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Functional Status of the Serotonin 5-HT_{2C} Receptor (5-HT_{2C}R) Drives Interlocked Phenotypes that Precipitate Relapse-Like Behaviors in Cocaine Dependence

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Relapse vulnerability in cocaine dependence is rooted in genetic and environmental determinants, and propelled by both impulsivity and the responsivity to cocaine-linked cues ('cue reactivity'). The serotonin (5-hydroxytryptamine, 5-HT) 5-HT_{2C} receptor (5-HT_{2C}R) within the medial prefrontal cortex (mPFC) is uniquely poised to serve as a strategic nexus to mechanistically control these behaviors. The 5-HT_{2C}R functional capacity is regulated by a number of factors including availability of active membrane receptor pools, the composition of the 5-HT_{2C}R macromolecular protein complex, and editing of the 5-HT_{2C}R pre-mRNA. The one-choice serial reaction time (1-CSRT) task was used to identify impulsive action phenotypes in an outbred rat population before cocaine self-administration and assessment of cue reactivity in the form of lever presses reinforced by the cocaine-associated discrete cue complex during forced abstinence. The 1-CSRT task reliably and reproducibly identified high impulsive (HI) and low impulsive (LI) action phenotypes; HI action predicted high cue reactivity. Lower cortical 5-HT_{2C}R membrane protein levels concomitant with higher levels of 5-HT_{2C}R:postsynaptic density 95 complex distinguished HI rats from LI rats. The frequency of edited 5-HT_{2C}R mRNA variants was elevated with the prediction that the protein population in HI rats favors those isoforms linked to reduced signaling capacity. Genetic loss of the mPFC 5-HT_{2C}R induced aggregate impulsive action/cue reactivity, suggesting that depressed cortical 5-HT_{2C}R tone confers vulnerability to these interlocked behaviors. Thus, impulsive action and cue reactivity appear to neuromechanistically overlap in rodents, with the 5-HT_{2C}R functional status acting as a neural rheostat to regulate, in part, the intersection between these vulnerability behaviors.
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INTRODUCTION

Cocaine dependence continues to extract considerable personal, health and societal tolls in the United States and worldwide. The cycling progressive nature of this chronic brain disorder stymies efforts to stay abstinent with vulnerability to abuse and relapse during abstinence often precipitated by impulsive behavior (Moeller *et al,* 2001a) and cocaine cue reactivity (Carpenter *et al,* 2006; Field and Cox, 2008). Impulsivity is a multifaceted construct that has been defined clinically as a predisposition toward rapid unplanned reactions to stimuli without regard to the negative consequences (Moeller *et al,* 2001b). Cue reactivity refers to the complex phenomenon comprising physiological (eg, heart rate), subjective (eg, craving), appetitive approach behaviors (eg, drug-seeking), and activation of specific corticostriatal subcircuits elicited by conditioned stimuli associated with motivationally salient events (Carter and Tiffany, 1999; Field and Cox, 2008; Koob and Volkow, 2010). We have recently demonstrated that levels of impulsive action correlate positively with cue reactivity as measured by attentional bias toward cocaine-associated cues in human cocaine-dependent subjects (Liu *et al,* 2011). There is evidence that high impulsivity (Green *et al,* 2009; Moeller *et al,* 2007) or cue reactivity (Carpenter *et al,* 2006) predicts reduced retention in outpatient treatment trials for cocaine dependence, and preclinical studies have established that high impulsive (HI) action predicts escalation of cocaine-taking (Dalley *et al,* 2007), progression to

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compulsive cocaine-taking (Belin *et al,* 2008), and punishment-resistant cocaine-seeking (Economidou *et al,* 2009). Further explorations are needed to delineate the interrelationship between impulsive action and cue reactivity, and understand the potential for shared neurobiology.

Corticostriatal connectivity is linked to both impulsive action and cue reactivity with medial prefrontal cortex (mPFC) of particular import (Chen *et al,* 2013; Dalley *et al,* 2011). The mPFC is densely innervated by serotonin (5-hydroxytryptamine; 5-HT) fibers in a distinct laminar organization (Linley *et al,* 2013), and 5-HT neurotransmission in the mPFC has been implicated in the control of impulsive action (Dalley *et al,* 2002) and cocaine-seeking (Bradberry and Rubino, 2004), particularly through the 5-HT$_{2C}$ receptor (5-HT$_{2C}$R) (Pentkowski *et al,* 2010). The ultimate level of functionality of the 5-HT$_{2C}$R is determined by a culmination of factors, including availability of active pools of receptors at the membrane and effective coupling to and activation of G-protein-dependent and -independent downstream signaling (Millan *et al,* 2008). The 5-HT$_{2C}$R functional status and subcellular localization can also be modulated by RNA editing, a mechanism that post-transcriptionally alters the coding properties of the RNA (Burns *et al,* 1997; Hoyer *et al,* 2002), and may impact alternative splicing mechanisms of the 5-HT$_{2C}$R (Martin *et al,* 2013). RNA editing of 5-HT$_{2C}$R reduces the efficiency of the interaction between the receptor and its signal transducers, which results in lower constitutive activity and signaling capacity (Burns *et al,* 1997). Thus, 5-HT$_{2C}$R neurotransmission is malleable at transcriptional (eg, editing and alternative splicing), translational (eg, synthesis and post-translational modifications), and topological (eg, trafficking and protein complex composition) levels, which are elegantly controlled and responsive to internal and external stimuli.

The importance of 5-HT$_{2C}$R function in the generation of impulsive action is supported by observations that the systemic administration of selective 5-HT$_{2C}$R agonists or 5-HT$_{2C}$R antagonists decrease and increase impulsive action, respectively (Anastasio *et al,* 2013a; Cunningham *et al,* 2012; Fletcher *et al,* 2011; Winstanley *et al,* 2004b). These effects reliably match the efficacy and directionality of the effects of these ligands in tasks that measure cocaine-seeking (Cunningham *et al,* 2011; Cunningham *et al,* 2012; Fletcher *et al,* 2008; Grottick *et al,* 2001). Despite the strong pharmacological data to support the involvement of the 5-HT$_{2C}$R in these behaviors (Cunningham and Anastasio, 2013), there is currently little to no information as to whether the functional status of the 5-HT$_{2C}$R in core brain circuits contributes to these vulnerability phenotypes. Here, we tested the hypothesis that dysregulation of 5-HT$_{2C}$R function in mPFC may represent a mechanism that confers high inherent impulsive action (but see, Robinson *et al,* 2008) and cocaine cue reactivity.

The present study investigated the mPFC 5-HT$_{2C}$R system as a neuromolecular driver of high inherent impulsive action and cocaine cue reactivity. The one-choice serial reaction time (1-CSRT) task, a simplified variant of the 5-CSRT task, is rapidly entrained and assesses deficits in behavioral control to appropriately withhold a prepotent response (action restraint) independent from complex visuospatial attentional processes (as in the 5-CSRT task) (Anastasio *et al,* 2011; Anastasio *et al,* 2013a; Cunningham *et al,* 2012; Dalley *et al,* 2002; Robbins, 2002). We tested the hypothesis that impulsive action and cocaine cue reactivity are interlocked constructs; the 1-CSRT task was used to identify impulsive action phenotypes in an outbred rat population before cocaine self-administration and assessment of cue reactivity in the form of lever presses reinforced by a cocaine-associated discrete cue complex during forced abstinence (FA) (Field and Cox, 2008). We then tested the hypothesis that phenotypic differences in inherent impulsive action associate with 5-HT$_{2C}$R protein expression and specific patterns of 5-HT$_{2C}$R mRNA variants in mPFC. Because the distribution pattern and functional status of the 5-HT$_{2C}$R is also modulated by association with a repertoire of co-expressed PDZ proteins including postsynaptic density protein 95 (PSD95) (Becamel *et al,* 2002; Gavarini *et al,* 2006), we also tested the hypothesis that the protein complex formed between 5-HT$_{2C}$R and PSD95 is differentially expressed in high and low impulsive (LI) action phenotypes. Finally, we tested the hypothesis that the genetic loss of 5-HT$_{2C}$R in the mPFC evokes high impulsive action concomitant with high cocaine cue reactivity. Taken together, we propose that dysregulation of cortical 5-HT$_{2C}$R may be a neurobiological mechanism underlying the intersection of impulsive action and cocaine cue reactivity.

MATERIALS AND METHODS

Animals

Male, Sprague–Dawley rats ($n = 176$; Harlan, Houston, TX) weighing 250–275 g at arrival were housed two/cage under a 12-h light–dark cycle at constant temperature (21–23 °C) and humidity (40–50%). For the duration of 1-CSRT task acquisition and maintenance, rats were food restricted to 90% free-feeding weight; water was available *ad libitum* except during daily operant sessions. Rats were weighed daily to ensure that their body weights were maintained at 90% of free-feeding levels. During the self-administration assay, food and water were available *ad libitum*. All experiments were carried out in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (2011) and with the University of Texas Medical Branch Institutional Animal Care and Use Committee approval.

General Methods

1-Choice serial reaction time task. Procedures occurred in standard five-hole nosepoke operant chambers equipped with a houselight, food tray, and an external pellet dispenser capable of delivering 45 mg pellets (Bio-Serv, Frenchtown, NJ) housed within a ventilated and sound-attenuated chamber (MedAssociates, St Albans, VT). The 1-CSRT task methodology has been described previously (Anastasio *et al,* 2011; Anastasio *et al,* 2013a). In the pre-training stage, rats ($n = 176$) were habituated to the test chamber and introduced to a nosepoke response for food pellets. During this stage, all responses made in the correctly lit (center) hole resulted in the illumination of the magazine light and presentation of a single food pellet. The training stages, thereafter, were each comprised of daily sessions of 100 trials to be completed in a maximum of 30 min; each

training stage involved incrementally lowering the stimulus duration with a 5-s limited hold and an intertrial interval (ITI) of 5 s. Thus, a maximum of 100 correct responses in a session resulted in a maximum of 100 reinforcers delivered; incorrect or premature responses or omissions resulted in a 5-s time-out period and a reduction in potential reinforcers obtained. Rats were required to meet an acquisition criteria of a minimum of 50 correct responses, $>80\%$ accuracy (correct responses/(correct + incorrect) $\times$ 100) and $<20\%$ omissions (omitted responses/trials completed $\times$ 100) to move from one training stage to the next (see Anastasio *et al,* 2011 for details).

The total number of responses (premature, correct, incorrect, and omissions) were recorded (Anastasio *et al,* 2011; Anastasio *et al,* 2013a; Cunningham *et al,* 2012). Premature responses were used to assess impulsive action. The number of reinforcers earned provides a measure of task competency and a secondary assessment of impulsive action, while percent accuracy is a general indication of attentional capacity. Percent omissions indicate failures of detection of the visual stimuli in the center hole as well as the motivation level to perform the task.

Cocaine self-administration assay. Rats ($n=64$) were anesthetized (i.m.) with a cocktail containing xylazine (8.6 mg/kg), acepromazine (1.5 mg/kg), and ketamine (43 mg/kg) in bacteriostatic saline. The catheter was inserted into the right jugular vein and exited dorsally (Cunningham *et al,* 2011; Cunningham *et al,* 2012). Daily flushes with a solution of heparinized saline (10 U/ml) with streptokinase (0.67 mg/ml) and ticarcillin disodium (66.67 mg/ml) were performed. Rats were allowed 7 recovery days after surgery before initiation of cocaine self-administration.

Self-administration studies took place in standard operant chambers equipped with two retractable levers, a stimulus light above each lever, and a houselight opposite the levers housed within a ventilated and sound-attenuated chamber (MedAssociates). Cocaine self-administration sessions were initiated 7 days post catheterization and consisted of daily 180-min sessions during which rats were trained to lever press for a cocaine infusion (0.75 mg/kg/inf) on a fixed-ratio (FR) FR1 schedule of reinforcement before progressing to an FR5. Schedule completions on the active lever resulted in simultaneous illumination of the house and stimulus lights, and activation of the infusion pump (discrete cue complex paired with delivery of cocaine); responses on the inactive lever were recorded but had no scheduled consequences. Cocaine infusions were delivered by a syringe attached to an infusion pump located outside the chamber. (−)-Cocaine (NIDA) was dissolved in 0.9% saline. After stable self-administration was established, rats were returned to their home cages, weighed and handled daily for 14 days of FA. On FA day 14, rats were reintroduced to the chambers for a single cue reactivity test session, and responses on the previously active lever were reinforced by presentation of the discrete cue complex on an FR1 (cue-reinforced lever presses). The responses recorded are therefore under the control of a stimulus complex that includes the contextual cues plus the discrete cue complex (light and sound of infusion pump), previously associated with cocaine delivery; inactive lever presses were recorded but produced no scheduled consequences.

5-HT$_{2C}$R protein analysis: immunoblotting and immunoprecipitation. The mPFC was homogenized in 10X w/v extraction buffer (10 mM HEPES, 1 mM EDTA, 2 mM EGTA, 1 mM DTT, 10 μl/ml protease inhibitor cocktail and 10 μl/ml phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich, St Louis, MO). The homogenate was centrifuged at 1000 *g* for 10 min at 4 °C to pellet the nuclear fraction. The supernatant was removed and centrifuged at 20 000 *g* at 4 °C for 30 min to pellet the membrane-bound protein fraction. The membrane-enriched pellet was washed once and then resuspended in 200 μl resuspension buffer (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, protease and phosphatase inhibitors, and 0.5% dodecyl maltoside) (Anastasio *et al,* 2010; Anastasio *et al,* 2013a).

Equal amounts of mPFC membrane protein were separated by SDS-PAGE and transferred to a PVDF membrane for immunoblotting with the following antibodies: 5-HT$_{2C}$R (sc-17797, 1 : 100; Santa Cruz Biotechnology, Dallas, TX), β-actin (MAB1501, 1 : 10000; Millipore, Billerica, MA), PSD95 (MAB1598, 1 : 1000; Millipore), or pan-cadherin (AB6528, 1 : 10000; Abcam, Cambridge, MA) (Anastasio *et al,* 2010; Anastasio *et al,* 2013a). Membranes were incubated with mouse IgG IRDye800 (1 : 10000) for detection by Odyssey Imaging System (LI-COR, Lincoln, NE). The integrated intensity of each band was analyzed with the Odyssey Software and normalized to pan-cadherin or β-actin immunoreactivity.

Co-immunoprecipitation methodology was used to assess the 5-HT$_{2C}$R protein complex with PSD95 in the mPFC of HI and LI rats; a synaptosomal preparation enriched for pre- and postsynaptic proteins (ie, presynaptic terminals, postsynaptic membranes, postsynaptic density, and synaptic protein complexes) was used (Anastasio *et al,* 2010). Briefly, the mPFC was homogenized (10X w/v) in ice-cold Krebs buffer containing 0.32 M sucrose plus protease inhibitor cocktail and phosphatase inhibitor 2 and 3 cocktails (10 μl/ml, Sigma-Aldrich). The homogenate was then centrifuged at 1000 *g* at 4 °C for 10 min. The supernatant was collected and centrifuged at 20 000 *g* at 4 °C for 30 min to pellet the crude synaptosomes; the resultant pellet was resuspended in Krebs buffer plus 1% dodecyl maltoside. The PSD95 antibody was covalently crosslinked onto protein A/G resin as previously described with minor modifications (Anastasio *et al,* 2010; Anastasio *et al,* 2013a). Synaptosomal protein was incubated with the antibody-crosslinked resin for 48 h at 4 °C with constant shaking. The eluted protein was resuspended in Laemmli buffer and subjected to SDS-PAGE. Immunoblotting for 5-HT$_{2C}$R and PSD95 was performed as described above.

5-HT$_{2C}$R mRNA analyses: isolation and isoform profile analyses. The mPFC was homogenized in TRI Reagent and RNA isolated using the RiboPure kit (Life Technologies, Grand Island, NY) (Lanfranco *et al,* 2009). Reverse transcription was performed on 250 ng RNA using SuperScript III First Strand Synthesis System (Life Technologies) with random hexamer primers. RT-PCR reactions were assayed in triplicate on a 7500 Fast RT PCR System using TaqMan Fast Advanced Master Mix and TaqMan gene-specific primer/probes (*Htr2c*: Rn00562748_m1; Cyclophilin A (*Ppia*): Rn00690933_m1; Life Technologies). Data are

presented in terms of crossing threshold (Ct), which was calculated as $\Delta Ct = Ct(Htr2c) - Ct(Cyclophilin)$.

Quantification of 5-HT$_{2C}$R mRNA isoform profiles was determined using a high-throughput sequencing strategy (Morabito *et al,* 2010). First-strand cDNA was synthesized with an 5-HT$_{2C}$R-specific antisense primer containing the adapter sequence required for the Illumina sequencing platform (5′-CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTATCAAAGCTTGACGGCGTAGGACGTAG-3′; Illumina adapter sequence is underlined) using avian myoblastosis virus reverse transcriptase (Promega, Madison, WI). The total reverse-transcription reaction was amplified by PCR with Phusion DNA polymerase (Finnzymes, Woburn, MA) using the same antisense primer and one of the twenty-four 5-HT$_{2C}$R-specific sense primers containing a six nucleotide bar-code (**NNNNNN**) that allowed for multiplex sample analysis and an adapter sequence (single underline) with a region complementary to the standard Illumina sequencing primer (double underline) (5′-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT**NNNNNN**GCTGGACCGGTATGTAGCA-3′). Amplified products were separated on a 2% agarose gel and purified from excised gel slices using the Wizard SV Gel and PCR Purification Kit (Promega); PCR amplicons corresponding to alternatively spliced 5-HT$_{2C}$R RNA isoform 2 (286 bp) were isolated for all experiments. The concentration of gel-purified fragments was measured by spectrophotometry (A_{260}) and ~20 ng of each individual sample was pooled with other products containing unique bar codes and subjected to single-end sequencing with the Illumina Genome Analyzer (Illumina, San Diego, CA) (Bentley *et al,* 2008). Data were filtered (Morabito *et al,* 2010) using a 72 nucleotide mouse 5-HT$_{2C}$R reference sequence (5′-**NNNNNN**ATTAACCCTCACTAAAGGGAGCTGGACCGGTATGTAGCA**R**T**R**CGT**RR**TCCT**R**TTGAGCATAGCCGG-3′; bar code sequence position indicated in bold **N**) that allowed for either an adenosine/guanosine at the five editing sites (**R**, purine).

shRNA design and production. A 24-nucleotide sequence within the coding region of the *Htr2c* was identified using methods we have previously reported (Hommel *et al,* 2003). Two sets of oligonucleotides (Integrated DNA Technology, Coralville, IA) for cloning were synthesized (*Htr2c* shRNA (top, 5′-TTGAATCCAGACGGGGCACAAATATCCTTCCTGTCAGATATTTGTGCCCCGTCTGGATTATTTTT-3′; bottom, 5′-CTAGAAAAATAATCCAGACGGGGCACAAATATCTGACAGGAAGGATATTTGTGCCCCGTCTGGATTC-3′); non-silencing control (NSC) shRNA (top, 5′-TTTGTGGAGCCGAGTTTCTAAATTCCGCTTCCTGTCACGGAATTTAGAAACCCGGCTCCAATTTTT-3′; bottom, 5′-CTAGAAAAATTGGAGCCGGGTTTCTAAATTCCGTGACAGGAAGCGGAATTTAGAAACTCGGCTCCAC-3′)). Oligonucleotides were designed with Sap1 and Xbal overhangs to allow ligation downstream of the mU6pro region of a modified pAAV-MCS vector, pAAV-shRNA, which was designed to coexpress hairpin RNAs, under the control of a mU6pro and an SV40 polyadenylation site, as well as eGFP controlled by an independent CMV promoter and hGH polyadenylation sequence (Hommel *et al,* 2003). To assess the extent of 5-HT$_{2C}$R knockdown *in vitro*, HEK293 cells were transiently co-transfected with the 5-HT$_{2C}$R and the 5-HT$_{2C}$R shRNA plasmid DNA or empty vector (1 : 10 μg); at 72 h post transfection, cells were harvested, RNA was extracted and RT-PCR was performed to quantify 5-HT$_{2C}$R mRNA expression as described above. Adeno-associated viral (AAV) serotype type 2 vectors were packaged using a helper-free packaging system (Life Technologies) and purified viral stocks were assayed in camptothecin-treated HT1080 cells to confirm titers of $1\text{-}2 \times 10^{11}$ transducing units/ml.

Viral-mediated gene transfer. Rats ($n = 48$) were anesthetized (i.m.) with a cocktail containing xylazine (8.6 mg/kg), acepromazine (1.5 mg/kg), and ketamine (43 mg/kg) in bacteriostatic saline and placed in a stereotaxic apparatus with the upper incisor bar at −3.8 mm below the interaural line. Two microsyringes (28 gauge, Hamilton Company, Reno, NV) were lowered bilaterally at 15° from the midsaggital plane relative to bregma (Paxinos and Watson, 1998) to target the mPFC encompassing the ventral prelimbic and dorsal infralimbic subnuclei (Supplementary Figure 2); the coordinates were anteroposterior +3 mm, mediolateral +1.8 mm, and dorsoventral −5.1 mm from the skull. The NSC shRNA-eGFP AAV ('control'; 1.5 μl) or 5-HT$_{2C}$R shRNA-eGFP ('5-HT$_{2C}$R knockdown'; 1.5 μl) AAV vectors were infused bilaterally at 0.1 μl/min over 15 min. Rats were allowed 3 weeks to recover and to allow for stable transgene expression. AAV infection has been well-characterized with stabilization of gene expression in rodent brain at 3 weeks and with stability for at least 12–18 months post infection (Daly, 2004; Leff *et al,* 1999).

Research Design

Identification of impulsive action phenotypes. (Cohorts 1–4; see experimental timelines, Figures 1–3). Rats ($n = 128$) were trained on the 1-CSRT task and were required to meet the acquisition criteria of a minimum of 50 correct responses, >80% accuracy and <20% omissions on the final training stage (0.5 s stimulus duration, 5 s limited hold, and 5 s ITI, ITI5) before being tested on an ITI8 challenge session (0.5 s stimulus duration, 5 s limited hold, and 8 s ITI) to more easily detect phenotypic differences in inherent impulsive action (Besson *et al,* 2013; Dalley *et al,* 2002). HI and LI rats were stratified as the upper and lower quartile based on the number of premature responses made during the ITI8 challenge session.

Inherent impulsive action predicts cocaine cue reactivity. (Cohort 2; see experimental timeline, Figure 2). Following phenotype identification, the 1-CSRT task sessions were terminated and HI rats ($n = 8$) and LI rats ($n = 8$) were retained in their home cages and allowed to free-feed for 7 days before surgical implantation of a jugular catheter. Cocaine self-administration commenced 7 days post catheterization; cue reactivity was assessed on FA day 14.

Functional 5-HT$_{2C}$R status in impulsive action phenotypes. (Cohorts 3 and 4; see experimental timeline, Figure 3). Following phenotype identification in two separate cohorts of rats ($n = 64$), the 1-CSRT task sessions were terminated and HI ($n = 16$) and LI rats ($n = 16$) were

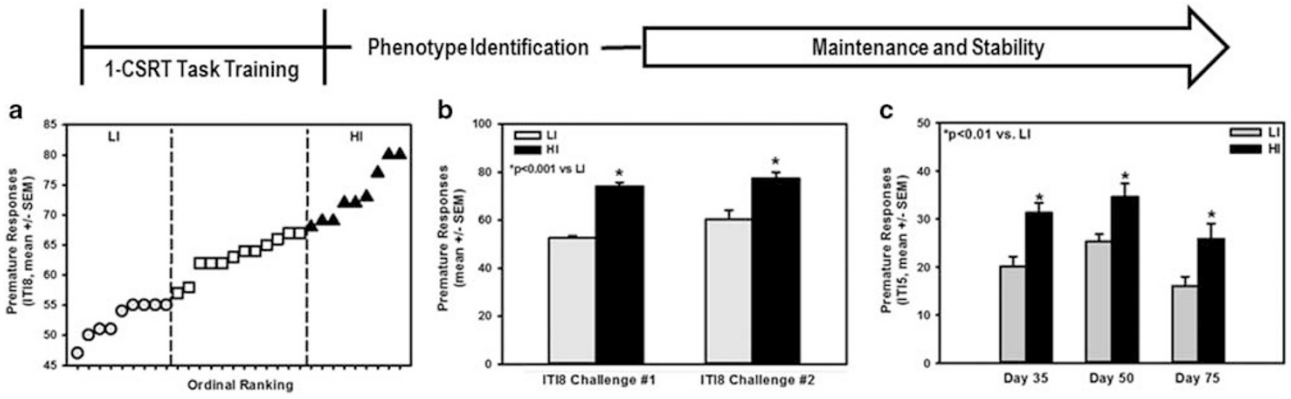

Figure 1 Impulsive action phenotypes are identifiable in an outbred rat population. Rats were required to meet an acquisition criteria of a minimum of 50 correct responses, >80% accuracy and <20% omissions on the final one-choice serial reaction time (1-CSRT) task training stage (0.5 s stimulus duration, 5 s limited hold and 5 s intertrial interval, ITI5) before being tested on an ITI8 challenge session to more easily detect phenotypic differences in inherent impulsive action. (a) The number of premature responses made during the ITI8 challenge session was used to stratify rats as high impulsive (HI) or low impulsive (LI) relative to rats in the interquartile range. (b) HI rats exhibited higher numbers of premature responses *vs* LI rats on two separate ITI8 challenge sessions conducted when the cohort initially met criteria (ITI8 challenge #1; day 25–30 on average) and then upon retest (ITI8 challenge #2; day 92 of 1-CSRT task maintenance), respectively (*$p<0.001$ *vs* LI). (c) The impulsive phenotype was identifiable and stable throughout >75 days of 1-CSRT sessions (ITI5) (HI *vs* LI at day 35 (*$p<0.05$ *vs* LI), day 50 (*$p<0.05$ *vs* LI) and day 75 (*$p<0.05$ *vs* LI)).

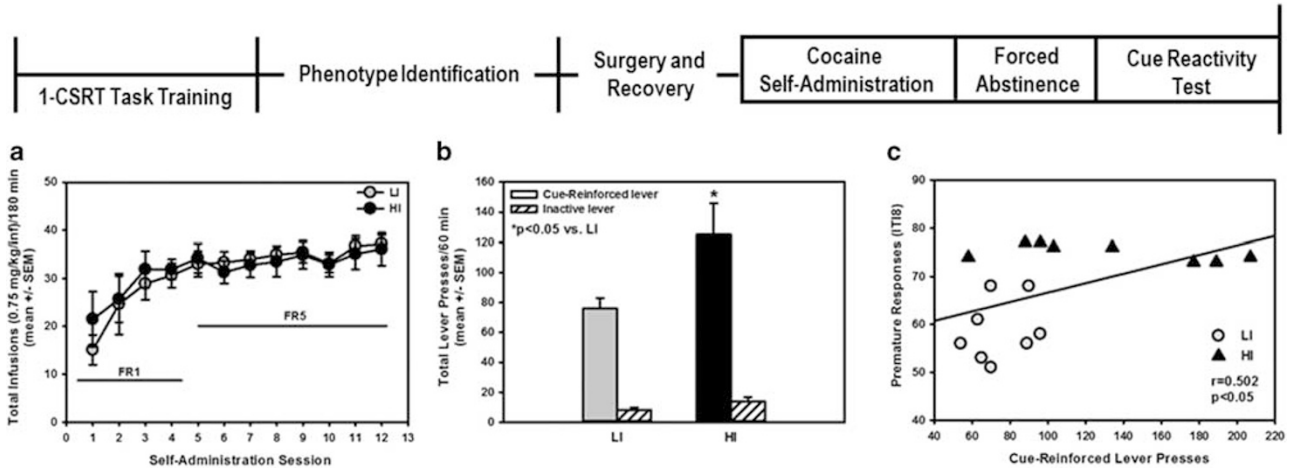

Figure 2 Inherent impulsive action predicts cocaine cue reactivity. Following phenotype identification, the one-choice serial reaction time (1-CSRT) task sessions were terminated, and HI rats and LI rats were retained in their home cages and allowed to free-feed for 7 days before surgical implantation of a jugular catheter. (a) In daily 180 min sessions, HI and LI rats readily acquired cocaine self-administration (0.75 mg/kg/0.1 ml infusion) to stability. (b) On forced abstinence (FA) day 14, HI rats exhibited a higher number of previously active (*$p<0.05$ *vs* LI), but not inactive lever presses (n.s.), during the cue reactivity test session *vs* LI rats. (c) There was a positive correlation between premature responses on the 1-CSRT task and cue-reinforced lever presses during the cue reactivity test session for individual subjects ($r=0.502$; $p<0.05$).

retained in their home cages (food restriction was maintained). Within 2–3 days, rats were anesthetized (chloral hydrate, 400 mg/kg) and decapitated, and the brain was microdissected immediately, flash frozen in liquid nitrogen, and stored at $-80\,^{\circ}$C for subsequent protein ($n=16$) (Anastasio *et al,* 2010; Anastasio *et al,* 2013a; Liu *et al,* 2007) or RNA analysis ($n=16$) (Lanfranco *et al,* 2009).

Genetic loss of mPFC 5-HT$_{2C}$R confers aggregate impulsive action/cocaine cue reactivity. (Cohort 5; see experimental timeline, Figure 4). Following intra-mPFC transgene delivery and stable viral vector expression, control and 5-HT$_{2C}$R knockdown rats ($n=48$) were trained to criteria on the 1-CSRT task. Upon completion of assessments on the final 1-CSRT task training stage (0.5 s stimulus duration, 5-s limited hold, ITI5), control and 5-HT$_{2C}$R knockdown rats were retained in their home cages and allowed to free-feed for 7 days before surgical implantation of a jugular catheter. Cocaine self-administration commenced 7 days post catheterization; cue reactivity was assessed on FA day 14. At the termination of the cue reactivity test, rats were removed from the operant chambers and returned to their home cages. Seven days later, rats were anesthetized (chloral hydrate; 400 mg/kg, i.p.) and killed, and tissue samples were taken for visualization, immunoblot analyses, or immunohistochemical verification of 5-HT$_{2C}$R protein knockdown. A 1-mm coronal section containing the mPFC was placed on

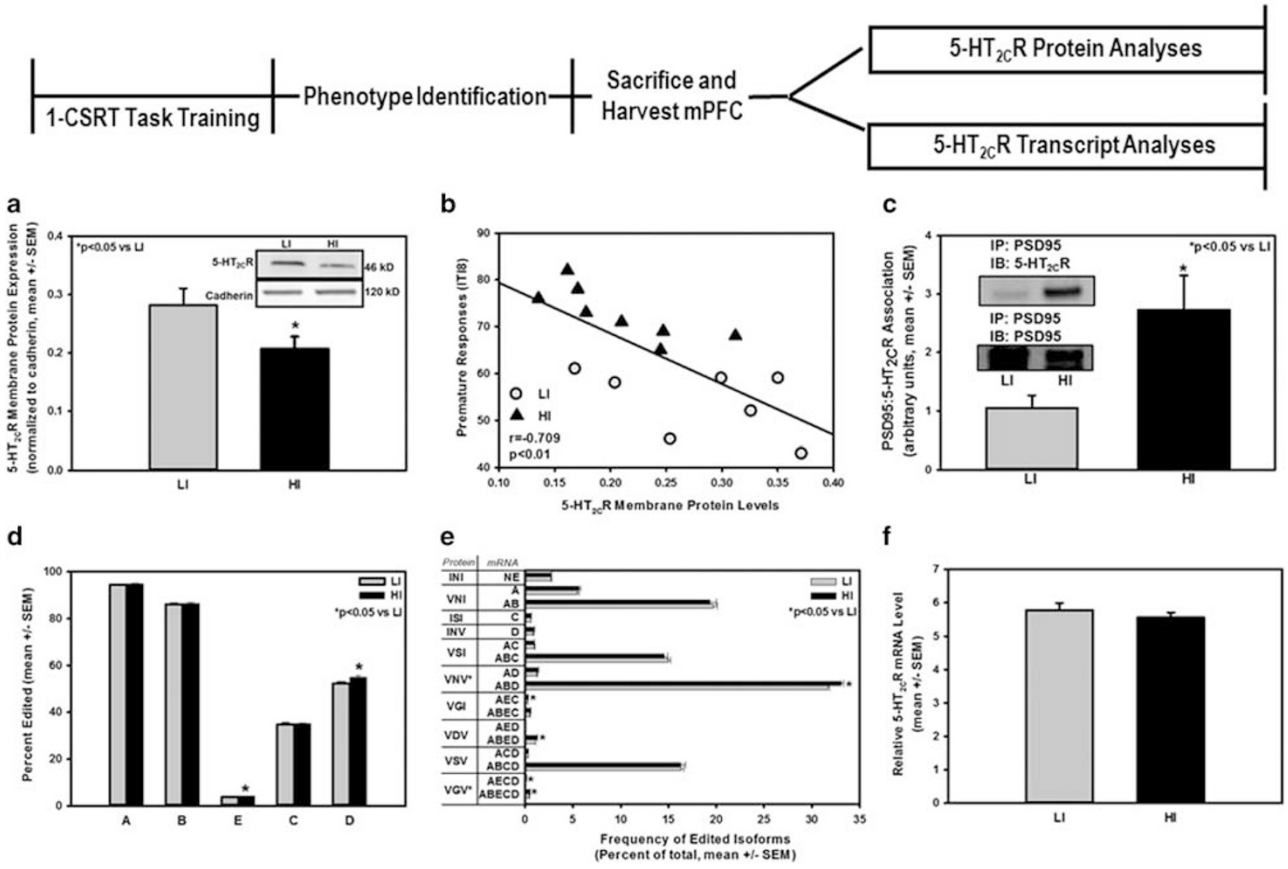

Figure 3 Functional status of the 5-HT$_{2C}$R is a neuromolecular substrate for inherent impulsive action. Following phenotype identification in two separate cohorts of rats, the one-choice serial reaction time (1-CSRT) task sessions were terminated, and HI and LI rats were retained in their home cages (food restriction was maintained) and killed within 2–3 days for *ex vivo* neurochemical analyses of 5-HT$_{2C}$R protein and mRNA. (a) Qualitative (inset) and quantitative demonstration that HI rats expressed lower levels of membrane medial prefrontal cortex (mPFC) 5-HT$_{2C}$R *vs* LI rats (*$p<0.05$ *vs* LI). (b) There was an inverse correlation between mPFC 5-HT$_{2C}$R membrane protein levels and premature responding on the 1-CSRT task for individual subjects ($r=-0.709$; $p<0.01$). (c) Immunoprecipitation (IP) for postsynaptic density 95 (PSD95) followed by immunoblot (IB) for 5-HT$_{2C}$R yielded 5-HT$_{2C}$R immunoreactivity in both HI and LI rats (inset). Synaptosomal 5-HT$_{2C}$R associated with PSD95 in the mPFC to a greater extent in HI *vs* LI rats (*$p<0.05$ *vs* LI); comparable levels of PSD95 were immunoprecipitated between HI and LI rats (inset). (d) The 5-HT$_{2C}$R mRNA editing efficiency was significantly different in HI *vs* LI rats; editing at the E and D sites was higher in HI *vs* LI rats (*$p<0.05$). (e) The frequencies of *ABD*, *AEC*, *ABED*, *AECD*, and *ABECD* 5-HT$_{2C}$R mRNA variants were elevated in HI rats compared with LI rats. HI rats expressed higher levels of predicted protein isoforms VNV and VGV *vs* LI rats (*$p<0.05$ *vs* LI). (f) Total mPFC 5-HT$_{2C}$R mRNA levels as assessed by RT-PCR did not differ between HI and LI rats (n.s.).

a cold glass slide and rapid visualization of eGFP *ex vivo* was accomplished with a DFP-1 Dual Fluorescent Protein Flashlight by the investigator wearing a pair of VG2 barrier filter glasses (Nightsea, Bedford, MA) (Li and Wolf, 2011). Photomicrographs of coronal sections were taken with a DSLR camera equipped with a macro lens and yellow filter (Li and Wolf, 2011). Fluorescent regions from the mPFC were then microdissected and assayed for immunoblotting to assess knockdown *ex vivo*. A subset of rats ($n=6$) were anesthetized (sodium pentobarbital; 100 mg/kg, i.p.) and perfused transcardially with 3% paraformaldehyde for immunohistochemical analyses (Bubar *et al,* 2011). Brains were removed, post fixed (2 h), and cryoprotected in 30% sucrose solution. Free-floating coronal sections at the level of the mPFC (30 μm) were incubated in 0.5% sodium borohydride to reduce autofluorescence. Sections were blocked (1.5% normal goat serum in 0.4% triton-PBS) before incubation with 5-HT$_{2C}$R antibody (1 : 100; 2 h 25 °C, 18 h 4 °C) followed by AlexaFluor 555 to mouse IgG (A21424, 1 : 2000; Life Technologies). Slides were cover-slipped with Vectashield fluorescent mounting medium with DAPI (Vector Laboratories, Burlingame, CA).

Statistical Analyses. Outcome measures from the 1-CSRT task, cocaine self-administration and cue reactivity assays, as well as 5-HT$_{2C}$R protein and mRNA analyses, were assessed by Student's *t*-test or ANOVA as appropriate to research design (Anastasio *et al,* 2011; Anastasio *et al,* 2013a; Cunningham *et al,* 2012; Keppel, 1973). Based upon preliminary data and published literature, *a priori* group comparisons were specifically defined before the start of the experiment and were assessed with the Tukey test (for multiple pairwise comparisons of means) (Keppel, 1973). To analyze the relationship between premature responses and cue-reinforced lever presses, or premature responses and protein expression, we used Pearson's partial correlation (Keppel, 1973). These analyses were performed in SAS (version 9.3) with an experimentwise error rate of $\alpha=0.05$.

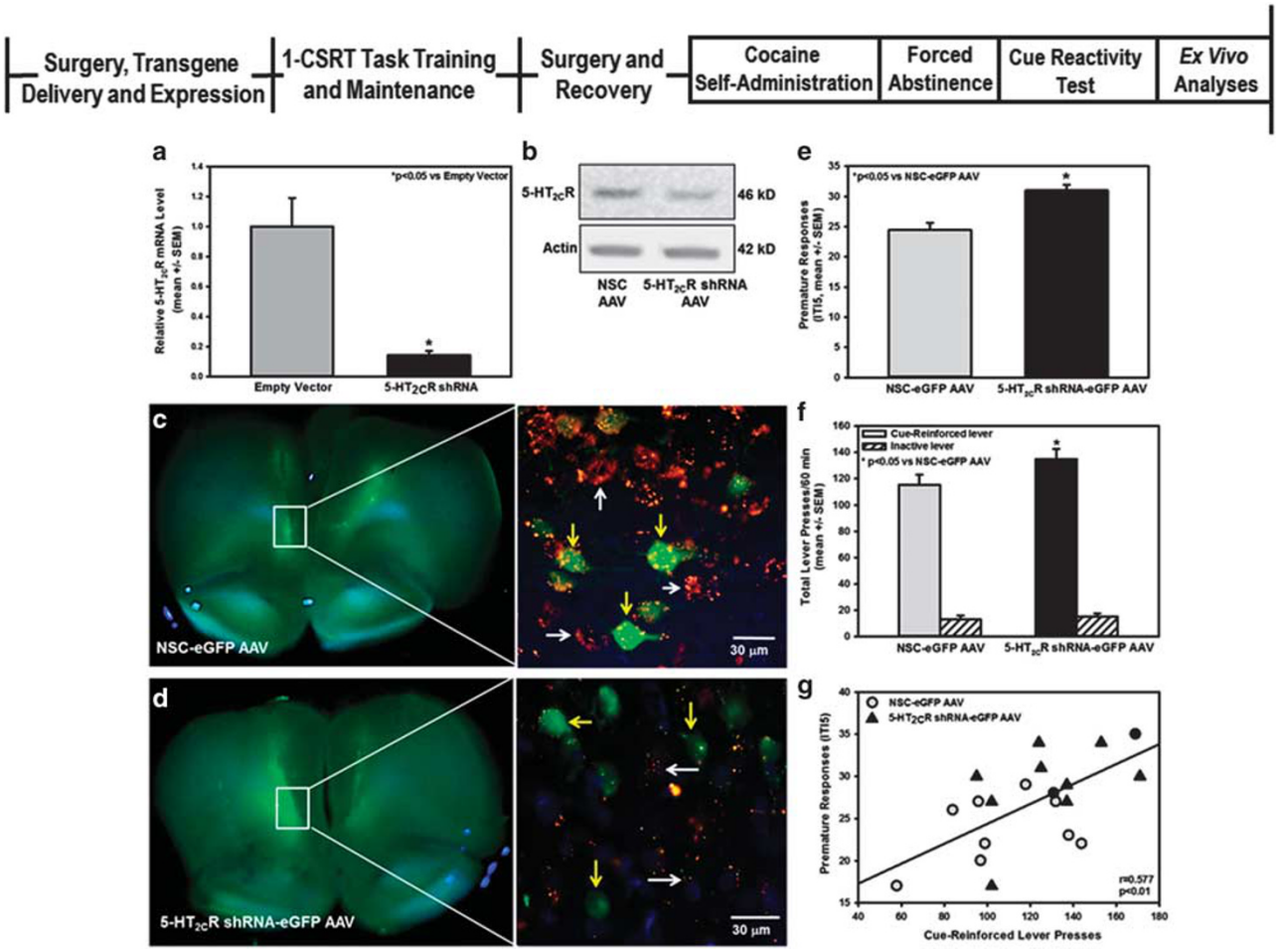

Figure 4 Genetic loss of 5-HT$_{2C}$R in medial prefrontal cortex (mPFC) confers aggregate impulsive action/cue reactivity. (a) The shRNA expression plasmid targeting the 5-HT$_{2C}$R efficiently silenced 5-HT$_{2C}$R mRNA in a cell line transiently expressing 5-HT$_{2C}$R (*$p<0.05$ *vs* LI). (b) The 5-HT$_{2C}$R shRNA-eGFP AAV knocked down 5-HT$_{2C}$R protein levels in the mPFC (~50% decrease) relative to the NSC-eGFP AAV. (c) Stereotaxic placement and NSC-eGFP AAV infection (green) in mPFC (left). The NSC-eGFP AAV (green) did not alter 5-HT$_{2C}$R protein expression (red) in infected neurons (yellow arrows) relative to non-AAV infected neurons (white arrows) (right). (d) Stereotaxic placement and 5-HT$_{2C}$R shRNA-eGFP AAV infection (green) in mPFC (left). The 5-HT$_{2C}$R shRNA-eGFP AAV (green) induced a significant knockdown of 5-HT$_{2C}$R protein (red) in infected neurons (yellow arrows) relative to non-infected neurons (white arrows) (right). (e) Following intra-mPFC transgene delivery and stable viral vector expression, control and 5-HT$_{2C}$R knockdown rats were subjected to the one-choice serial reaction time (1-CSRT) task. The 5-HT$_{2C}$R knockdown rats expressed significantly higher premature responses *vs* control rats on the final 1-CSRT task training stage (0.5 s stimulus duration, 5 s limited hold and 5 s intertrial interval (ITI5)) (*$p<0.05$ *vs* NSC-eGFP AAV). Upon completion of 1-CSRT task assessments, control and 5-HT$_{2C}$R knockdown rats were retained in their home cages and allowed to free-feed for 7 days before surgical implantation of a jugular catheter. Cocaine self-administration commenced 7 days post catheterization. (f) On forced abstinence (FA) day 14, 5-HT$_{2C}$R knockdown rats exhibited higher cue-reinforced lever presses (*$p<0.05$ *vs* NSC-eGFP AAV), but not inactive, lever presses (n.s.), during a cue reactivity test session *vs* control rats. (g) There was a positive correlation between premature responses and cue-reinforced lever presses during the cue reactivity test session for individual subjects ($r=0.577$; $p<0.01$).

RESULTS

Impulsive Action Phenotypes are Stable in the 1-CSRT Task

Four separate cohorts of outbred rats were trained on the 1-CSRT task and, within each cohort ($n=32$), HI ($n=7$-8/cohort) and LI rats ($n=7$-9/cohort) were stratified as the top and bottom 25% of rats based upon premature responses on the ITI8 challenge session. Supplementary Table 1 illustrates the consistency of premature responses, reinforcers earned, accuracy and percent omissions on the ITI8 challenge observed across the four cohorts of HI and LI rats used for these analyses. There was no main effect of cohort on premature responses for LI rats ($F_{(3,27)}=2.47$, n.s.) or HI rats ($F_{(3,27)}=2.47$, n.s.; Supplementary Table 1). Accuracy averaged 95–99% for all cohorts and did not differ between HI and LI rats; levels of premature responses, reinforcers earned, and percent omissions were consistently different in HI *vs* LI rats in all four cohorts (statistical analyses presented in Supplementary Table 1). The results across the four cohorts support the utility, reliability, and consistency of the 1-CSRT task to identify phenotypic differences in impulsive action.

Cohort 1 was used to test the hypothesis that individual differences in levels of impulsive action were observable and stable on the 1-CSRT task (Figure 1a–c; experimental timeline). The ordinal distribution of individual rats in Cohort 1 are plotted by premature responses to illustrate the upper (HI) and lower (LI) quartile of rats relative to those in the interquartile range (Figure 1a). HI rats exhibited

higher numbers of premature responses *vs* LI rats on two separate ITI8 challenge sessions conducted when each rat initially met criteria (25–30 sessions) and then upon retest on session 92, respectively (Figure 1b; $p<0.05$). For rats phenotypically identified on the ITI8 challenge session, impulsive action was significantly higher in HI *vs* LI rats on their ITI5 maintenance sessions on day 35 ($p<0.05$), day 50 ($p<0.05$), and day 75 ($p<0.05$) (Figure 1c). Both HI and LI rats exhibited characteristically high accuracy and low omissions (Supplementary Table 1), indicating that all rats detected the task stimuli and performed effectively. HI rats earned fewer reinforcers and displayed lower percent omissions relative to LI rats (Supplementary Table 1), suggesting that HI rats may have greater motivational drive to perform the task *vs* LI rats (Frijda, 2010). Taken together, these data support the identification of a stable, trait-like impulsive action phenotype in an outbred rat population.

Impulsive Action and Cocaine Cue Reactivity are Interlocked Phenotypes

Cohort 2 was used to test the hypothesis that inherent impulsive action predicted levels of cue reactivity as measured by cue-reinforced lever presses (Figure 2, experimental timeline). After screening in the 1-CSRT task, HI and LI rats were trained to self-administer cocaine and then subjected to a 14-day FA. HI and LI rats readily acquired cocaine self-administration to stability (Figure 2a; Supplementary Figure 1); across the last three sessions, there was no main effect of phenotype ($F_{(1,47)}=0.56$, n.s.), session ($F_{(2,47)}=0.99$, n.s.), or a phenotype × session interaction ($F_{(2,47)}=1.00$, n.s.) for the total number of infusions (Figure 2a). Average total daily cocaine intake over the last three self-administration sessions did not differ between phenotypes (HI = 10.3 ± 1.1 mg/kg/day; LI = 9.4 ± 1.7 mg/kg/day; n.s.). On FA day 14, HI rats exhibited higher cue-reinforced (Figure 2b; $p<0.05$), but not inactive, lever presses (Figure 2b; n.s.) *vs* LI rats; a positive correlation was observed between premature responses and cue-reinforced lever presses in individual rats (Figure 2c; $r=0.502$; $p<0.05$). Notably, LI rats (gray circles) expressed the lowest levels of cue reactivity and did not overlap with HI rats (black triangles) (Figure 2c). In contrast, HI rats consistently exhibited high levels of cue reactivity with exceedingly high levels (⩾180–210 lever presses/60 min) expressed in some HI rats (Figure 2c). Thus, inherent impulsive action predicted the level of cocaine cue reactivity in an outbred strain of rats.

Impaired 5-HT$_{2C}$R Functional Status may Underlie Impulsive Action

The ultimate level of functionality of the 5-HT$_{2C}$R is determined by a culmination of factors, including the availability of active pools of receptors in the synaptic compartment and effective coupling to, and activation of, downstream signaling and/or scaffolding components. In Cohorts 3 and 4 of HI and LI rats, we evaluated 5-HT$_{2C}$R protein and transcript expression in the mPFC to gain insight into the functionality of this receptor as a neuromolecular substrate for impulsive action.

In Cohort 3, we tested the hypothesis that HI and LI rats would exhibit differential expression of the 5-HT$_{2C}$R protein in a crude membrane preparation of the mPFC. We observed that 5-HT$_{2C}$R membrane protein expression in the mPFC was lower in HI *vs* LI rats (Figure 3a; $p<0.05$) and that 5-HT$_{2C}$R membrane protein expression in individual rats was inversely correlated to premature responses (Figure 3b; $r=-0.709$; $p<0.01$). Notably, levels of 5-HT$_{2C}$R protein expression in HI rats did not overlap with those in LI rats (Figure 3b).

The 5-HT$_{2C}$R in the membrane exists in a macromolecular complex (Becamel *et al,* 2002) and one key regulatory partner is PSD95, which, through its association with the 5-HT$_{2C}$R, promotes internalization and desensitization of the receptor, thereby attenuating the responsivity of 5-HT$_{2C}$R signaling (Abbas *et al,* 2009; Becamel *et al,* 2002; Gavarini *et al,* 2006). Thus, we tested the hypothesis that, in addition to lower membrane 5-HT$_{2C}$R in HI *vs* LI rats, HI rats may present with a greater association of PSD95 with 5-HT$_{2C}$R, which would be expected to further reduce the responsivity of 5-HT$_{2C}$R signaling. We used co-immunoprecipitation techniques to assess this physical association of PSD95 with the 5-HT$_{2C}$R in the synaptosomal fraction of the mPFC of HI and LI rats. The synaptosome contains the presynaptic molecular machinery for the uptake, storage, and release of neurotransmitters, as well as the postsynaptic milieu that is composed of receptors and downstream effectors of neuronal transmission without contamination from membranous organelles found in crude membrane protein fractions (Breukel *et al,* 1997). Immunoprecipitation (IP) for PSD95 followed by immunoblot (IB) for 5-HT$_{2C}$R yielded 5-HT$_{2C}$R immunoreactivity in both HI and LI rats (Figure 3c), indicating that a 5-HT$_{2C}$R:PSD95 protein complex is assembled in native mPFC tissue and can be examined *ex vivo* (Anastasio *et al,* 2010). In addition, HI rats exhibited higher levels of the 5-HT$_{2C}$R:PSD95 complex in the synaptosomal fraction of the mPFC *vs* LI rats (Figure 3c; $p<0.05$). These data suggest that the functionality of available receptors may be reduced by higher coupling of PSD95 to the 5-HT$_{2C}$R in the mPFC of HI *vs* LI rats (Abbas *et al,* 2009; Becamel *et al,* 2002; Gavarini *et al,* 2006). Immunoblot for PSD95 after IP for PSD95 indicated that comparable levels of PSD95 protein were immunoprecipitated between HI and LI rats (Figure 3c). No difference in synaptosomal PSD95 protein levels in the mPFC were detected between HI (9.9 ± 0.4 arbitrary units) and LI rats (11.6 ± 1.4 arbitrary units; n.s.). Taken together, the lower 5-HT$_{2C}$R membrane protein expression and the higher 5-HT$_{2C}$R:PSD95 complex in mPFC of HI rats suggest that reduced responsivity of the 5-HT$_{2C}$R in mPFC may confer in part high inherent impulsive action.

The functional capacity of the 5-HT$_{2C}$R protein and its signaling network is regulated by editing of the 5-HT$_{2C}$R pre-mRNA, which generates up to 32 mRNA isoforms that encode up to 24 predicted receptor protein isoforms (Burns *et al,* 1997; Herrick-Davis *et al,* 1999; Marion *et al,* 2004). Editing drives localization of the receptor (eg, cellular membrane *vs* endocytic vesicles), constitutive activity and trafficking, and responsiveness to ligands with the degree of editing generally associated with the degree of receptor stimulation (Marion *et al,* 2004); editing can also influence alternative splicing of the 5-HT$_{2C}$R (Martin *et al,* 2013). The

nonedited isoform (5-HT$_{2C\text{-}INI}$R) exhibits the highest constitutive activity, whereas the fully edited isoform (5-HT$_{2C\text{-}VGV}$R) is incapable of coupling to its G protein and exhibits no constitutive activity (Burns *et al*, 1997; Hoyer *et al*, 2002; Marion *et al*, 2004). Partially edited isoforms exhibit profiles between these two extremes. Although these multiple isoforms are predicted as key in 5-HT$_{2C}$R function, there are no published methods to separately analyze expression of isoform-specific proteins; however, high-throughput sequencing provides the opportunity to evaluate expression of mRNA for all 5-HT$_{2C}$R isoforms across phenotypes (Morabito *et al*, 2010; Zhu *et al*, 2012).

In Cohort 4, we tested the hypothesis that the editing efficiency and 5-HT$_{2C}$R mRNA profiles would distinguish inherent impulsive action phenotypes, providing a window on the predicted protein isoforms that may contribute to differential 5-HT$_{2C}$R protein expression seen in HI *vs* LI rats. RNA extracted from the mPFC collected from HI and LI rats was analyzed for efficiency of editing at each of the five editing sites (A, B, E, C, D) as well as the frequency of mRNA variants using high-throughput multiplexed transcript analysis (Morabito *et al*, 2010; Zhu *et al*, 2012); all 32 possible mRNA variants were detected in the rat mPFC (Supplementary Table 2). A main effect of phenotype ($F_{(1,56)} = 9.86$, $p < 0.01$), site ($F_{(4,56)} = 22744.6$, $p < 0.001$), and a phenotype × site interaction ($F_{(4,56)} = 3.22$, $p < 0.05$) on editing efficiency at the five editing sites was observed; HI rats expressed higher levels of editing at the E and D sites *vs* LI rats (Figure 3d; $p < 0.05$). We observed a trend toward a main effect of phenotype ($F_{(1,399)} = 4.00$, $p = 0.06$), a main effect of mRNA variant ($F_{(31,399)} = 4372.9$, $p < 0.001$), and a phenotype × mRNA variant interaction ($F_{(31,399)} = 1.58$, $p < 0.05$) on the frequency of expressed mRNA variants; HI rats expressed higher levels of mRNA variants *ABD*, *ABECD*, *ABED*, *AEC*, and *AECD* *vs* LI rats (Figure 3e; $p < 0.05$), all of which include editing at the E and/or D sites.

The 32 possible 5-HT$_{2C}$R mRNA variants can lead to a predicted 24 resultant 5-HT$_{2C}$R protein isoforms (Supplementary Table 2). A main effect of phenotype ($F_{(1,313)} = 5.3$, $p < 0.05$), a main effect of predicted protein isoform ($F_{(23,313)} = 4452.58$, $p < 0.001$) and a trend toward a phenotype × predicted protein isoform interaction ($F_{(23,313)} = 1.43$, $p = 0.09$) was observed on the frequency of predicted protein isoforms; the frequency of the predicted isoforms for the most abundant, partially edited 5-HT$_{2C\text{-}VNV}$R (encoded by *AD* and *ABD*), and the fully edited 5-HT$_{2C\text{-}VGV}$R (encoded by *AECD* and *ABECD*) were higher in HI *vs* LI rats (Figure 3e; $p < 0.05$). Although statistical differences in the *AEC* and *ABED* mRNA variants between HI and LI rats were detected, there was no difference in the predicted protein isoforms (5-HT$_{2C\text{-}VGI}$R and 5-HT$_{2C\text{-}VDV}$R, respectively) in HI *vs* LI rats. A probe spanning the boundary of exons 4 and 5 of translated mRNA was used to detect total 5-HT$_{2C}$R (full-length and all splice variants) mRNA levels in the mPFC of HI and LI rats; total 5-HT$_{2C}$R mRNA levels did not differ between HI and LI rats (Figure 3f; n.s.), suggesting that differential protein expression profiles in HI *vs* LI rats (above) is under the regulatory control of post-transcriptional, rather than transcriptional, mechanisms. Thus, the frequency of edited 5-HT$_{2C}$R mRNA variants was elevated with the predicted protein population in HI rats, favoring the isoforms linked to reduced signaling capacity and constitutive activity.

Loss of 5-HT$_{2C}$R in mPFC Evokes Aggregate Impulsive Action/Cocaine Cue Reactivity

Cohort 5 was used to test the hypothesis that genetic knockdown of the 5-HT$_{2C}$R in the mPFC in behavioral- and drug-naive rats would recapitulate high inherent impulsive action and cocaine cue reactivity (Figure 4, experimental timeline). To study the functional consequences of disrupted 5-HT$_{2C}$R function directly in the mPFC *in vivo*, we used an RNA interference strategy (Hommel *et al*, 2003) to engineer AAV vectors to suppress all endogenous 5-HT$_{2C}$R RNA variants. *In vitro* analyses indicated that the selected 5-HT$_{2C}$R shRNA plasmid effectively silenced 5-HT$_{2C}$R RNA expression *vs* the empty vector in HEK293 cells expressing the 5-HT$_{2C}$R (Figure 4a; $p < 0.05$). *Ex vivo* analyses indicated that after microinfusion into the mPFC, the 5-HT$_{2C}$R shRNA-eGFP AAV curtailed 5-HT$_{2C}$R protein production (~50% decrease) relative to the NSC-eGFP AAV (Figure 4b). Cross-sections through the mPFC of a rat infused with NSC-eGFP AAV (Figure 4c) or 5-HT$_{2C}$R shRNA-eGFP AAV (Figure 4d) demonstrated reduced 5-HT$_{2C}$R immunoreactivity in infected neurons (compare yellow arrows in Figure 4c and d); analysis of microinfusion placements in mPFC of individual rats indicated that viral infection was localized within the mPFC along the border of the infralimbic/prelimbic subnuclei (Supplementary Figure 2).

Knockdown of the 5-HT$_{2C}$R in the mPFC enhanced premature responses in the 1-CSRT task *vs* control (Figure 4e; $p < 0.05$). Descriptive statistics for the effects of 5-HT$_{2C}$R knockdown on additional 1-CSRT task measures are presented in Supplementary Figure 3. Of note, the number of reinforcers earned, accuracy, percent omissions, and latency to start the task did not differ between knockdown and control rats (Supplementary Figure 3), suggesting that the 5-HT$_{2C}$R in the mPFC selectively regulates prepotent responding in the 1-CSRT task, a key facet of impulsive action. Both 5-HT$_{2C}$R knockdown and control rats readily acquired cocaine self-administration to stability; across the last three sessions of stable self-administration, no main effect of phenotype ($F_{(1,81)} = 0.63$, n.s.), session ($F_{(2,81)} = 0.21$, n.s.) and no phenotype × session interaction ($F_{(2,81)} = 1.24$, n.s.) was observed for total number of cocaine infusions (Supplementary Figure 4). Total daily cocaine intake did not differ between treatment groups (knockdown = 10.5 ± 0.5 mg/kg/day; control = 11.3 ± 0.5 mg/kg/day; n.s.). On FA day 14, knockdown rats displayed higher cue-reinforced lever presses (Figure 4f; $p < 0.05$) *vs* controls; inactive lever presses during the cue reactivity test did not differ between 5-HT$_{2C}$R knockdown and control rats. There was a positive correlation between premature responses and cue-reinforced lever presses in individual rats (Figure 4g; $r = 0.577$; $p < 0.01$). Thus, genetic loss of the 5-HT$_{2C}$R in mPFC induces aggregate impulsive action/cocaine cue reactivity, suggesting that depressed 5-HT$_{2C}$R tone in this region confers vulnerability to these interlocked behaviors.

DISCUSSION

We provide the first direct evidence that 5-HT$_{2C}$R neurotransmission in the mPFC is an important neuromolecular driver of inherent impulsive action and cocaine cue

reactivity in an outbred population of rats. Our results demonstrate that high inherent impulsive action is associated with lower cortical 5-HT$_{2C}$R membrane protein and higher 5-HT$_{2C}$R:PSD95 complex expression, and a shift toward edited and less functional 5-HT$_{2C}$R isoforms. We are the first to engineer and use virally mediated 5-HT$_{2C}$R genetic deletion methods to discover that the loss of 5-HT$_{2C}$R in the mPFC results in aggregate impulsive action/cocaine cue reactivity. Thus, we demonstrate that phenotypes in rats that correlate in humans and drive relapse-like behaviors appear to neuromechanistically overlap, with the 5-HT$_{2C}$R functional status acting as a neural rheostat to regulate inherent impulsive action, as well as the intersection between impulsive action and cocaine cue reactivity.

Control of impulsive behavior is a critical factor in the propensity to relapse (Moeller *et al,* 2001b). Tasks of impulsive action can be reasonably matched to provide valid translational research approaches (Robbins, 2002; Voon *et al,* 2013), although few studies have used such tasks to address questions concerning the relationship between impulsive action and cue-reinforced cocaine-seeking. Here, we evaluated the relationship between impulsive action and cocaine-seeking, following a period of FA that is more similar to the situation in cocaine addicts that do not experience extinction training or seeking-taking chained paradigms (Belin *et al,* 2008; Economidou *et al,* 2009). Trait impulsive action does not consistently predict higher levels of cocaine intake (eg, current study; Besson *et al,* 2013), although HI rats acquire cocaine self-administration at a faster rate with higher intake levels than LI rats when trained on submaximal doses of cocaine (Anastasio and Cunningham, unpublished observations; Belin *et al,* 2008; Dalley *et al,* 2007) (but, see Besson *et al,* 2013). Trait impulsive action has also been linked to the persistence of cocaine-seeking behavior in the absence of cocaine (Belin *et al,* 2008) or following punishment (Economidou *et al,* 2009). To our knowledge, our findings comprise the first report that HI animals exhibit elevated cocaine-seeking under conditions in which each lever press is reinforced by discrete cocaine-paired cues; no association was observed under conditions in which lever presses were not reinforced by discrete cocaine-paired cues. These data support a direct relationship between inherent impulsive action and the motivational attributes of discrete cocaine-associated cues. Thus, impulsive action and cue reactivity are related processes that may be mediated by a common underlying neurobiology and mutual 'top-down' cortical circuitry (Chen *et al,* 2013; Dalley *et al,* 2011).

The mPFC is a major neural director of reward-driven behavior, impulse control, and integration of internal states with environmental cues (Kalivas and Volkow, 2005; Koob and Volkow, 2010). Disruptions in brain glutamate and dopamine homeostasis within the corticolimbic circuit are involved in impulsive action and cue reactivity (Dalley *et al,* 2007; Koob and Volkow, 2010; Volkow *et al,* 2009). Both of these core neurotransmitter systems and the response of the neurocircuitry to acute and chronic exposure to cocaine are under the regulatory control of the 5-HT system (for review, see Cunningham and Anastasio, 2013). Integration of the few studies that have directly addressed the involvement of 5-HT in impulsive action (Dalley *et al,* 2002; Winstanley *et al,* 2004a) and cocaine-seeking (Bradberry and Rubino, 2004; Parsons *et al,* 1995) is hampered not only by the complexity of the tasks used and the state in which these behaviors are assessed, but also by the tonic and phasic mechanisms by which the 5-HT system modulates the mPFC microcircuitry (Komlosi *et al,* 2012) and output to subcortical regions (Warden *et al,* 2012). Nonetheless, previous findings coupled with the present results suggest that heterogeneity in 5-HT neurotransmission and perturbations in its balance may contribute to impulsive action and cocaine-seeking, driven in part by altered forebrain 5-HT$_{2C}$R function.

The sites of action and regulatory processes underlying 5-HT$_{2C}$R functional status have not yet been fully characterized in the impulsive action phenotype, and few have evaluated the integrity and fidelity of the 5-HT$_{2C}$R system in impulsive action *ex vivo.* As shown here, differences in total 5-HT$_{2C}$R mRNA levels in the mPFC are not evident between HI and LI rats (Besson *et al,* 2013). Multiple factors impact functionality of 5-HT$_{2C}$R, including (but not limited to) divergent and convergent signaling through G-protein-dependent and -independent mechanisms, constitutive activity, availability of active pools of receptors at the membrane, and the composition of the associated macromolecular protein complex. An important regulator of 5-HT$_{2C}$R constitutive activity is RNA editing. There are no reports of how editing alters the coupling efficiency between the receptor and its G-protein *in vivo;* however, editing of the 5-HT$_{2C}$R restricts its ability to activate intracellular cascades, and also affects receptor desensitization and internalization in cellular models (Werry *et al,* 2008). Further, RNA editing may interact with the alternative splicing machinery of the 5-HT$_{2C}$R, thereby shifting the expression ratio of the full-length protein variant relative to the truncated variant, a mechanistic process that has been proposed to influence the density of 5-HT$_{2C}$R in the plasma membrane *in vitro* (Martin *et al,* 2013). We suggest that differential expression of greater edited 5-HT$_{2C}$R variants and lower 5-HT$_{2C}$R membrane protein may translate into depressed basal levels of 5-HT$_{2C}$R intracellular signaling and subsequent dampened activation of cortical neurons, which could account for the HI phenotype. In keeping with this concept, the functional status of the 5-HT$_{2C}$R is also modulated by association with a repertoire of co-expressed PDZ proteins, such as PSD95 (Becamel *et al,* 2002; Gavarini *et al,* 2006). Given that the association of 5-HT$_{2C}$R with PSD95 promotes desensitization and internalization of the 5-HT$_{2C}$R (Becamel *et al,* 2002; Gavarini *et al,* 2006), our discovery of an elevated cortical 5-HT$_{2C}$R:PSD95 complex in HI rats may reflect phenotypic distinctions in neurobiological trait (inherent) or state (ligand-mediated) markers of 5-HT$_2$R coupling to its G-proteins or downstream signaling capacity. Taken together, these multi-dimensional neurochemical facets that dictate 5-HT$_{2C}$R functional status would be expected to contribute to generation of impulsive action through control of neuronal firing and mPFC output.

Activation of the mPFC is required for suppression of impulsive action (Dalley *et al,* 2011; Murphy *et al,* 2012) and cocaine-seeking behavior (Koya *et al,* 2009; LaLumiere *et al,* 2012). Our observation that 5-HT$_{2C}$R knockdown in the mPFC resulted in elevated impulsive action and cocaine cue reactivity suggests that loss of the 5-HT$_{2C}$R inactivates the mPFC suppression circuit to drive aggregate expression of these behaviors. Of note intra-mPFC administration of the

selective 5-HT$_{2C}$R antagonist SB242084 did not significantly alter impulsive action under training conditions in the 5-CSRT task (Robinson *et al,* 2008). Differences in the sensitivity of the task parameters as well as the tools used to manipulate 5-HT$_{2C}$R function (genetic *vs* pharmacological) most likely underlie the discrepancies between the present study and previous work (Robinson *et al,* 2008). Nonetheless, it is tempting to speculate that 5-HT$_{2C}$R signaling skewed toward a lower basal tone, and/or less constitutively active 5-HT$_{2C}$R may account in part for a shift in the 5-HT$_{2C}$R homeostatic framework that governs output from the mPFC to generate relapse-predictive behaviors in cocaine-dependent individuals. This is an entirely innovative concept, and taken together, our data support the idea that the functional status of the 5-HT$_{2C}$R is differentially regulated in impulsive action phenotypes, and may contribute to a mechanistic imbalance within the mPFC that confers interlocked impulsive action and cue reactivity.

This contribution is significant because it is the first step in a continuum of research that will lead to the targeted development of pharmacological and molecular strategies to restore serotonergic function and minimize deleterious behaviors that promote relapse. Furthermore, this study provides not only insight into neural dysfunction in cocaine dependence but also into the molecular basis of normal brain function, as well as insight into the therapy of other related diseases associated with 5-HT$_{2C}$R dysfunction (eg, obesity and eating disorders) and impulse control deficits.

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