylphenidate, but not Cocaine

Dopamine uptake rates are greater in the DMS than the NAc (Cragg et al., 2000; Calipari et al., 2012; Siciliano et al., 2014a). Therefore, effects of psychostimulants were compared between these two regions in GH and SI reared rats (Figure 4). Two way ANOVA revealed a main effect of amphetamine concentration on dopamine uptake inhibition ($F_{4,45} = 72.68$; $p < 0.0001$), with greater inhibition in the DMS versus the NAc in GH rats ($F_{1,4} = 9.31$; $p = 0.0038$; Figure 4A,D). There was also a significant Region X Concentration interaction ($F_{4,45} = 3.042$; $p = 0.0266$) in GH rats. In SI rats, amphetamine (Figure 4A,D) induced uptake inhibition (Concentration, $F_{4,39} = 53.69$; $p < 0.0001$) was greater in the DMS versus the NAc (Region, $F_{1,4} = 9.485$; $p = 0.0038$), with a Region X Concentration interaction ($F_{4,39} = 2.926$; $p = 0.033$).

In GH rats, methylphenidate (Figure 4B,E) inhibited dopamine uptake in a concentration dependent manner ($F_{4,38} = 37.40$; $p < 0.0001$), with higher uptake-inhibition potency in the DMS than

NAc brain region ($F_{1,4}$ = 9.278; p = 0.0042), with a significant Concentration X Region interaction ($F_{4,38}$ = 3.035; p = 0.0289). In SI rats, methylphenidate reduced uptake (Figure 4B,E) in a concentration dependent manner ($F_{4,58}$ = 276.9; p < 0.0001) with greater inhibition in the DMS compared to the NAc (Region, $F_{1,4}$ = 8.389; p = 0.0053). There was also a significant Concentration X Region interaction for methylphenidate in SI rats ($F_{4,58}$ = 3.317; p = 0.0163).

In contrast to the effects of amphetamine and methylphenidate, cocaine's effects on dopamine uptake do not appear to be directly related to uptake rates (Calipari et al., 2013). Consistent with previous results, cocaine inhibition of dopamine uptake was concentration dependent (GH, $F_{4,27}$ = 407.5; p < 0.0001; SI, $F_{4,60}$ = 191.2; p < 0.0001) and was similar between the DMS and NAc in GH (Figure 4C,F) and SI (Figure 4C,F) rats (p > 0.05).

3.4. Dopamine Uptake Rates Correlate with Psychostimulant Drug Potency

Next, baseline uptake V_{max} values from the DMS and NAc of SI and GH rats were plotted against apparent dopamine K_m values for amphetamine (10 μM; Figure 5A), methylphenidate (30 μM; Figure 5B) and cocaine (30 μM; Figure 5C) to examine correlations between these DAT function and drug effects. Uptake V_{max} positively correlated with K_m for amphetamine (r_{19} = 0.8544, p < 0.0001), methylphenidate (r_{20} = 0.8498, p < 0.0001) and cocaine (r_{23} = 0.442, p = 0.0394). In addition, the slopes of the regressions were significantly different from a slope of zero for amphetamine (β = 7.146 ± 0.9970, $F_{1,19}$ = 51.37, p < 0.0001), methylphenidate (β = 6.075 ± 0.7855, $F_{1,23}$ = 59.80, p<0.0001) and cocaine (β = 1.175 ± 0.5330, $F_{1,20}$ = 4.857, p = 0.0394). Further analysis revealed that the slopes for amphetamine and methylphenidate were significantly greater than for cocaine (Amphetamine vs Cocaine, $F_{1,39}$ = 28.718, p < 0.0001; Methylphenidate vs Cocaine, $F_{1,43}$ = 23.132, p < 0.0001; Figure 5D), suggesting that although DAT levels may play a role in cocaine uptake inhibition, the relationship between uptake and potency for amphetamine/methylphenidate is more robust.

3.5. Social Isolation Rearing increases the ability of Cocaine, but not Methylphenidate or Amphetamine, to Enhance Evoked Dopamine Release

In voltammetric measurements, the measured peak concentration of dopamine is a result of the opposing processes of release and uptake; thus, elevations in peak dopamine may be attributed to increased release and/or decreased uptake (Wightman and Zimmerman, 1990; Yorgason et al., 2011). To determine the effects of SI on psychostimulant-induced increases in peak dopamine, dopamine release amplitude in response to amphetamine, methylphenidate and cocaine was measured (Figure 6). Amphetamine decreased dopamine release in the NAc ($F_{4,32} = 117.3$, $p < 0.0001$; Figure 6A) and DMS ($F_{4,36} = 110.1$, $p < 0.0001$; Figure 6D), with no significant difference in evoked dopamine release detected between rearing conditions ($p > 0.05$). In contrast, methylphenidate increased evoked dopamine release, with an inverted-U shaped curve, and increases at 300 nM-10 μM concentrations in the NAc ($F_{4,48} = 16.27$, $p < 0.0001$; Figure 6B) and DMS ($F_{4,40} = 16.76$, $p < 0.0001$; Figure 6E). ANOVA failed to reveal any significant differences between rearing conditions for methylphenidate experiments ($p > 0.05$). Cocaine also augmented dopamine release in an inverted-U dependent manner, with large increases at 300 nM-10 μM concentrations, and decreases at 30 μM concentrations. Two-way ANOVA revealed significant effects of cocaine in the NAc ($F_{4,44} = 8.844$, $p < 0.0001$; Figure 6C) and DMS ($F_{4,40} = 50.32$, $p < 0.0001$; Figure 6F). In contrast to results from amphetamine and methylphenidate experiments, there was a main effect of rearing conditions in cocaine induced increases in release, with greater dopamine enhancement in SI compared to GH rats in the NAc ($F_{1,11} = 7.383$, $p = 0.02$; Figure 6C), but not the DMS ($p > 0.05$). A summary table is included, describing the peak effects of psychostimulants on dopamine uptake and release in GH and SI rats (Table 1).

4. Discussion

Here we show that SI enhances psychostimulant effects on dopamine terminals, which provides a mechanism for the increase reinforcement of psychostimulants following early life stress. In the present study, SI rearing increased striatal dopamine release and increased uptake rates in the NAc and DMS. Similar to previous studies (Chadchankar et al., 2012; Calipari et al., 2013; Siciliano et al., 2014b; Ramsson et al., 2011; Avelar et al., 2013), the psychostimulants tested all produced robust inhibition of dopamine uptake, but different effects on release. While amphetamine decreased evoked dopamine release, methylphenidate and cocaine generally enhanced release, demonstrating the diverse nature of the effects of these drugs. The potencies of the dopamine releaser, amphetamine, and the DAT blocker methylphenidate, but not cocaine, were greater in SI reared rats in the NAc and DMS. Amphetamine and methylphenidate's effects on uptake were strongly correlated to baseline uptake rates, suggesting that DAT function is involved in the greater psychostimulant effects observed in SI animals. While cocaine effects at the DAT were unchanged following SI, cocaine-induced enhancement of dopamine release was greater in SI than GH rats, whereas methylphenidate and amphetamine had similar release profiles between rearing conditions, providing further evidence that the mechanism by which SI increases the reinforcing efficacy of these drugs differs. The present results suggest that increased DAT function is a potential mechanism by which SI results in increased stimulant potencies and reinforcing properties. Given the increased rates of stimulant abuse in individuals that have been exposed to early life stress, an understanding of mechanisms involved in this drug abuse vulnerability may allow for the development of therapeutic interventions (Scheller-Gilkey et al., 2004; Enoch, 2012).

SI resulted in increased stimulated dopamine release in SI compared to GH animals, suggesting increased overall dopamine signaling. This suggests that more dopamine may be released in response to salient events via phasic burst firing in SI animals, resulting in more potent associations between cues and the environment. This is particularly relevant in drug abuse, as cue-reward associations are thought to play an integral role in addiction and relapse (Koob and Volkow, 2010). Indeed, these results may help explain the well documented increases in reinforcement of psychostimulants in SI rats (Schenk et al.,

1987; Bozarth et al., 1989; Yajie et al., 2005; McCool and Chappell, 2009; Whitaker et al., 2013). Dopamine transmission is a tightly regulated process that is controlled by auto- and hetero-receptor activity, exocytotic release regulating machinery, as well as uptake processes (for review see Zhang and Sulzer, 2012; Ferris et al., 2013). SI has been shown to produce changes in dopamine function that would suggest increases in extracellular dopamine levels (Hall, 1998; Heidbreder et al., 2000; Barr et al., 2004; Roncada et al., 2009; Fabricius et al., 2010; Whitaker et al., 2013). However, microdialysis studies have failed to demonstrate large increases in extracellular dopamine levels after SI (Wilkinson et al., 1994; Miura et al., 2002a; Miura et al., 2002b; Brenes and Fornaguera, 2009). The increased evoked release in SI rats reported herein is consistent with previous studies that have suggested overall increased dopamine terminal activity. Furthermore, faster uptake rates in SI rats may normalize differences in dopamine tone between SI and GH rats in microdialysis studies; however the increased release capacity likely leads to increased phasic responses, which are not observable with microdialysis due to the low temporal resolution.

Increases in DAT function appear to be central in the context of stimulant releasers, such as amphetamine (Calipari et al., 2013; Siciliano et al., 2014a). Therefore, in order to determine how DAT mediated processes contribute to amphetamine vulnerability in early life stress models, amphetamine induced dopamine uptake inhibition was examined in SI and GH reared rats. DAT function was highly correlated with drug potency, with greater amphetamine potency in SI than GH rats. Similar to amphetamine, methylphenidate, a DAT blocker structurally homologous to amphetamine, also exhibited increased potency for inhibiting uptake in SI rats, with greatest inhibition observed in the DMS. This finding corroborates results from other studies, in that higher DAT function in the DMS appears to increase the potency of amphetamine and methylphenidate for uptake inhibition (Calipari et al., 2013; Siciliano et al., 2014a). While results from these previous studies mechanistically link increases in DAT levels to potency for releasers, it is still unknown how increases in DAT function can selectively predict potency for the DAT blocker methylphenidate, but not cocaine. Since methylphenidate and amphetamine share some structural homology, the mechanism underlying this effect may relate to how these drugs

interact with the DAT. However, whether this similarity in function is due to an allosteric DAT modification, or through a competition effect at the dopamine binding pocket remains unclear and will need to be tested.

Although increased DAT function seems to be the mechanism underlying increased potency for amphetamine and methylphenidate, this cannot explain the increased cocaine self-administration (Schenk et al., 1987) and cocaine-induced dopamine release (Howes et al., 2000) in SI animals, as cocaine potency for uptake inhibition was not different between rearing conditions. Previous work has established that increases in DAT levels, either by self-administration or by genetic manipulation have no effects on the behavioral or neurochemical effects of cocaine (Calipari et al., 2013). However, the current work provides initial evidence for a possible mechanism by which cocaine reinforcement is increased in SI animals, specifically the differences observed in NAc dopamine release across all cocaine concentrations. In addition to cocaine's direct effects on DAT-mediated uptake, there are also many other direct and indirect effects that may influence dopamine transmission, including increased D2 autoreceptor activity (Gantz et al., 2013), voltage gated Na+ channel blockade (Wang, 1988), synapsin dependent dopamine pool mobilization (Venton et al., 2006; Kile et al., 2010; Federici et al., 2014) and heterosynaptic activity from feedback circuits to name a few (Zhang and Sulzer, 2012; Ferris et al., 2013). Considering these many effects of cocaine, it is difficult to know what differences between SI and GH rats may be driving greater cocaine-induced release in the NAc of SI rats. Since differences were only observed in the NAc, future studies examining sensitivity to cocaine's dopamine-enhancing across these brain regions may reveal important differences in cocaine sensitive release regulating proteins. Also, since all the psychostimulants tested have various uptake dependent and independent effects, and since differences in release were only observed in response to cocaine, future studies may benefit by comparing/contrasting the known effects of these psychostimulants. For instance, while cocaine is non-selective for the DAT, norepinephrine and serotonin transporters, methylphenidate is more selective towards the DAT and norepinephrine transporter.

5. Conclusions

The current studies have demonstrated that SI produces robust changes in dopamine terminal activity, including increased release and uptake, and DAT expression in the dorsal and ventral striatum. SI-induced increases in dopamine uptake in the NAc may be due to elevations in DAT levels or changes in DAT function. These results suggest that individuals who have suffered from early life stress have elevated dopamine release and uptake throughout the striatum. Higher DAT function in SI rats was accompanied by larger psychostimulant effects on dopamine terminal function. For amphetamine and methylphenidate, these changes were manifested as increases in potency for DAT inhibition. The greater dopamine enhancing effects of cocaine in the NAc of SI rats were due to increased release and not uptake inhibition. Together, these data provide a site of action of increased psychostimulant effects in SI rats, and suggest that individuals with increased early life stress may be hyperresponsive to psychostimulant actions on dopamine terminals throughout the striatum. These data are of particular importance as they suggest a possible mechanism for increased susceptibility to the addictive properties of psychostimulants in individuals who have experienced severe early life stress. Furthermore, since SI rearing also increases anxiety-like behaviors (Da Silva et al., 1996; McCool and Chappell, 2009; Chappell et al., 2013; Yorgason et al., 2013) it will be important to know if increases in psychostimulant sensitivity are specific to early life stress conditions, or if this effect is generalizable to high anxiety phenotypes.

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Nonstandard Abbreviations:

DAT – Dopamine transporter

DMS – Dorsal medial striatum

GH – Group housed rearing

App K_m – Apparent affinity of dopamine for the dopamine transporter

NAc – Nucleus accumbens

PD – Postnatal day

SERT – Serotonin transporter

SI – Social isolation rearing

V_{max} – Maximal rate of dopamine uptake

Keywords

Dopamine; psychostimulant; voltammetry; accumbens, striatum; early life stress

Figure Legends

Figure 1. Dopamine release and uptake were measured in the core of the nucleus accumbens (NAc) and dorsal medial striatum (DMS) of socially isolated (SI) and group housed (GH) rats.

Figure 2. Dopamine release and uptake are increased in the nucleus accumbens (NAc) of socially isolated (SI) compared to group housed (GH) rats. Rats were reared in SI or GH conditions from postnatal day 21 to 77. Voltammetry experiments were performed at the end of the rearing period to determine dopamine release and uptake kinetics. **A**, representative raw dopamine traces recorded in the NAc of SI and GH rats. **B**, mean (± SEM) stimulated dopamine release for SI and GH rats. **C**, mean (± SEM) maximal rate of dopamine uptake (V_{max}) in the NAc of GH and SI rats, as determined by Michaelis-Menten based kinetic modeling. **D**, representative NAc western blots from SI and GH rats. **E**, mean (± SEM) DAT (~80kDa) expression in the NAc, normalized to GH values, as determined from western blotting experiments. *p<0.05, **p<0.01, ***p<0.0001

Figure 3. Dopamine release and uptake are increased in the dorsal medial striatum (DMS) of socially isolated (SI) versus group housed (GH) rats. Rats were reared in SI or GH conditions from postnatal day 21 to 77. Voltammetry experiments were performed at the end of the rearing period to determine dopamine release and uptake kinetics. **A**, representative raw dopamine traces recorded in the DMS of SI and GH rats. **B**, mean (± SEM) stimulated dopamine release for SI and GH rats. **C**, mean (± SEM) maximal rate of dopamine uptake (V_{max}) in the DMS of GH and SI rats, as determined by Michaelis-Menten based kinetic modeling. **D**, representative CPu western blots from SI and GH rats. **E**, mean (± SEM) DAT (~80kDa) expression in the NAc, normalized to GH values, as determined from western blotting experiments. *p<0.05, ***p<0.0001

Figure 4. Psychostimulant potency for uptake inhibition is increased in socially isolated (SI) compared to group housed (GH) rats. Electrically stimulated dopamine release was measured in the nucleus

accumbens (NAc) and dorsal medial striatum (DMS) of GH and SI rats while bath applying increasing concentrations of psychostimulants. Dopamine uptake inhibition, as reported by changes in the apparent affinity of dopamine (app. K_m) for the dopamine transporter (DAT), was determined using Michaelis-Menten based kinetic modeling. **A,D**, mean (± SEM) amphetamine-induced (100 nM-10 μM) uptake inhibition in the NAc (**A**) and DMS (**D**) of SI and GH rats. **B,E**, mean (± SEM) methylphenidate-induced (300 nM-30 μM) uptake inhibition in the NAc (**B**) and DMS (**E**) of SI and GH rats. **C,F**, mean (± SEM) cocaine-induced (300 nM-30 μM) uptake inhibition in the NAc (**C**) and DMS (**F**) of SI and GH rats. *p<0.05, **p<0.01, ***p<0.0001

Figure 5. Pyschostimulant potency for dopamine uptake inhibition is directly related to baseline uptake rates. Maximal rate of dopamine uptake (V_{max}) and apparent affinity of dopamine for the dopamine transporter (app. K_m) was determined by Michaelis-Menten based kinetic modeling. **A**, amphetamine-induced (10 μM) uptake inhibition correlated to V_{max} values. **B**, methylphenidate-induced (30 μM) uptake inhibition correlated to V_{max} values. **C**, cocaine-induced (30 μM) uptake inhibition correlated to V_{max} values. **D**, combined data from **A-C**. amphetamine and methylphenidate-induced uptake inhibition versus V_{max} was compared to that of cocaine. ***p<0.0001

Figure 6. The effects of psychostimulants on evoked dopamine release in socially isolated (SI) and group housed (GH) rats from the nucleus accumbens (NAc) and dorsal medial striatum (DMS). **A,D**, mean (± SEM) amphetamine-induced (100 nM-10 μM) changes in evoked dopamine release in the NAc (**A**) and DMS (**D**). **B,E**, mean (± SEM) methylphenidate-induced (300 nM-30 μM) changes in evoked dopamine release in the NAc (**B**) and DMS (**E**). **C,F**, mean (± SEM) cocaine-induced (300 nM-30 μM) changes in evoked dopamine release in the NAc (**C**) and DMS (**F**). *p<0.05

Table 1

Table 1. Effects of Psychostimulants on Dopamine Uptake and Release in GH and SI Rats (Mean ± SEM)

		NAc		DMS	
		GH	**SI**	**GH**	**SI**
Baseline	**Release (µM)**	1.091±0.101	1.426±0.140*	1.617±0.206	2.184±0.194*
	Uptake (V_{max})	1.899±0.117	2.840±0.171*	3.023±0.256	4.812±0.194*
Uptake Inhibition at Max (Km, µM)	**Amphetamine**	13.388±1.204	24.689±3.698*	19.785±1.866	36.715±4.331*
	Methylphenidate	17.411±2.412	29.089±1.973*	20.047±3.133	34.807±2.697*
	Cocaine	17.156±0.548	16.816±0.794	16.508±0.753	19.521±2.354
Dopamine Release at Peak (3 µM)	**Amphetamine**	0.460±0.093	0.463±0.048	0.309±0.077	0.261±0.063
	Methylphenidate	1.728±0.193	1.783±0.240	1.622±0.159	1.749±0.295
	Cocaine	1.464±0.153	2.266±0.245*	1.621±0.096	1.551±0.182

*, $p<0.05$

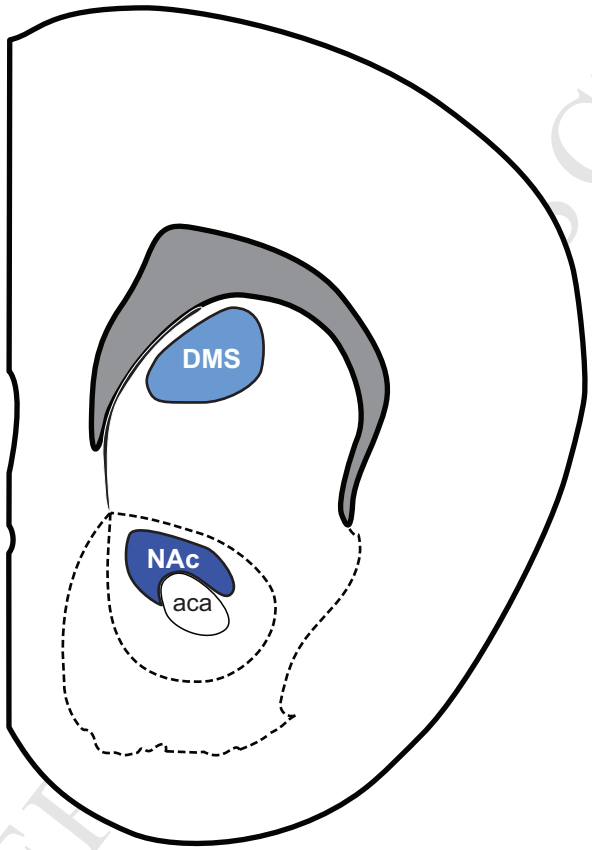


figure 1

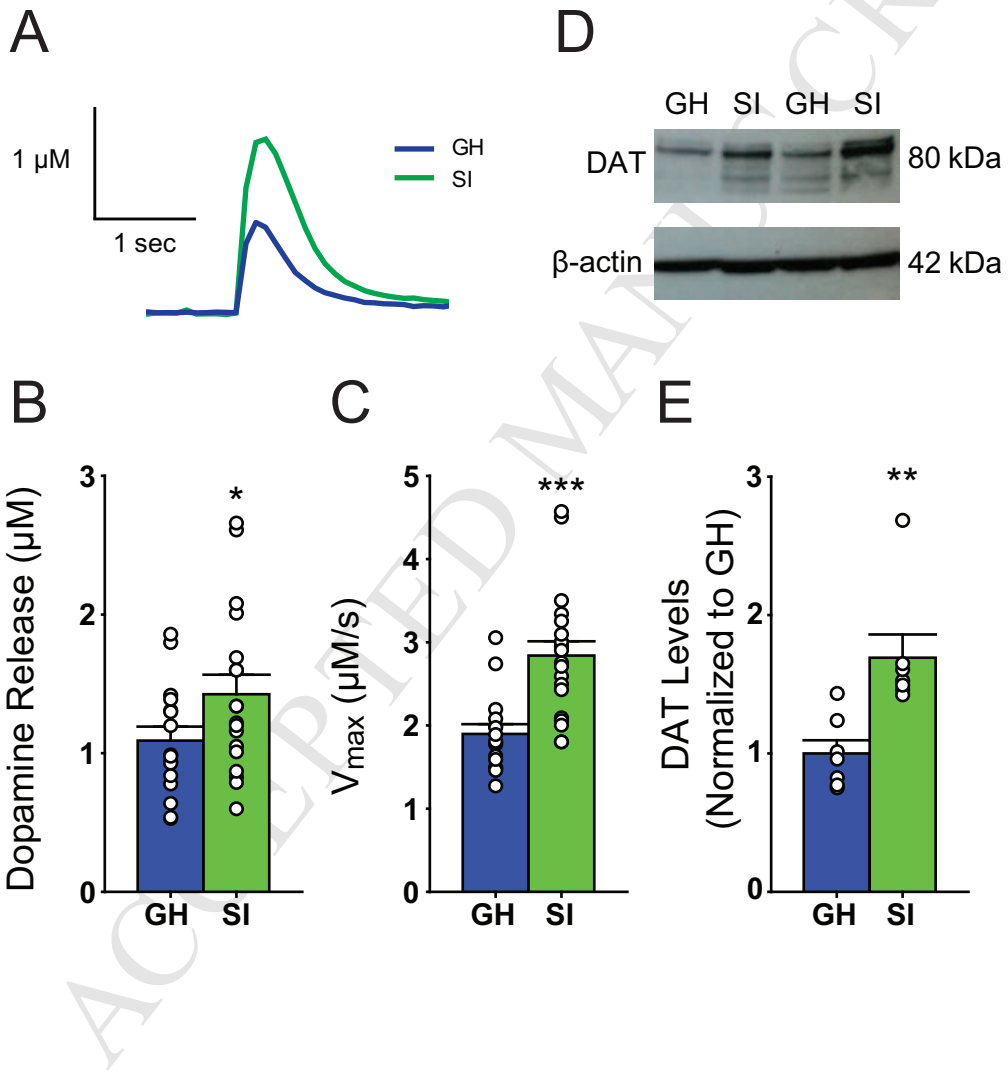
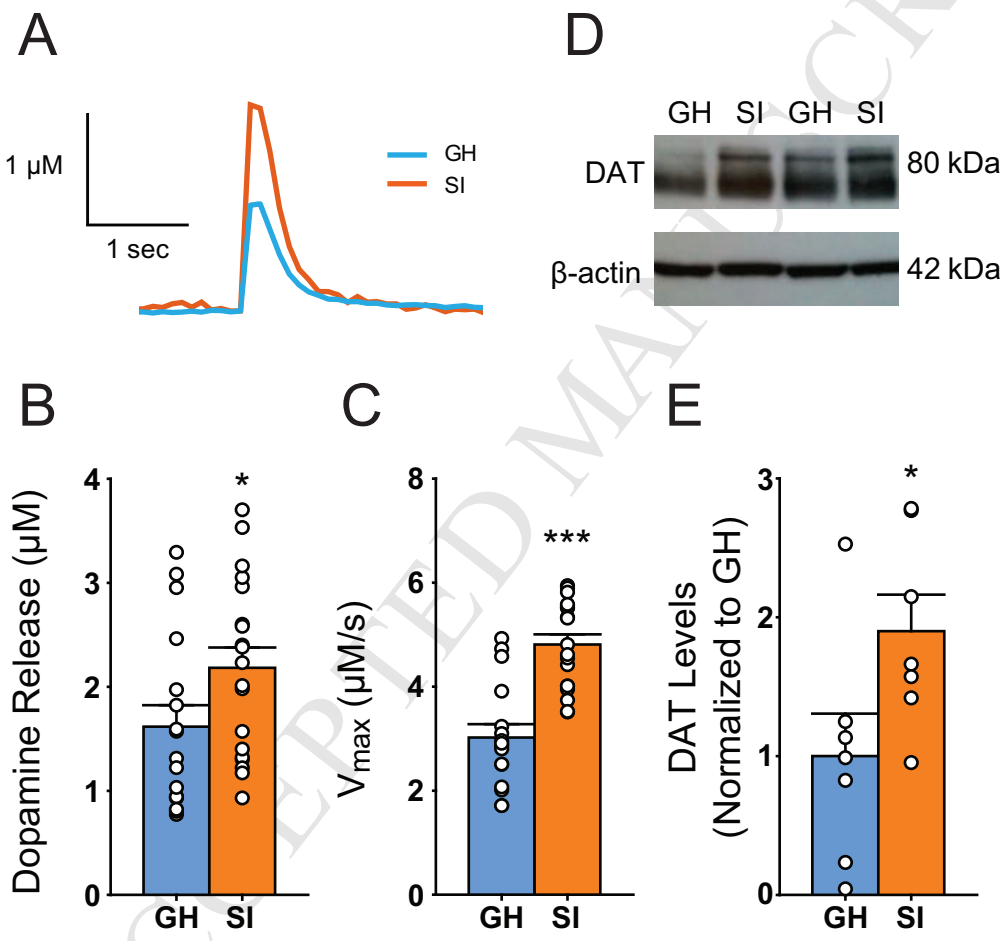


figure 2



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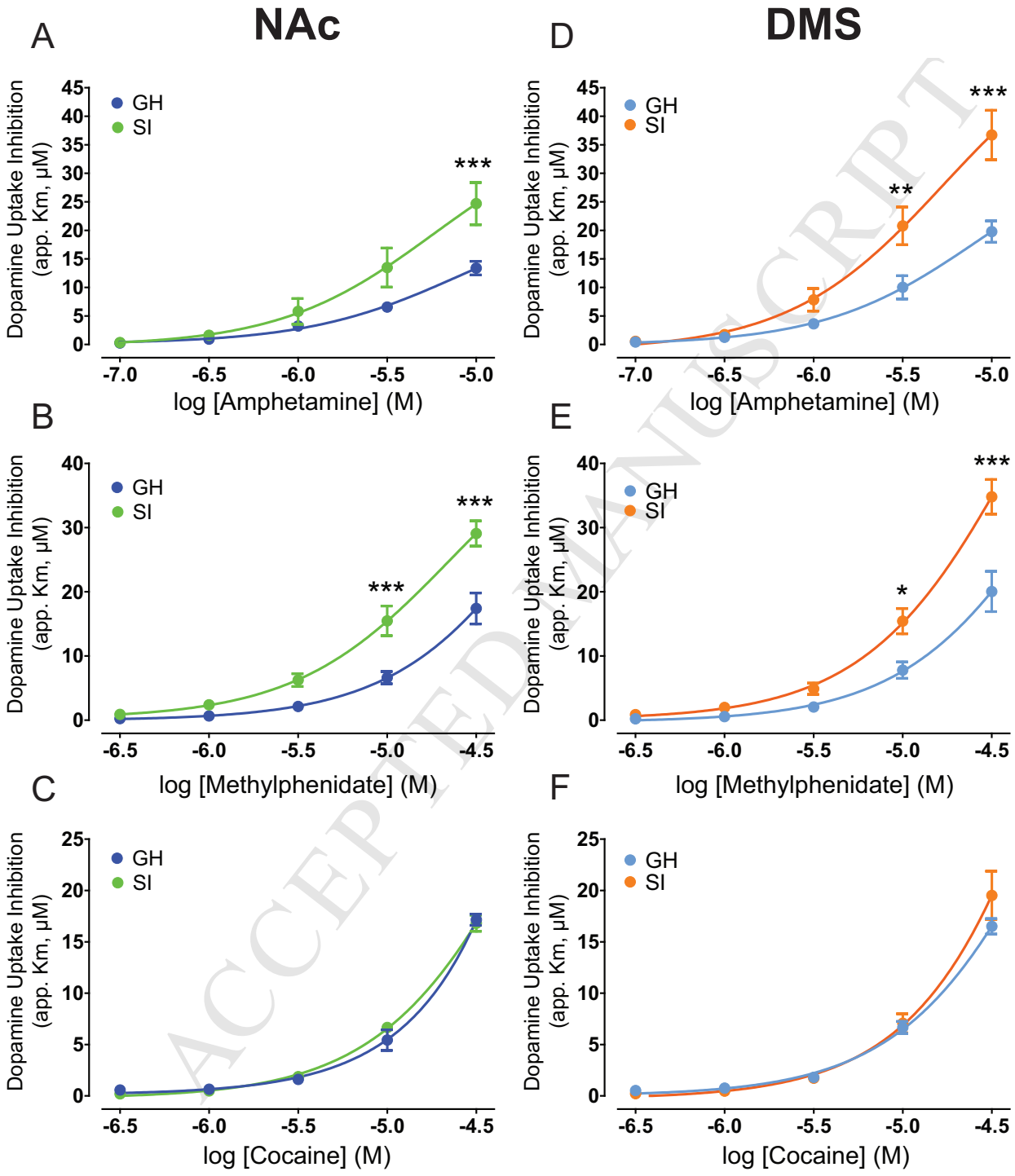

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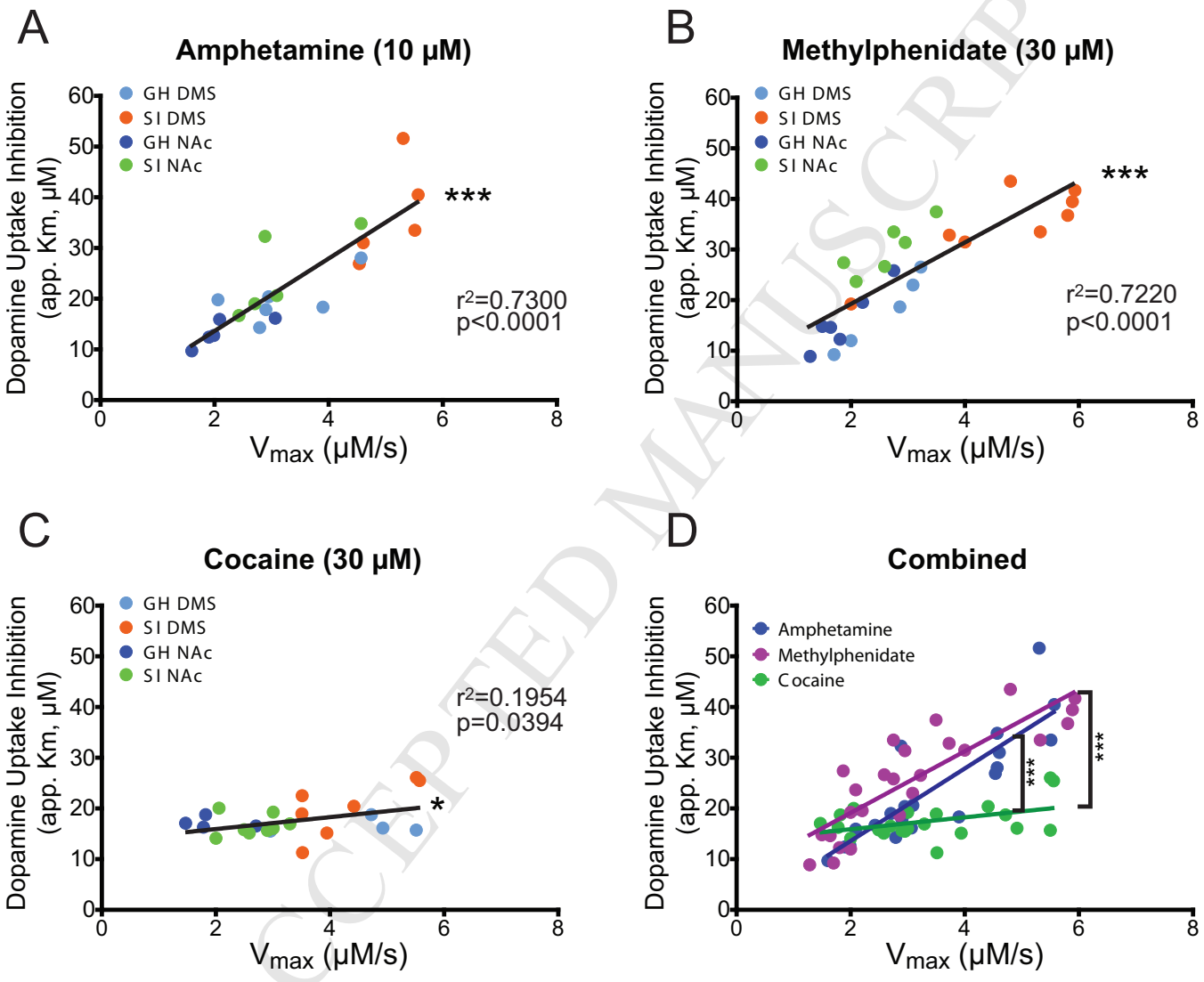

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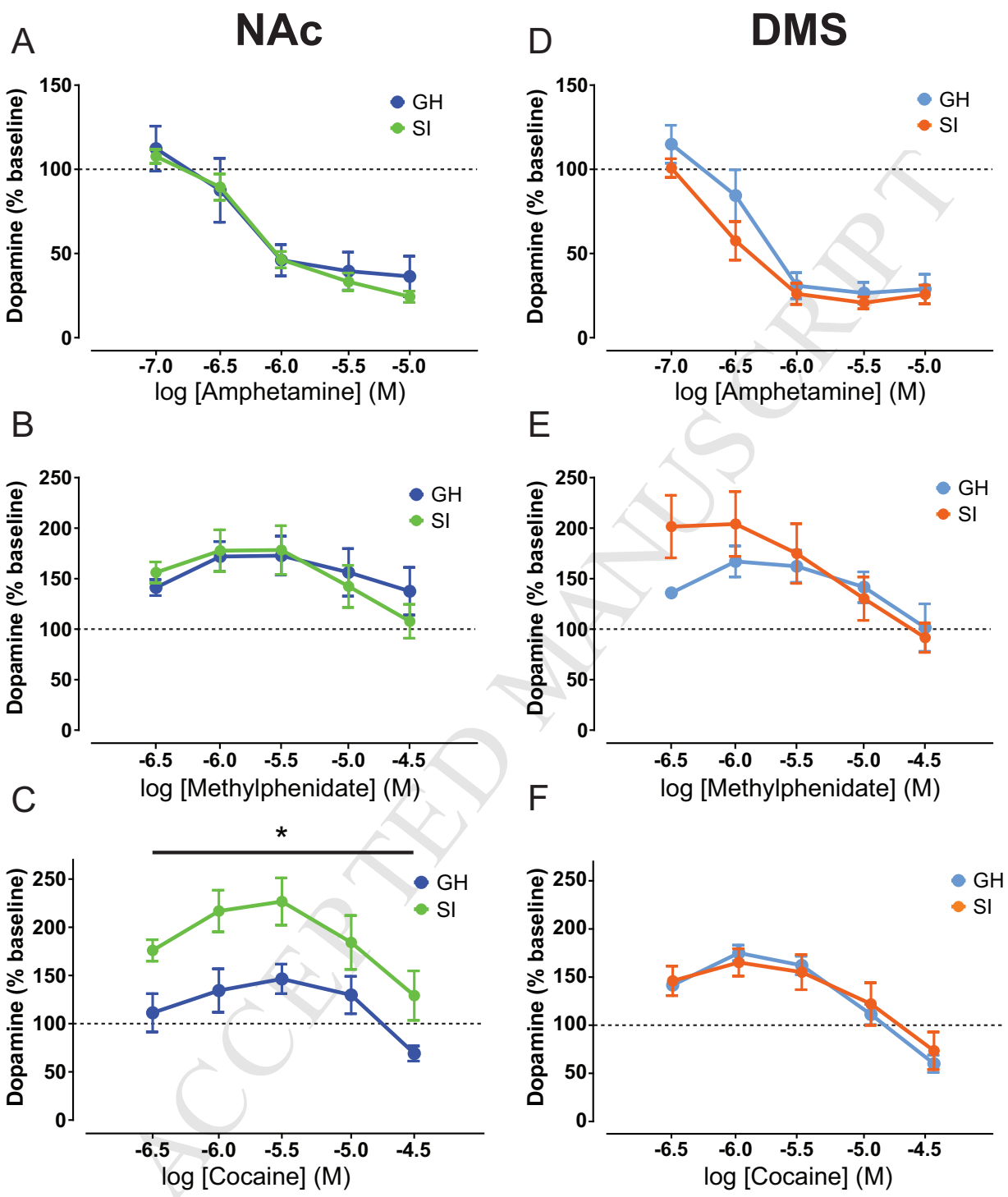

figure 6

Isolation rearing enhances accumbal and striatal dopamine release and uptake

Isolation rearing also increases psychostimulant effects on dopamine

Amphetamine and methylphenidate inhibit uptake greater in isolates

Cocaine does not inhibit uptake differently between isolates and grouped animals

However, cocaine increases evoked dopamine release greater in isolates