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ORIGINAL INVESTIGATION

Contributions of prolonged contingent and non-contingent cocaine exposure to escalation of cocaine intake and glutamatergic gene expression

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Abstract

Similar to the pattern observed in people with substance abuse disorders, laboratory animals will exhibit escalation of cocaine intake when the drug is available over prolonged periods of time. Here, we investigated the contribution of behavioral contingency of cocaine administration on escalation of cocaine intake and gene expression in the dorsal medial prefrontal cortex (dmPFC) in adult male rats. Rats were allowed to self-administer intravenous cocaine (0.25 mg/infusion) under either limited cocaine—(1 h/day), prolonged cocaine—(6 h/day), or limited cocaine—(1 h/day) plus yoked cocaine-access (5 h/day); a control group received access to saline (1 h/day). One day after the final self-administration session, the rats were euthanized and the dmPFC was removed for quantification of mRNA expression of critical glutamatergic signaling genes, *Homer2*, *Grin1*, *and Dlg4*, as these genes and brain region have been previously implicated in addiction, learning, and memory. All groups with cocaine-access showed escalated cocaine intake during the first 10 min of each daily session, and within the first 1 h of cocaine administration. Additionally, the limited-access + yoked group exhibited more non-reinforced lever responses during self-administration sessions than the other groups tested. Lastly, *Homer2*, *Grin1*, and *Dlg4* mRNA were impacted by both duration and mode of cocaine exposure. Only prolonged-access rats exhibited increases in mRNA expression for *Homer2*, *Grin1*, and *Dlg4* mRNA. Taken together, these findings indicate that both contingent and non-contingent "excessive" cocaine exposure supports escalation behavior, but the behavioral contingency of cocaine-access has distinct effects on the patterning of operant responsiveness and changes in mRNA expression.

Keywords Cocaine · Self-administration · Escalation · Contingent access · Non-contingent access

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Introduction

Cocaine addiction is a chronic disorder that persists in spite of negative interpersonal, professional, and physical consequences, with the development of tolerance and increased cocaine intake serving as central diagnostic criteria for stimulant use disorders (APA 2013). It has been argued that one major reason for the escalation of cocaine intake relates to gradual changes in motivation and learning processes associated with responses to the positive and negative reinforcing properties of cocaine (Koob 2004). In order to model the differences in cocaine use observed in humans, differential access to cocaine self-administration has been employed as an avenue to study the behavioral and neurobiological aspects of drug-taking. Ahmed and Koob (1998) demonstrated that "limited" daily-access

(1 h/ day) and "prolonged" daily-access conditions (6 h/ day) are distinct in their ability to model aspects of drug abuse/addiction in two major ways: (1) rats with prolonged cocaine-access escalate their cocaine intake across daily sessions, whereas the limited-access rats exhibit stable intake for several weeks and 2) rats with daily "prolonged" cocaine-access dramatically increase intake of cocaine for the 1st 10 min of self-administration while "limited" access rats do not escalate cocaine intake within the 1st 10 min of self-administration (Ahmed and Koob 1998). This differential pattern of responding has since been replicated with a number of procedural variations to determine the underlying changes in drug-taking behavior (Ahmed and Koob 1999), neurocircuitry (Ahmed et al. 2002; Ben-Shahar et al. 2012; Robinson and Kolb 2004), and cellular/ molecular function in rats (Ben-Shahar et al. 2009, 2013), as well as in other species (Kirkland Henry et al. 2009; Nakamura et al. 2011).

Despite substantial study, it remains to be determined whether escalated cocaine intake (and the neurobiological changes associated there with) is dependent upon the amount of cocaine exposure or the act of self-administering the cocaine. Indeed, escalated responding and intake of non-drug reinforcers can be achieved by prolonged access to liquid food, suggesting that the escalation phenomenon may be mediated by behavioral processes underlying the self-administration of appetitive stimuli (Goeders et al. 2009). One way to dissociate the relative contribution of total drug exposure from the behavioral contingency of that exposure is to employ yoked-access procedures (i.e., the administration of cocaine under the control of a separate self-administering rat). For instance, Hemby et al. (1997) investigated the effects of cocaine under response-dependent and -independent conditions in a yoked-triad. Both self-administering and yoked-access rats exhibited elevated levels of dopamine in the nucleus accumbens during the first hour of self-administration, but the self-administering animals exhibited greater dopamine levels than their yoked-counterparts (Hemby et al. 1997). Additionally, relative to rats self-administering cocaine, yoked rats exhibit differential corticosterone levels both in systemic plasma (Galici et al. 2000) and brain (Palamarchouk et al. 2009), have a higher morbidity rate (Dworkin et al. 1995), and exhibit higher indices of distress (measured by ultrasonic vocalization) (Mutschler and Miczek 1998). These studies demonstrate that, despite equivalent cocaine dosing, animals given contingent-access to cocaine exhibit distinct behavioral and neurobiological effects compared to animals with non-contingent access.

To expand upon the role of behavioral contingency in the behavioral sequelae of cocaine exposure, Kippin et al. (2006) employed a novel mixed self-administration/yoked cocaine exposure procedure in which rats received 1-h access to cocaine self-administration before receiving non-contingent cocaine infusions via yoking procedures during the last 5 h of cocaine self-administration. Although escalated cocaine intake was not observed in this earlier study, both the contingent and non-contingent excessive cocaine exposure groups exhibited greater cue-induced reinstatement than rats with a history of limited cocaine-access only. However, only the prolonged contingent-access rats exhibited greater cocaine-primed reinstatement of responding. In the present study, we employ the prolonged-access and limited-access + yoked procedures to determine the impact of contingent and non-contingent "excessive" cocaine exposure on the escalation of cocaine intake and operant responding for cocaine to determine how behavioral contingency of cocaine delivery influences these aspects of cocaine addiction-related behavior.

The dorsomedial prefrontal cortex (dmPFC) is dysregulated in human cocaine addicts (Verdejo-Garcia et al. 2015), and this dysregulation is linked to aberrant learning and plasticity that is attributed to anomalies in glutamate transmission (Kalivas et al. 2005; Pascoli et al. 2014; Ruan and Yao 2017b). To explore the potential neurobiology underpinning the effects of cocaine on the dmPFC as a function of contingency, we measured the levels of mRNA for *Homer2*, *Grin1*, and *Dlg4* within the dorsomedial prefrontal cortex (dmPFC). Homer2 is a glutamate receptor scaffolding protein that is up-regulated within PFC by both non-contingent and contingent cocaine administration (Ary and Szumlinski 2007; Ary et al. 2013; Ben-Shahar et al. 2009; Gould et al. 2015). While it remains to be determined whether or not cocaine-induced increases in PFC Homer2 protein expression reflects increased gene transcription, Homer2 expression bi-directionally regulates both basal and cocaine-induced changes in extracellular glutamate levels within PFC (Ary et al. 2013) to influence cocaine-conditioned approach behavior in place-conditioning models (Ary et al. 2013), and cocaine-primed reinstatement of lever-pressing behavior in operant-conditioning models (Gould et al. 2015). *Grin1* mRNA encodes the obligatory N-methyl-D-aspartate (NMDA) receptor subunit GluN1 within the mammalian brain (Bai and Hoffman 2009); this receptor is widely implicated and critical for many forms of plasticity (Bear 1996; Hopf 2017; Sweatt 2016; Thiels et al. 1996), and is up-regulated in the PFC following cocaine exposure (Ary and Szumlinski 2007; Blanco et al. 2014; Hemby et al. 2005). *Dlg4* encodes the sequence for postsynaptic density-95 (PSD-95), a receptor scaffolding protein that regulates plasticity and learning through its interactions with the NMDA receptor (Wang and Peng 2016). PSD-95 expression in the PFC is increased after prolonged withdrawal from cocaine (Ghasemzadeh et al. 2009; McIntosh et al. 2013) and following extinction testing during prolonged exposure to cocaine self-administration (Ghasemzadeh et al. 2011).

Methods

Subjects

Male Sprague-Dawley rats were pair-housed in a 12-h reverse light-dark cycle room and had ad libitum access to food and water (except as noted below). The housing and care of the rats followed the guidelines set forth by the "Guide for the Care and Use of Laboratory Rats, 8th Edition" (NIH 2011).

Surgery

Male Sprague-Dawley rats weighing 300–350 g were deeply anesthetized using ketamine (60 mg/kg) and xylazine (10 mg/kg). Chronic indwelling catheters were constructed using a bent steel cannula with a screw-type connector (Plastics One, Roanoke, VA), SILASTIC tubing (11 cm, i.d. 0.64 mm, o.d. 1.19 mm, Dow Corning, Midland, MI), Prolite polypropylene monofilament mesh (Atrium Medical Corporation, Hudson, NH), a silicon ball 2.5 cm from the end, and methyl methacrylate dental cement. The catheters were implanted and maintained as we have reported previously (Ben-Shahar et al. 2013; Kerstetter et al. 2008). Naïve rats were left in the vivarium and handled daily, but had no access to behavioral training or surgery. They were euthanized at the same age as the rats undergoing behavioral training.

Behavioral training

Food training and cocaine self-administration utilized standard operant chambers (Med Associates Inc., St. Albans, VT, USA) and were conducted during a fixed time in the dark phase of the rat's circadian cycle each day. Before surgical implantation of the jugular catheters, the rats were restricted to 20 g of food for 1 week and trained on a fixed ratio 1 (FR1) schedule of food reinforcement for two 16-h training sessions where each right lever press was associated with a 45 mg food pellet (Ben-Shahar et al. 2012). After recovery from the surgery, the rats were placed on a fixed ratio 1 (FR1) schedule of reinforcement for intravenous (IV) cocaine (0.1 mL at 0.25 mg/infusion in 0.9% saline) or saline for 1 h/day for 5 days. Each active lever-press was associated with a 4-s infusion of cocaine or saline and a 20-s timeout was signaled with a 20-s light cue above the active lever (Ben-Shahar et al. 2012). On the 6th day, the cocaine rats were divided into limited (1 h/day) cocaine-access, prolonged (6 h/day) cocaine-access, and limited + yoked-access (1 h access followed by 5 h of yoked exposure) treatment groups and continued the FR1 schedule of reinforcement for an additional 15 days. During yoked exposure, rats remained in their chambers for an additional 5 h with the levers retracted to eliminate the opportunity to perform the operant response. During this time, yoked rats received a 4-s cocaine infusion every time a paired prolonged-access rat self-administered an infusion, but without cue light presentation.

Tissue collection and mRNA quantification

Twenty-four hours after the last self-administration session, the animals were sacrificed via rapid decapitation, and their brains were frozen over ice and dissected into 0.5-mm sections with a metal brain mold (Braintree Scientific, Braintree, MA). The dmPFC was dissected out at 3.24 to 2.74-mm anterior to bregma and stored at – 80° C (Fig. 1). The frozen dmPFC was added to 600 μL of buffer RLT (Qiagen DNA/RNA/Protein extraction kit) and homogenized with the Qiagen TissueRuptor for 30 s. RNA was then extracted through use of the AllPrep DNA/RNA/protein extraction kit provided by Qiagen in accordance to the protocol provided by the manufacturer. RNA was eluted from the spin column with 50 μL of nuclease-free water. RNA (500 ng/sample) was incubated with 2 μL of gDNA Wipeout Buffer (Qiagen) at 42 °C for 2 min then cooled over ice. One microliter of Reverse Transcription Master Mix (Qiagen), 1 μL of RT primer mix (Qiagen), and 4 μL of Quantiscript RT Buffer (Qiagen) were added to the reaction mixture and incubated in an Eppendorf MasterCycler at 42 °C for 18 min to amplify the product, then incubated at 95 °C for 3 min to inactivate the reverse transcriptase. A reverse transcriptase-negative reaction was carried out in parallel with the samples from 500 ng of pooled sample RNA.

Levels of mRNA were assessed in triplicate using quantitative real-time PCR (qRT-PCR) (Biorad) on the BioRad CFX96 Touch Real-Time system. Negative controls consisted of a DNA-negative sample and a reverse transcriptase free sample. Standard curves were run on each PCR plate with ×3 serial dilutions ranging from 50.0 to 1.85 ng/uL. The data were normalized using three control genes (Gapdh, βActin,

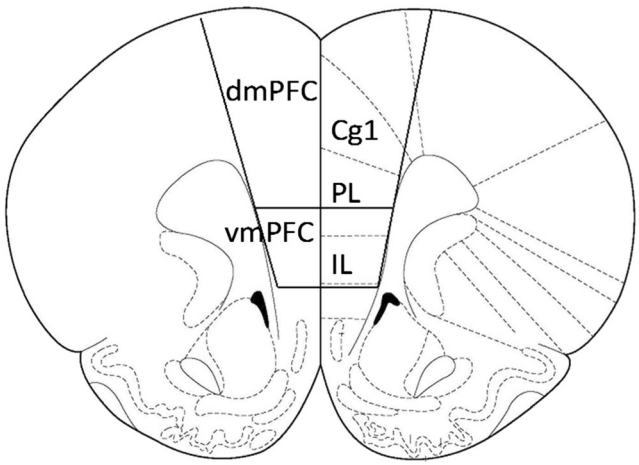

Fig. 1 dmPFC dissection of the rat brain. The brain was dissected horizontally between bregma + 3.24 and bregma + 2.74 (Paxinos and Watson 2006)

and Tubb5) and dmPFC tissue from naïve age-matched rats according to the equations outlined by Hellemans et al. 2007 (Table 1).

Statistical analyses

The self-administration data for individual sessions during differential access to cocaine were compared to baseline responding (average of days 6, 7, and 8 of differential access) at 10 min (i.e., loading phase; e.g., Ahmed and Koob 1998) and 1-h intervals to compare across all four conditions, as well as across the entire 6-h sessions for the prolonged-access and limited-access + yoked groups. Separate two-way, between-within (group X day), repeated measures ANOVAs, were conducted for cocaine intake followed by Dunnett post-hoc comparisons to deconstruct significant interactions/main effects using the Prism 6 statistical software (Graphpad). Non-reinforced responding (i.e., during the time-out period) was analyzed separately from total responding via a two-way, between-within (group X day), repeated measures ANOVAs followed by Tukey's post-hoc comparison for the first 10 min and 1 h of self-administration to assess the efficiency of behavioral responding before (days 4 and 5) and after differential access (days 6 to 20). Inactive lever presses were also analyzed but no significant effects or interactions were detected, and in all cases, mean inactive lever presses were < 5 (Fig. 2c). Additionally, self-administration data was separated into separate 10-min blocks on day 6 and day 20 to assess loading during initial daily access. A two-way ANOVA (time block X condition) was conducted to assess the differences in cocaine intake during 10-min time blocks for the first hour of self-administration in all four experimental groups during the 6th day and 20th day of self-administration; Tukey's post-hoc comparison was used to deconstruct significant interactions using the Prism 6 software. Lastly, analyses of normalized quantitative PCR data were performed by one-way MANOVA for *Homer2*, *Grin1*, and *Dlg4* mRNA and decomposed via post-hoc LSD tests using SPSS Statistics 24 (IBM). All graphics were plotted by using the Prism 6 statistical software (Graphpad).

Results

Cocaine intake

A two-way repeated measures ANOVA of cocaine intake (mg/kg) during the loading phase of self-administration sessions (first 10 min) revealed significant within-group effects of time ($F_{15,\ 2055} = 8.049$, $p < 0.0001$ Fig. 2a), between-group effects of treatment ($F_{3,\ 137} = 82.32$, $p < 0.0001$, Fig. 2a), and an interaction between time and treatment ($F_{45,\ 2055} = 2.88$, $p < 0.0001$ Fig. 2a). Within-group (aka time) differences were

Table 1 Primer concentrations, size, and run conditions for qPCR experiments. Primers were created using Primer3 software and verified with PrimerBLAST and a 2% agarose gel

Target	Accession number	Forward (5′ ≥ 3′)	Reverse (5′ ≥ 3′)	Amplicon length (bp)	Annealing temp	Primer concentration
Homer2 mRNA	NM_053309	GAGTGCTGCCAATGTGAAGA	TTGATCTCACCGCACTGTTC	195	61° C	30 nM
Grin1 mRNA	NM_001270610.1	CGGCTCTTGGAAGATACAGC	GTGGGAGTGAAGTGGTCGTT	156	60° C	20 nM
Dlg4 mRNA	NM_019621.1	CCGACAAGTTTGGATCCTGT	CCCATAGAGGTGGCTGTTGT	164	60° C	20 nM
Gapdh mRNA	NM_017008.4	AGAACATCATCCCTGCATCC	AGGAGACAACCTGGTCCTCA	240	61° C	20 nM
βActin mRNA	NM_031144	TGTCACCAACTGGGACGATA	GGGGTGTTGAAGGTCTCAAA	165	63° C	20 nM
Tubb5 mRNA	NM_139254	TGAGGCCTCCTCTCACAAGT	TGCAGGCAGTCACAATTCTC	237	62° C	20 nM

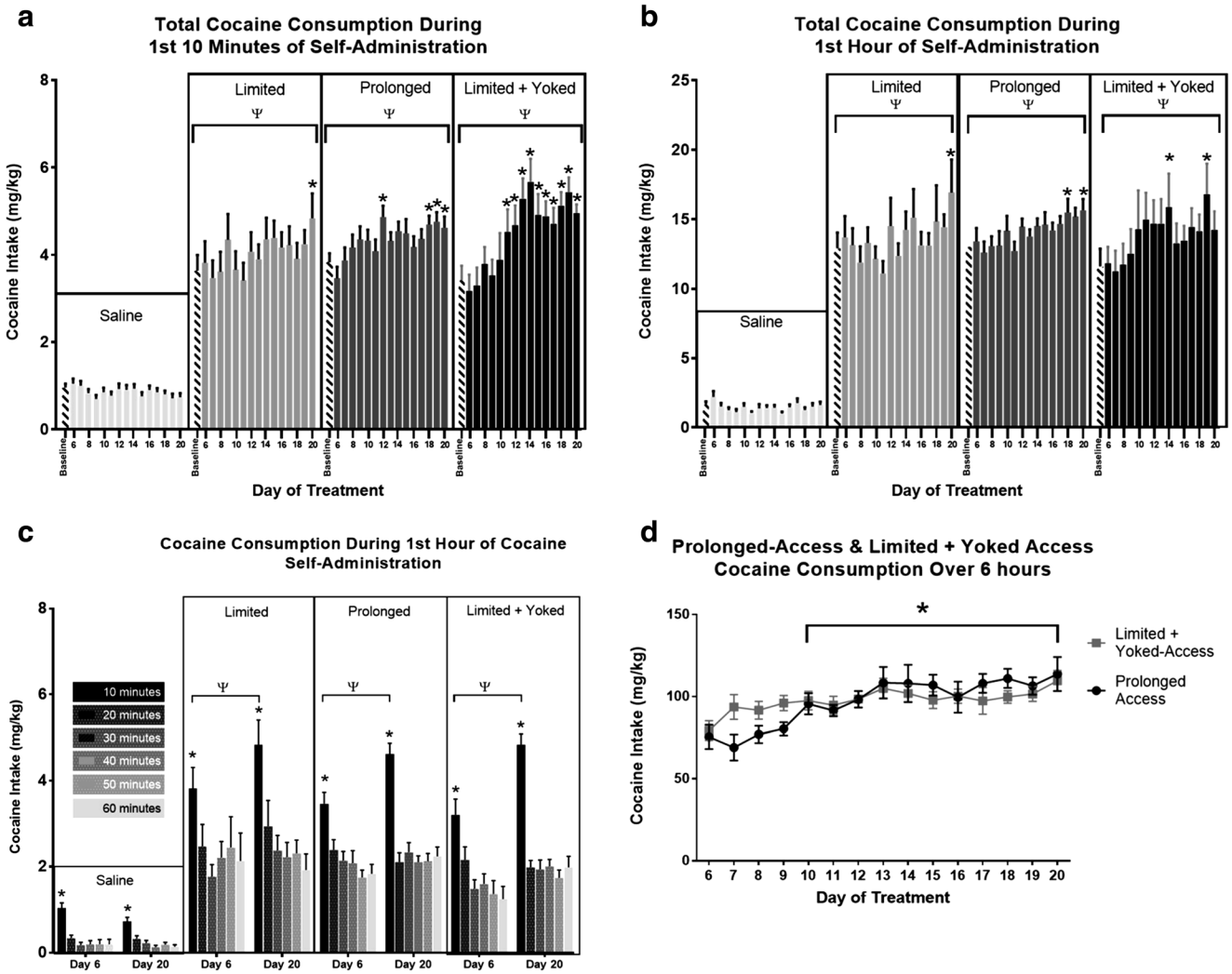

Fig. 2 Cocaine intake during self-administration. * within-group differences, ψ between-group differences. **a** 1st 10 min of self-administration. Limited-access rats escalated cocaine intake on day 20 of self-administration. Prolonged-access rats exhibited escalated cocaine intake on days 12, 18, 19, and 20 of self-administration. Limited + yoked-access rats escalated cocaine intake in between days 11 and 20 of self-administration (*$p < 0.05$). All cocaine-access groups had higher intake than saline-access groups (ψ, $p < 0.0001$). **b** 1st 1 h of self-administration. Limited-access rats escalated cocaine intake on day 20. Prolonged-access rats escalated cocaine intake of days 18 and 20. Limited + yoked-access rats escalated cocaine in take days 14 and 19 (*$p < 0.05$). All cocaine-access groups had higher intake than saline-access groups (ψ, $p < 0.0001$). **c** Cocaine intake recorded every 10 min during 1st hour of self-administration. Limited-access, prolonged-access, and limited + yoked-access rats all displayed increased cocaine intake during the first 10 min of self-administration sessions on both day 6 and day 20 of self-administration. Additionally, prolonged-access and limited + yoked-access rats exhibited increased cocaine intake within the 1st 10 min of self-administration between day 6 and day 20. (*$p < 0.05$, ψ $p < 0.05$). **d** Total daily cocaine intake for prolonged- and limited + yoked-access rats. Cocaine intake escalated in both groups beginning on the 10th day of extended access to cocaine (*$p < 0.05$). Total cocaine intake did not differ prolonged- and limited + yoked-access rats

assessed via Dunnett's post-hoc test and revealed increases in cocaine intake for day 20 vs baseline in the limited-access group, days 12, 18, 19, and 20 vs baseline in prolonged-access group ($p < 0.05$), and days 11 through 20 vs baseline in limited + yoked-access rats ($p < 0.05$) (Fig. 2a). These results indicate an escalation of cocaine intake in all cocaine-access groups for the first 10 min of self-administration. The limited + yoked-access rats exhibited escalation first (day 11), which was slightly faster than prolonged-access rats (day 12), and much faster than limited-access rats (day 20). However, limited + yoked-access rats had a slightly lower baseline than prolonged-access rats, so the difference in escalation between the two groups is likely negligible. Additionally, between-group (aka treatment) differences were assessed via Tukey's post-hoc comparison test and revealed that all cocaine-access conditions had significantly higher intake than the saline-access condition (Fig. 2a).

The first hour of drug intake (mg/kg) during self-administration was also assessed via a two-way repeated measures ANOVA and revealed significant within-group effects

of time ($F_{15, 1875} = 4.121$, $p < 0.0001$ Fig. 2b), between-group effects of treatment ($F_{3, 125} = 88.64$, $p < 0.0001$ Fig. 2b), and an interaction between time and treatment ($F_{45, 1875} = 1.385$, $p < 0.05$; Fig. 2b). Dunnett's multiple comparisons post-hoc analysis showed increased cocaine intake in the limited-access rats for day 20 vs baseline ($p < 0.05$), prolonged-access rats for days 18 and 20 vs baseline ($p < 0.05$), and in limited + yoked-access rats for days 14 and 19 vs baseline ($p < 0.05$). These data indicate a distinct difference in the time of onset for escalated cocaine intake between prolonged-access and limited + yoked-access rats.

Additionally, we divided cocaine intake for the first 1 h of self-administration into 10-min blocks and assessed intake on day 6 and day 20 of self-administration between different conditions (within-subjects effects), as well as cocaine intake between days and conditions (between-subjects effects) with a three-way repeated measures ANOVA. Within-subjects tests revealed significant effects of minutes (aka 10-min time blocks, $F_{5, 270} = 61.940$, $p < 0.0001$), minutes × condition ($F_{5, 272} = 8.771$, $p < 0.0001$), minutes × day ($F_{5, 270} = 3.975$, $p < 0.0001$), and minutes × day × condition ($F_{5, 272} = 3.439$, $p < 0.005$) (Fig. 2c). Within-subject effects were assessed via Dunnett's post-hoc test and revealed significant increases in cocaine intake during the first 10 min of self-administration for all cocaine groups compared to other time points ($p < 0.05$). Between-subjects tests revealed a significant effect of day ($F_{1, 274} = 4.171$, $p < 0.05$), where day 20 has higher intake than day 6, as well as significant effects of condition ($F_{3, 274} = 68.624$, $p < 0.0001$). Tukey's post-hoc comparison revealed significantly higher levels of intake for all cocaine-access conditions compared to the saline-access condition ($p < 0.0001$). These results indicate that cocaine intake was highest during the first 10 min of self-administration (aka binge behavior), and that intake increased between day 6 and day 20 for all cocaine-access conditions (aka escalation, Fig. 2c).

Lastly, we looked at total cocaine intake between prolonged-access and limited + yoked-access rats. A two-way repeated measures ANOVA was run to assess any differences in total cocaine intake (mg/kg) over the full 6 h of the experiment (Fig. 2d). The results revealed a significant effect of time ($F_{14, 392} = 6.874$, $p < 0.0001$), but no effect for treatment ($F_{1, 28} = 0.0318$, $p = 0.8597$); however, there was a significant interaction between time and treatment ($F_{14, 392} = 1.779$, $p < 0.05$). Dunnett's post-hoc comparison revealed that both the prolonged-access and limited + yoked-access conditions exhibited escalated cocaine intake from day 12 to day 20. Additionally, there was no difference between prolonged-access and limited + yoked-access conditions. These data indicate that both groups had increased cocaine exposure from baseline and, expectedly, as the majority of daily intake in both groups was controlled by prolonged-access rats, there is no observable difference in cocaine exposure between these two conditions (Fig. 2d).

Non-reinforced lever responding

In addition to cocaine intake, we analyzed non-reinforced lever responding. A two-way repeated measures ANOVA of the numbers of non-reinforced active lever responses (aka active lever responses – number of infusions) during the first 10 min of self-administration revealed an effect of treatment ($F_{3, 134} = 5.236$, $p < 0.005$) and an interaction between time and treatment ($F_{51, 2278} = 2.183$, $p < 0.0001$, Fig. 3a). Dunnett's post-hoc comparison was used to assess within-group differences and indicated a significant increase in non-reinforced lever responding for only the limited + yoked-access rats on days 12, 13, 14, 15, 18, and 19 versus baseline ($p < 0.05$, Fig. 3a). Furthermore, between-group differences were analyzed via Tukey's post-hoc test; limited + yoked-access rats exhibited significantly higher non-reinforced lever responding than other groups on days 10 to 19 ($p < 0.05$, Fig. 3a). These data indicate that for the first 10 min of self-administration, the limited + yoked-access rats exhibited significantly more non-reinforced lever responding than limited-access and prolonged-access conditions well after differential access to cocaine was initiated.

Non-reinforced lever responding was also assessed over the course of the first 1 h of self-administration. A two-way repeated measures ANOVA of the non-reinforced lever responding during the first 1 h of self-administration revealed a significant effect of time ($F_{17, 2210} = 4.31$, $p < 0.001$), and effect of treatment ($F_{3, 130} = 7.979$, $p < 0.0001$), and an interaction between time and treatment ($F_{51, 2210} = 1.379$, $p < 0.05$; Fig. 3b). Dunnett's multiple comparisons post-hoc analysis was used to break down within-group effects and revealed a significantly larger number of non-reinforced active lever responses for limited-, prolonged-, and limited + yoked-access conditions on the 4th day of self-administration ($p < 0.05$). In addition, Tukey's post-hoc comparisons revealed significant between-group effects; all cocaine rats engaged in more non-reinforced lever responses than saline-access rats on day 4 of self-administration, and the limited + yoked-access rats had more non-reinforced lever responses than all other groups on days 12, 14, and 18 of self-administration (Fig. 3b). These data indicated two main points: (1) before differential access, all cocaine-access rats exhibited non-reinforced lever responding at day 4 of self-administration and (2) only limited-access + yoked-access rats continued non-reinforced lever responding later in the experiment.

Lastly, inactive-lever responding was analyzed via a two-way repeated measures ANOVA. There was no effect of time nor was there an interaction between time and treatment, but there was a main effect of treatment ($F_{3, 133} = 9.769$, $p < 0.0001$). Tukey's post-hoc test was used to assess between-group differences and revealed more inactive-lever responding in the saline-access rats compared to the prolonged-access rats on days 10, 11, 12, 14, and 19 of self-

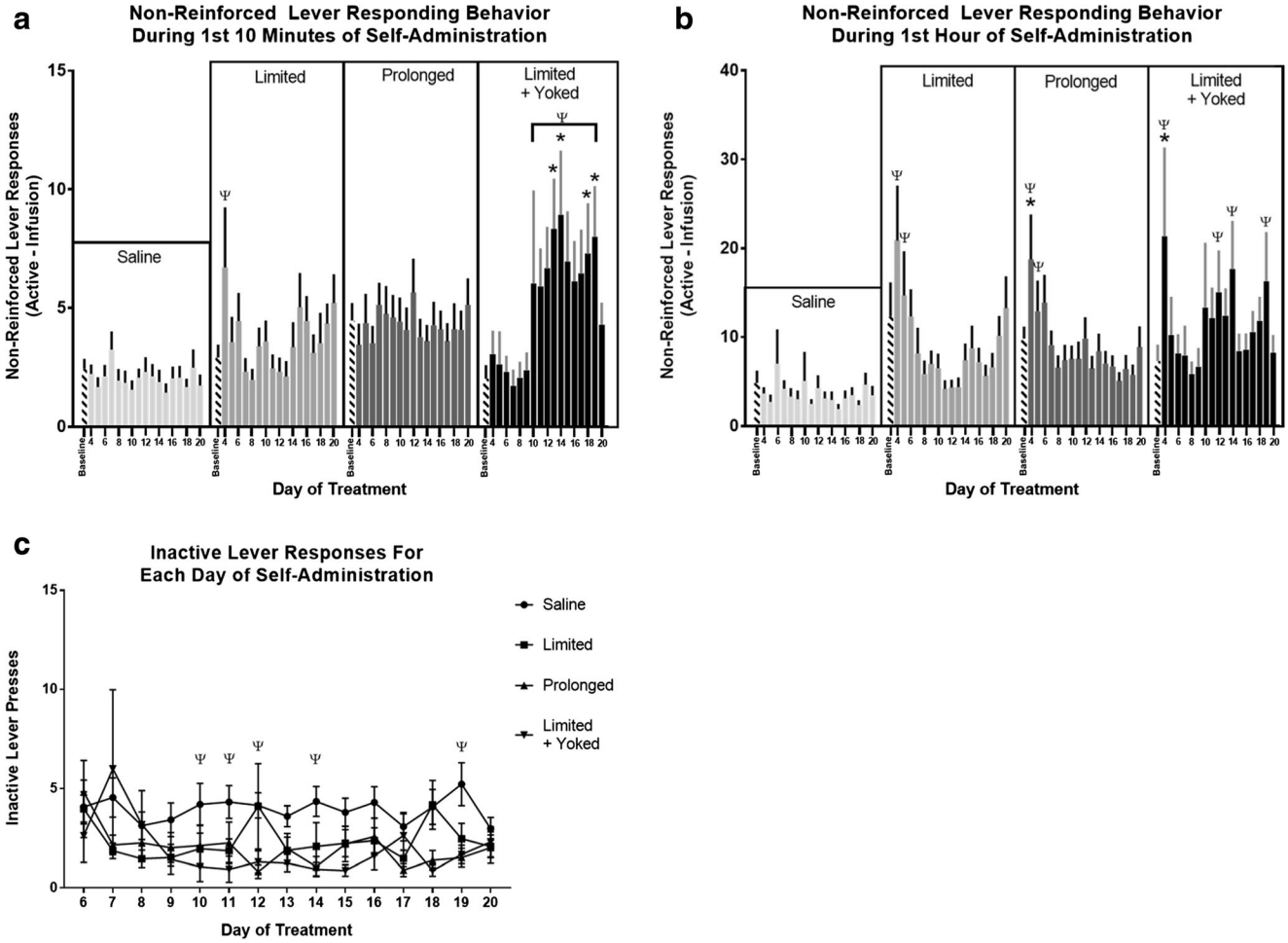

Fig. 3 Non-reinforced lever responding behavior. * within-group differences, ψ between-group differences. **a** 1st 10 min of self-administration. Only limited + yoked-access rats exhibited high levels of non-reinforced cocaine responding on days 12, 13, 14, 15, 18, and 19 compared to baseline (* < 0.05), and compared to other treatment conditions on days 10–19 (ψ < 0.05). **b** 1st 1 h of self-administration. All cocaine-access groups showed initially high non-reinforced active-lever responding on day 4 of self-administration (* < 0.05, ψ < 0.05), and limited + yoked-access rats exhibited more non-reinforced lever responding than other conditions for the first 1 h of self-administration on days 12, 14, and 18 (ψ < 0.05). **c** Inactive lever responses over entire day. Saline-access rats exhibited higher inactive lever responding than prolonged-access rats on days 10, 11, 12, 14, and 19 of self-administration (ψ < 0.05)

administration (Fig. 3c). However, the mean differences (~ 3 lever responses) between these two conditions are not particularly meaningful in comparison to the total number of lever responses for cocaine administration. Therefore, these data indicate a minor increase in inactive lever responses within the saline-access rats.

Quantitative real-time PCR of mRNA

A one-way MANOVA of mRNA expression for *Homer2*, *Grin1*, and *Dlg4* resulted in a significant main effect of condition (Hotelling's trace = 0.652, $F_{12,\ 110}) = 1.991$, $p < 0.05$). LSD post-hoc pair-wise comparisons revealed greater *Homer2* mRNA expression in the prolonged-access group relative to the naïve, saline, and limited-access + yoked groups (Fig. 4a). LSD post-hoc pair-wise comparisons also revealed increased *Grin1* mRNA expression in the prolonged-access group compared to naïve, saline, limited-access, and limited + yoked-access groups (Fig. 4b). Lastly, LSD post-hoc pair-wise comparisons revealed increased levels of *Dlg4* mRNA expression in the prolonged-access group compared to the naïve and limited-access + yoked-access groups (Fig. 4c). These data indicate that prolonged-access rats have a unique molecular phenotype, even though rats in the limited + yoked-access group received equivalent amounts of cocaine and escalated cocaine intake at about the same rate as the prolonged-access rats.

Additionally, Pearson correlation coefficients were calculated for total cocaine intake versus mRNA expression. *Homer2* expression was correlated positively with cocaine exposure for both prolonged-access groups ($R^2 = 0.1975$, $p < 0.05$) and limited-access ($R^2 = 0.4763$, $p < 0.05$) groups, whereas mRNA expression correlated negatively for the limited + yoked-access group ($R^2 = 0.4046$,

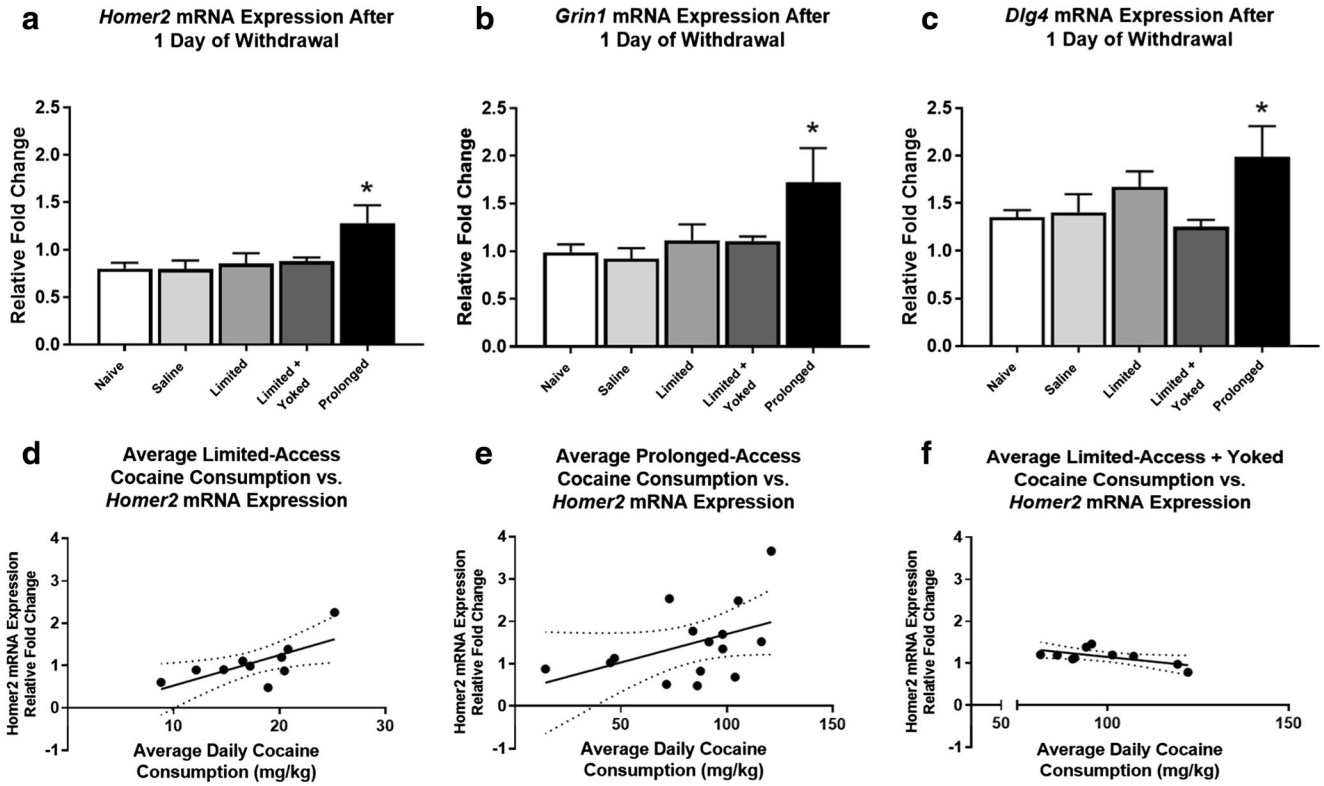

Fig. 4 mRNA expression for glutamatergic genes in the dmPFC. **a** Prolonged access to cocaine resulted in increased levels of *Homer2* mRNA within the dmPFC after 1 day of withdrawal (*$p < 0.05$). **b** Prolonged access to cocaine resulted in increased levels of *Grin1* mRNA within the dmPFC after 1 day of withdrawal (*$p < 0.05$). **c** Prolonged access to cocaine resulted in increased levels of *Dlg4* mRNA within the dmPFC after 1 day of withdrawal (* $p < 0.05$). **d** *Homer2* mRNA was positively correlated with total cocaine infusions in the prolonged access group ($R^2 = 0.1975$, $p < 0.05$). **e** *Homer2* mRNA was positively correlated with total cocaine infusions in the limited-access group ($R^2 = 0.4763$, $p < 0.05$). **f** *Homer2* mRNA was negatively correlated with total cocaine infusions in the limited + yoked-access group ($R^2 = 0.4046$, $p < 0.05$)

$p < 0.05$) (Fig. 4d, e, f). Pearson correlation coefficients failed to reveal significant correlations between total cocaine intake versus *Grin1* and *Dlg4* mRNA (p's > 0.05).

Discussion

The major finding of the present study is that rats self-administering cocaine under all three cocaine-access conditions exhibited escalation of cocaine intake, but with distinct temporal and molecular profiles. Rats in all self-administration conditions escalated their cocaine intake during the "loading phase" of self-administration (i.e., the first 10 min, Fig. 2a, c), during the first 1 h of self-administration, and over entire daily sessions (Fig. 2b, d). Additionally, both prolonged-access and limited + yoked-access conditions escalated cocaine intake faster than limited-access animals. However, despite equivalent "excessive" cocaine exposure, the associated patterns of active lever responding are distinct between prolonged-access and limited + yoked-access groups, with the limited + yoked-access condition exhibiting more non-reinforced lever responding on the active lever during the first 10 min of self-administration (i.e., during the time-out period which did not result in cocaine infusion; see Fig. 3). This suggests that partially distinctive behavioral mechanisms may underlie the escalation of cocaine intake induced by contingent versus non-contingent cocaine exposure. Furthermore, we observed an overall increase in *Homer2*, *Grin1*, and *Dlg4* mRNA expression only in the prolonged-access rats, and total cocaine exposure and *Homer2* mRNA expression are positively correlated in both prolonged- and limited-access conditions but negatively correlated in the limited + yoked-access condition indicating distinct neurobiological consequences of both amount and mode of cocaine exposure. Thus, the present study demonstrates that escalation of cocaine-taking induced by differential cocaine-access, both with respect to session duration and behavioral contingency of cocaine delivery, can produce distinct behavioral and neurobiological consequences.

The finding that all cocaine-taking groups exhibited an escalation of drug intake, but at different rates and to different degrees, is generally consistent with prior findings. Although the capacity of prolonged drug-access to escalate drug-taking, over that observed under limited-access conditions, is a highly

replicable finding (e.g., Ahmed and Koob 1998; Ben-Shahar et al. 2004; Ben-Shahar et al. 2013), an escalation of cocaine intake is also reported in rats self-administering cocaine during slightly longer (2 h) daily sessions, albeit to a lesser extent than counterparts with daily 6-h access (Mandt et al. 2015). Further, some rat strains exhibit escalation under daily 1-h sessions of cocaine-access (Perry et al. 2006), particularly when allowed to self-administer cocaine over a protracted test period (e.g., 75 days) (Belin et al. 2009). The present report extends the literature on escalation by demonstrating that cocaine intake during the initial 10 min of the self-administration paradigm (i.e., the loading phase; see Ahmed and Koob 1998) also escalates with drug experience in limited-, prolonged-, and limited + yoked-access rats, but at somewhat different rates (Fig. 2). Thus, it appears that the loading phase (i.e., first 10 min) is more sensitive to escalation of intake than the overall duration of daily access.

The escalation of cocaine intake observed in the limited + yoked-access condition was additionally associated with a pronounced, but transient, increase in non-reinforced lever responding during cocaine-access (Fig. 3 and Supplemental Fig. 1). Marked differences in non-reinforced responding have been reported in the absence of differences in cocaine intake; e.g., in female relative to male rats (Fuchs et al. 2005; Kippin et al. 2006; Kosten and Zhang 2009); thus, the two measures appear generally dissociable. In the case of the limited + yoked-access rats in the present study, the rats had acquired responding for cocaine, accompanied by low levels of non-reinforced responding which was markedly elevated, particularly during the loading phase (Fig. 3a), following exposure to the yoking procedure. This disruption of "efficient" (i.e., lever presses are not tied to the drug reinforcer) operant behavior elicited by the addition of non-contingent cocaine exposure is likely due to discontinuous reinforcement schedules. Further, contextual cues can modulate escalation behavior; when rats are allowed alternating days of 1- and 6-h access to cocaine with differential cues, they only escalate during the 6-h sessions (Beckmann et al. 2012). An equally viable explanation for increased non-reinforced lever responding induced by non-contingent drug exposure may pertain to differences in the aversive, stress-inducing, and glucocorticoid-releasing properties of cocaine delivered under yoked procedures (Twining et al. 2009) which have been implicated in the processes underlying escalation (Mantsch et al. 2007, 2008). Thus, the combination of yoked cocaine with subsequent reintroduction to the operant chamber with differential cues (i.e., lever extension and operant light) appears to serve as a potent elicitor of lever responding. Furthermore, although it appears that limited + yoked-access rats escalate intake faster than prolonged-access rats, it is evident that escalation within the limited + yoked-access rats occurs alongside excessive non-reinforced lever responding (i.e., on days 12, 14, and 18, Fig. 3a, b).

Given the central role of drug-induced neuroadaptations, particularly alterations in glutamate function, in theories of addiction (see e.g., Kalivas and Volkow 2005), it is critical to discern the role of behavioral contingency in neurobiological changes associated with escalating drug intake. Here, we identified differences between the prolonged-access and limited + yoked-access conditions; we examined the dmPFC which has been implicated in the escalation of cocaine intake (Smith et al. 2008) for changes in the expression levels of molecular markers implicated in addiction (i.e., *Homer2*, *Grin1*, and *Dlg4* mRNA). Consistent with prior findings (Ben-Shahar et al. 2009), we observed increased levels of *Homer2* mRNA only in the prolonged-access condition, (Fig. 4a). Furthermore, the level of intake in the limited- and prolonged-access conditions correlated positively with *Homer2* mRNA expression; whereas, cocaine exposure in the limited + yoked-access group correlated negatively with *Homer2* mRNA expression (Fig. 4d–f). In addition, we also observed increases in *Grin1* and *Dlg4* mRNA within the dmPFC of the prolonged-access rats only (Figs. 4b–c). Overall, the differences between prolonged-access and limited + yoked-access conditions are consistent with other studies employing yoked procedures (Krawczyk et al. 2013; Ma et al. 2013; McFarland et al. 2003; Radley et al. 2015). However, the present finding furthers this literature by demonstrating that contingent and non-contingent cocaine exposure induces distinct neurobiological changes even when both are associated with escalation of cocaine intake, with only the prolonged-access condition producing elevation of several genes that are suggestive of enhanced glutamatergic signaling.

The increases in expression of glutamate-related genes observed, specifically following prolonged, contingent-access to cocaine, relates to the current literature concerning enhanced glutamate neurotransmission and cocaine-specific neuroplasticity in the prefrontal cortex. Repeated contingent-access to cocaine results in cocaine-specific synaptic plasticity including: increased dendritic spine density (Frankfurt et al. 2011), increased long-term potentiation (LTP) in the PFC (Huang et al. 2006), and lowered induction threshold for inducing cocaine-specific LTP (Ruan and Yao 2017a). Further, cocaine self-administration results in increased excitatory postsynaptic currents (EPSCs) between mPFC and NAC D1 medium spiny neuron synapses, and optogenetic inhibition of these synapses eliminates cocaine-seeking behaviors (Pascoli et al. 2014). Lastly, we have previously demonstrated that prolonged-access to cocaine enhances cue-elicited glutamate release during protracted withdrawal (Shin et al. 2016), and increased expression of the NMDA GluN2b receptor subunits during early and later withdrawal (Szumlinski et al. 2016). Other groups have also shown increases in NMDA, as well as AMPA and Kainate, receptor subunits after withdrawal from contingent cocaine self-administration (Crespo et al. 2002; Ghasemzadeh et al. 2009; Tang et al. 2004). These

previous studies, as well as the current report (Fig. 3), indicate that cocaine-specific plasticity of glutamatergic receptors develops with repeated contingent-access to cocaine and is necessary for cocaine-seeking behavior.

Briefly, *Homer2* is a gene encoding for a scaffolding protein that interacts with Group1 metabotropic glutamate receptors (mGluRs) and NMDA receptors and has been implicated in addictive behaviors (c.f., Szumlinski et al. 2008). Homer2a/b protein within the mPFC is increased following prolonged-access to cocaine (Ben-Shahar et al. 2009) and repeated cocaine injections (Ary and Szumlinski 2007). Further, viral-mediated *Homer2b* overexpression in the mPFC increases basal glutamate levels and cocaine-conditioned reward in mice; whereas, *Homer2b* knockdown reduces basal glutamate in this area (Ary et al. 2013). Additionally, *Grin1* encodes for the obligatory NR1 subunit of NMDA receptors, while *Dlg4* encodes for PSD-95, a scaffolding protein that regulates NMDA receptor function (Bai and Hoffman 2009). Increases in NR1 protein within the PFC have previously been observed in response to repeated cocaine injections (Kovacs et al. 2010) and cocaine self-administration (Hemby et al. 2005). Furthermore, NR1 is essential for cocaine-mediated learning; mice expressing a mutant version of the NR1 subunit (which reduces calcium flow through the NMDA receptor) fail to form conditioned place preference and locomotor sensitization in response to repeated cocaine exposure (Heusner and Palmiter 2005). PSD-95 is critical for synaptic plasticity and regulation of NMDA receptor location and function (Wang and Peng 2016). PSD-95 is also implicated in behavioral plasticity associated with chronic cocaine administration (Yao et al. 2004) and extinction of cocaine self-administration (Knackstedt et al. 2010; Ghasemzadeh et al. 2011), as well as prolonged withdrawal from cocaine (Ghasemzadeh et al. 2009; McIntosh et al. 2013). Thus, our RNA data is generally consistent with findings examining protein levels of glutamatergic signaling molecules with the increases in RNA observed here coinciding or preceding latent increases in protein.

Escalation of drug intake is an important diagnostic criterion of addiction in humans and an integral component in various theories of addiction. Therefore, understanding the behavioral and neurobiological underpinnings of escalation is likely to facilitate addiction management programs in humans. In addition to facilitating cocaine intake, prolonged daily access to cocaine is associated with several behavioral changes, such as reduced brain reward function (Ahmed et al. 2002; Ahmed and Koob 2005), increased breakpoints for cocaine reinforcement under progressive ratio schedules (Paterson and Markou 2004; Wee et al. 2009), diminished aversive properties of cocaine (Ben-Shahar et al. 2008), and increased extinction responding during protracted withdrawal (Ferrario et al. 2005), as well as increased responding during cocaine-primed and cue-induced reinstatement of cocaine-seeking (Ahmed and Cador 2006; Kippin et al. 2006; Knackstedt and Kalivas 2007; Mantsch et al. 2004). Similarly, other approaches to modeling "excessive" intake also produce increases in measures of cocaine-taking and -seeking behaviors (Deroche-Gamonet et al. 2004; Roberts et al. 2007). Further study is required to determine the relations between the nature of cocaine exposure and induction of addiction-like behavior, as well as between behavioral and molecular outcomes. To this end, the present study demonstrates behavioral contingency plays an important role in the nature of the behavioral and molecular changes induced by cocaine exposure.

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