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Serotonin (5-HT) 5-HT$_{2A}$ Receptor (5-HT$_{2A}$R):5-HT$_{2C}$R Imbalance in Medial Prefrontal Cortex Associates with Motor Impulsivity

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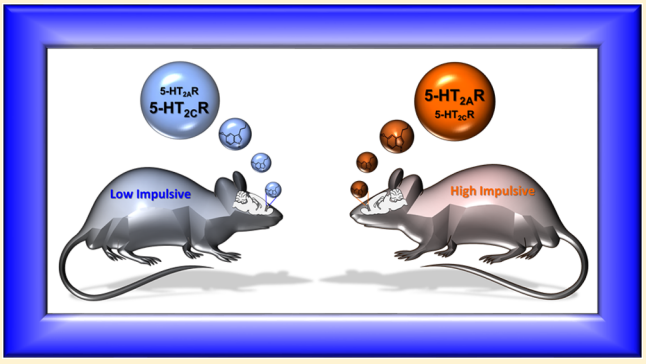
ABSTRACT: A feature of multiple neuropsychiatric disorders is motor impulsivity. Recent studies have implicated serotonin (5-HT) systems in medial prefrontal cortex (mPFC) in mediating individual differences in motor impulsivity, notably the 5-HT$_{2A}$R receptor (5-HT$_{2A}$R) and 5-HT$_{2C}$R. We investigated the hypothesis that differences in the ratio of 5-HT$_{2A}$R:5-HT$_{2C}$R protein expression in mPFC would predict the individual level of motor impulsivity and that the engineered loss of the 5-HT$_{2C}$R would result in high motor impulsivity concomitant with elevated 5-HT$_{2A}$R expression and pharmacological sensitivity to the selective 5-HT$_{2A}$R antagonist M100907. High and low impulsive rats were identified in a 1-choice serial reaction time task. Native protein levels of the 5-HT$_{2A}$R and the 5-HT$_{2C}$R predicted the intensity of motor impulsivity and the 5-HT$_{2A}$R:5-HT$_{2C}$R ratio in mPFC positively correlated with levels of premature responses in individual outbred rats. The possibility that the 5-HT$_{2A}$R and 5-HT$_{2C}$R act in concert to control motor impulsivity is supported by the observation that high phenotypic motor impulsivity associated with a diminished mPFC synaptosomal 5-HT$_{2A}$R:5-HT$_{2C}$R protein:protein interaction. Knockdown of mPFC 5-HT$_{2C}$R resulted in increased motor impulsivity and triggered a functional disruption of the local 5-HT$_{2A}$R:5-HT$_{2C}$R balance as evidenced by a compensatory upregulation of 5-HT$_{2A}$R protein expression and a leftward shift in the potency of M100907 to suppress impulsive behavior. We infer that there is an interactive relationship between the mPFC 5-HT$_{2A}$R and 5-HT$_{2C}$R, and that a 5-HT$_{2A}$R:5-HT$_{2C}$R imbalance may be a functionally relevant mechanism underlying motor impulsivity.



KEYWORDS: *1-Choice serial reaction time task, 5-HT$_{2A}$ receptor, 5-HT$_{2C}$ receptor, motor impulsivity, medial prefrontal cortex, serotonin*

Impulsivity is a complex, multifaceted personality construct[1] and is recognized as a symptomatic element of multiple neuropsychiatric disorders (e.g., attention deficit/hyperactivity disorder, autism, drug addiction).[2] Motor impulsivity (difficulty in withholding a prepotent motor response) and impulsive choice (preference for small immediate rewards over large delayed rewards) are two primary facets of impulsivity which have been reliably assayed with self-report questionnaires and laboratory measures in humans and animals (for reviews).[1,3] Analyses employing choice serial reaction time (CSRT) tasks in outbred rat strains implicate catecholamine, γ-aminobutyric acid (GABA), glutamate, and serotonin (5-HT) systems in corticostriatal circuits in inherent motor impulsivity.[4−10] The 5-HT$_{2A}$ receptor (5-HT$_{2A}$R) and 5-HT$_{2C}$R are G protein-coupled receptors (GPCRs) demonstrated to control motor impulsivity. Systemic administration of selective 5-HT$_{2A}$R antagonists (e.g., M100907)[9,11−14] or selective 5-HT$_{2C}$R agonists (e.g., Ro 60-0175, WAY163909)[11,12,14−17] consistently reduces while the preferential 5-HT$_{2A}$R agonist 2,5-dimethoxy-4-iodoamphetamine (DOI)[9,18−26] or the 5-HT$_{2C}$R antagonist SB242084 enhances motor impulsivity.[12,27] The observation that motor impulsivity was synergistically suppressed by the combination of subthreshold doses of M100907 *plus* WAY163909 raises the possibility that the 5-HT$_{2A}$R and 5-HT$_{2C}$R may act in concert to regulate impulsive responding.[15]

The control of motor impulsivity by the 5-HT$_{2A}$R and 5-HT$_{2C}$R systems intersects within the medial PFC (mPFC), a

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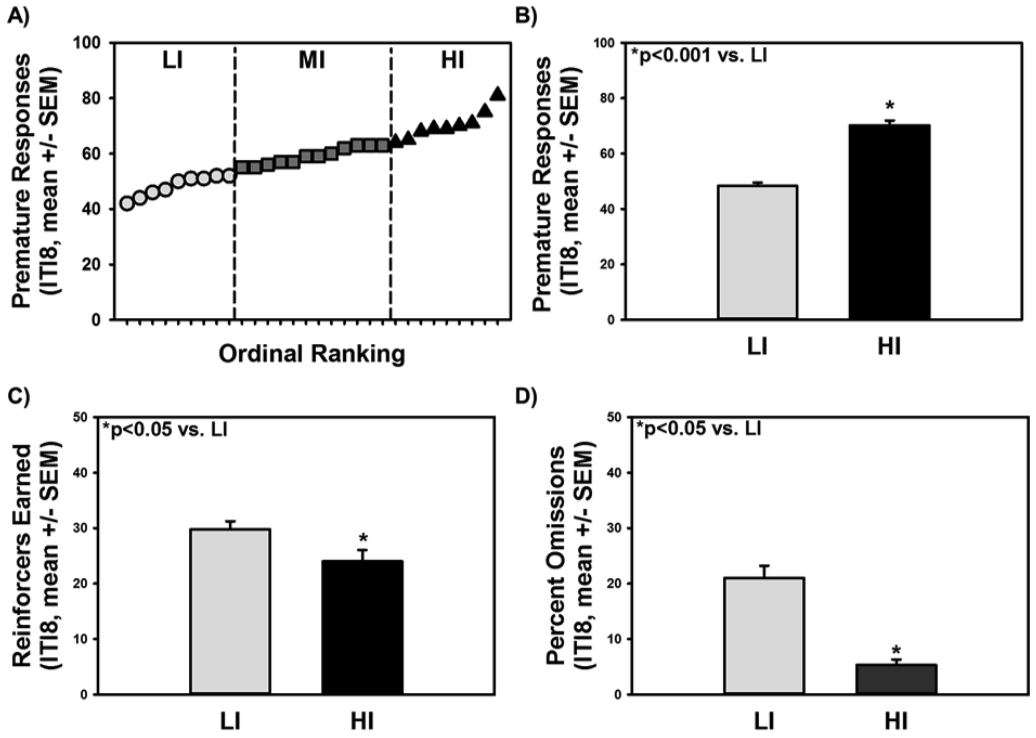

Figure 1. Phenotypic stratification of motor impulsivity with the 1-CSRT task. (A) Number of premature responses made during the ITI8 challenge session was used to stratify rats as high impulsive (HI, upper quartile) or low impulsive (LI, lower quartile) relative to mid impulsive (MI, middle two quartiles) rats. (B) HI rats displayed higher premature responses (*$p < 0.001$ vs LI), (C) earned fewer reinforcers (*$p < 0.05$ vs LI), and (D) made fewer omissions relative to LI rats (*$p < 0.05$ vs LI).

critical neurobiological substrate of motor impulsivity.[10,28−31] The mRNA and/or protein for both the 5-HT_{2A}R and 5-HT_{2C}R are found in glutamatergic and GABAergic neurons in the mPFC.[32−36] Localized infusion of DOI into the mPFC enhances[37] while intra-mPFC M100907[38] suppresses premature responding assessed in the 5-CSRT task. The density of 5-HT_{2A}R[9] as well as 5-HT_{2C}R[6] protein expression in the mPFC predicts premature responses in the 1-CSRT task in outbred rats. High impulsive rats exhibit a greater 5-HT_{2A}R-mediated head-twitch response and are more sensitive to the suppressive effects of the selective 5-HT_{2A}R antagonist M100907,[9] while virally mediated 5-HT_{2C}R knockdown in the mPFC generates elevated premature responses in the 1-CSRT task.[6] Taken together, these data suggest that dysregulation of 5-HT_{2A}R and 5-HT_{2C}R neuronal signaling in the mPFC contributed to high levels of inherent motor impulsivity.

The present study was designed to extend previous findings and investigate the hypothesis that the status and balance of the 5-HT_{2A}R and 5-HT_{2C}R in mPFC constitute neurobiological markers of inherent motor impulsivity in an outbred rodent population. We hypothesized that high impulsive (HI) rats, identified based upon levels of premature responses in the 1-CSRT task,[6,9,13,15,17] would exhibit a higher ratio of 5-HT_{2A}R to 5-HT_{2C}R (5-HT_{2A}R:5-HT_{2C}R) expression in the mPFC, along with our previously observed higher and lower levels of 5-HT_{2A}R[9] and 5-HT_{2C}R,[6] respectively, and a disruption in the 5-HT_{2A}R:5-HT_{2C}R protein:protein interaction, relative to low impulsive (LI) rats. Lastly, we tested the hypothesis that the genetic knockdown of 5-HT_{2C}R in the mPFC will evoke high motor impulsivity concomitant with elevated 5-HT_{2A}R expression and pharmacological sensitivity to the suppressive effects of the selective 5-HT_{2A}R antagonist M100907 relative to control rats. The observed differential ratio of native 5-HT_{2A}R:5-HT_{2C}R in high vs low impulsive outbred rats as well as the observations from an engineered imbalance in the 5-HT_2R system suggest that cortical 5-HT_{2A}R and 5-HT_{2C}R homeostasis is a key regulatory factor in motor impulsivity.

RESULTS AND DISCUSSION

Phenotypic Stratification of Motor Impulsivity Is Achievable with the 1-CSRT Task. The motor impulsivity phenotype is reliably identifiable in CSRT tasks.[4−9] Here, a cohort of outbred rats was stratified for the motor impulsivity phenotype using the 1-CSRT task. High (HI; $n = 9$) and low impulsive rats (LI; $n = 9$) were classified as the upper and lower quartile based upon premature responses on an ITI8 challenge session; the ITI8 challenge promotes premature responding and thus more easily allows for the detection of phenotypic differences.[4−9] Accuracy on the ITI5 during task training and maintenance sessions and on the ITI8 challenge session averaged 97−98% indicating that rats detected the stimuli and performed effectively under both ITI conditions.[6,9] Figure 1A illustrates the ordinal distribution of individual rats plotted by premature responses for the upper (HI) and lower (LI) quartile of rats relative to those in the mid (MI) range (middle two quartiles). HI rats engendered more premature responses vs LI rats (Figure 1B; $p < 0.001$). HI rats earned fewer reinforcers (Figure 1C; $p < 0.05$) and exhibited lower percent omissions (Figure 1D; $p < 0.05$) vs LI rats. No differences between LI and HI on ITI8 were observed in accuracy (97.3% ± 1.3 vs 97.8% ± 1.1) or the latency to the first pellet (1.6 ± 0.7 s vs 0.6 ± 0.2). There was no significant difference in 1-CSRT task performance or phenotypic identification between the three cohorts of rats (data not shown) employed in the present studies (see Research Design subsection).[6,9] Thus, the

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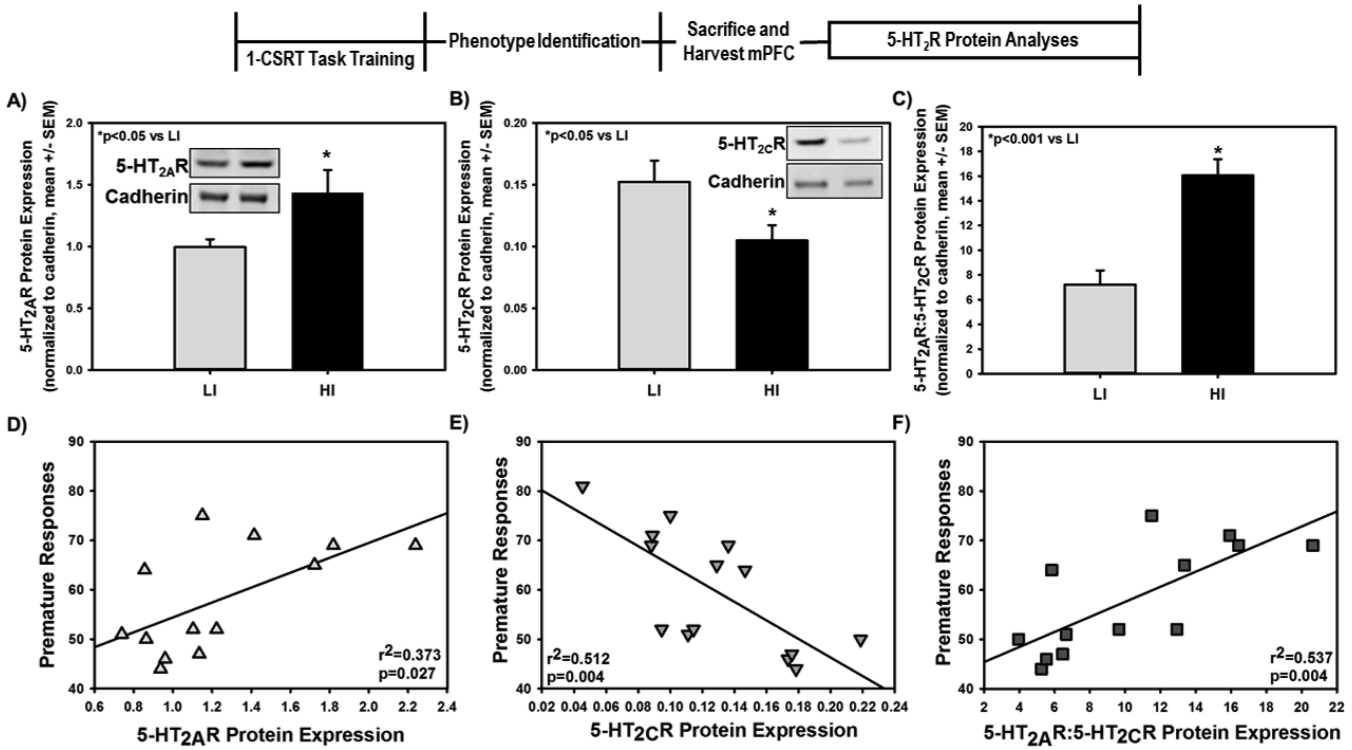

Figure 2. Inherent motor impulsivity predicts mPFC 5-HT_{2A}R:5-HT_{2C}R synaptosomal protein profile. Following 1-CSRT task training and phenotypic identification, the mPFC was collected for biochemical analysis. Immunoblots for the (A inset) 5-HT_{2A}R, (B inset) 5-HT_{2C}R, and (A and B inset) cadherin loading control were performed using crude synaptosomal protein from the mPFC. Densitometric quantitation revealed (A) higher 5-HT_{2A}R, (B) lower 5-HT_{2C}R, and (C) a heightened ratio of 5-HT_{2A}R:5-HT_{2C}R protein expression in HI relative to LI rats (*$p < 0.05$ vs LI). There was a correlation between (D) premature responses and 5-HT_{2A}R protein expression ($r^2 = 0.373$; $p < 0.05$), (E) premature responses and 5-HT_{2C}R protein expression ($r^2 = 0.512$; $p < 0.001$), and (F) premature responses and the 5-HT_{2A}R:5-HT_{2C}R ratio ($r^2 = 0.537$; $p < 0.001$).

natural variation in levels of motor impulsivity in an outbred rat population is quantifiable in the 1-CSRT task.[6,9]

The mPFC 5-HT_{2A}R:5-HT_{2C}R Synaptosomal Protein Profile Distinguishes Motor Impulsivity Phenotypes. The 5-HT_{2A}R and 5-HT_{2C}R regulate the excitatory/inhibitory balance in the mPFC, and the net consequence of 5-HT_{2A}R and 5-HT_{2C}R activation on cortical neurons is interactive as shown by the observation that constitutive knockout of the 5-HT_{2A}R upregulates 5-HT_{2C}R control over the excitability of mPFC pyramidal neurons.[39] Rodent studies provide the opportunity to link individual differences in motor impulsivity with the functional capacity of cortical 5-HT_{2A}R and 5-HT_{2C}R ex vivo. The 5-HT_{2A}R and 5-HT_{2C}R are detected postsynaptically with a smaller proportion found in the presynaptic milieu in forebrain.[40−42] To assess the available synaptosomal pool of receptors as a possible factor in differential receptor functionality and a neurobiological substrate of inherent motor impulsivity, we evaluated 5-HT_{2A}R and 5-HT_{2C}R synaptosomal protein expression in the mPFC in individual HI and LI rats. The synaptosomal protein fraction employed is enriched for the presynaptic and postsynaptic compartments.[43] High impulsive rats (determined from the ITI8 challenge) demonstrated higher synaptosomal mPFC 5-HT_{2A}R (Figure 2A, $p < 0.05$)[9] and lower synaptosomal mPFC 5-HT_{2C}R (Figure 2B, $p < 0.05$)[6], resulting in a higher ratio of 5-HT_{2A}R to 5-HT_{2C}R (5-HT_{2A}R:5-HT_{2C}R; Figure 2C, $p < 0.001$). Premature responses positively correlated with mPFC 5-HT_{2A}R synaptosomal protein levels (Figure 2D; $r^2 = 0.373$; $p = 0.027$), inversely correlated with mPFC 5-HT_{2C}R synaptosomal protein levels (Figure 2E, $r^2 = 0.512$; $p = 0.004$) and positively correlated with the mPFC 5-HT_{2A}R to 5-HT_{2C}R ratio (Figure 2F, $r^2 = 0.537$; $p = 0.004$). In contrast, the reinforcers earned on the ITI8 challenge did not correlate with either 5-HT_{2A}R ($r^2 = 0.0647$; $p = 0.380$) or 5-HT_{2C}R ($r^2 = 0.0684$; $p = 0.367$) protein expression in mPFC; a trend toward a correlation between reinforcers earned and the 5-HT_{2A}R:5-HT_{2C}R ratio was seen ($r^2 = 0.252$; $p = 0.067$). The percent omissions positively correlated with 5-HT_{2C}R ($r^2 = 0.647$; $p < 0.001$) and inversely correlated with the 5-HT_{2A}R:5-HT_{2C}R ratio ($r^2 = 0.484$; $p = 0.006$), but not with 5-HT_{2A}R expression ($r^2 = 0.101$; $p = 0.267$). Taken together, these data indicate that the 5-HT_{2A}R:5-HT_{2C}R balance is a neurobiological substrate underlying both prepotent responding and motivational drive in high inherent motor impulsivity.

High Motor Impulsivity Associates with Lower 5-HT_{2A}R:5-HT_{2C}R Protein Complex Formation in the mPFC. The 5-HT_{2A}R and 5-HT_{2C}R transcript and/or protein have been localized to both glutamate and GABA neurons in the mPFC,[32−35,44−47] and has most recently been found to colocalize in GABA neurons, and perhaps pyramidal neurons of the mPFC.[36] Given the association of 5-HT_{2A}R and 5-HT_{2C}R proteins in mPFC with levels of motor impulsivity (Figure 2),[6,9] we further considered the possibility that a 5-HT_{2A}R:5-HT_{2C}R protein complex may exist in the mPFC and that the pattern of complex formation may track with inherent motor impulsivity. Co-immunoprecipitation protocols[6,9,17,40] were employed to assess the protein:protein interaction of the 5-HT_{2A}R and 5-HT_{2C}R in the mPFC. This technique is based on the ability of an antibody to capture the primary target (e.g., 5-HT_{2A}R or 5-HT_{2C}R) as well as other macromolecules that are

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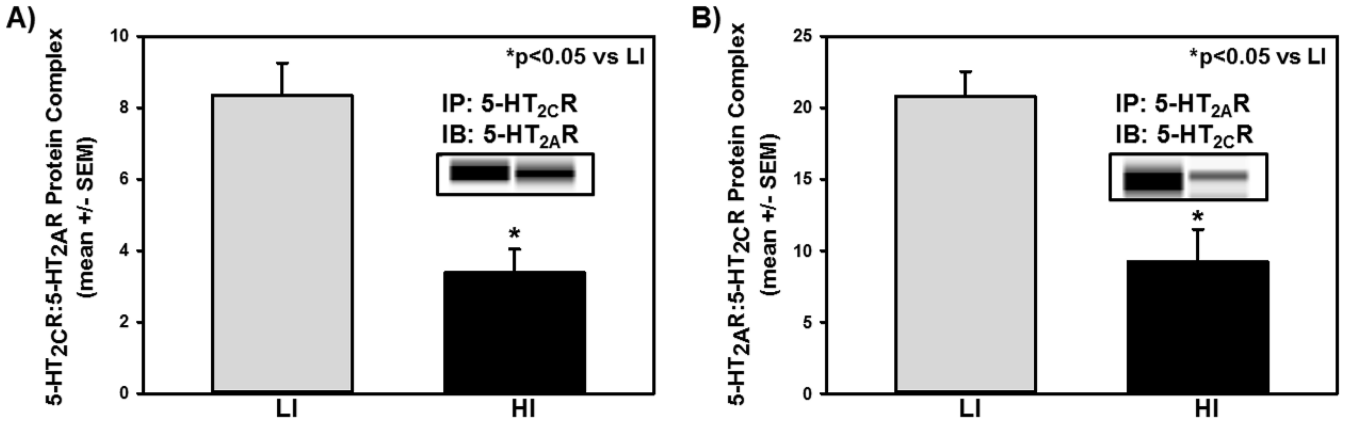

Figure 3. The 5-HT_{2A}R:5-HT_{2C}R mPFC protein complex is disrupted in high motor impulsivity. Following 1-CSRT task training and phenotypic identification, the mPFC was collected for biochemical analysis. (A) Immunoprecipitation (IP) for 5-HT_{2C}R followed by immunoblot (IB) for 5-HT_{2A}R yielded 5-HT_{2A}R immunoreactivity in both HI and LI rats. (B) Immunoprecipiation for 5-HT_{2A}R and IB for 5-HT_{2C}R yielded 5-HT_{2C}R immunoreactivity in both HI and LI rats. (A, B) Qualitative (insets) and quantitative demonstration that synaptosomal 5-HT_{2C}R associates with 5-HT_{2A}R in the mPFC to a lesser extent in HI relative to LI rats (*$p < 0.05$). Insets are representative electrophoretic bands. Arbitrary units (A.U.) of densitometry are presented.

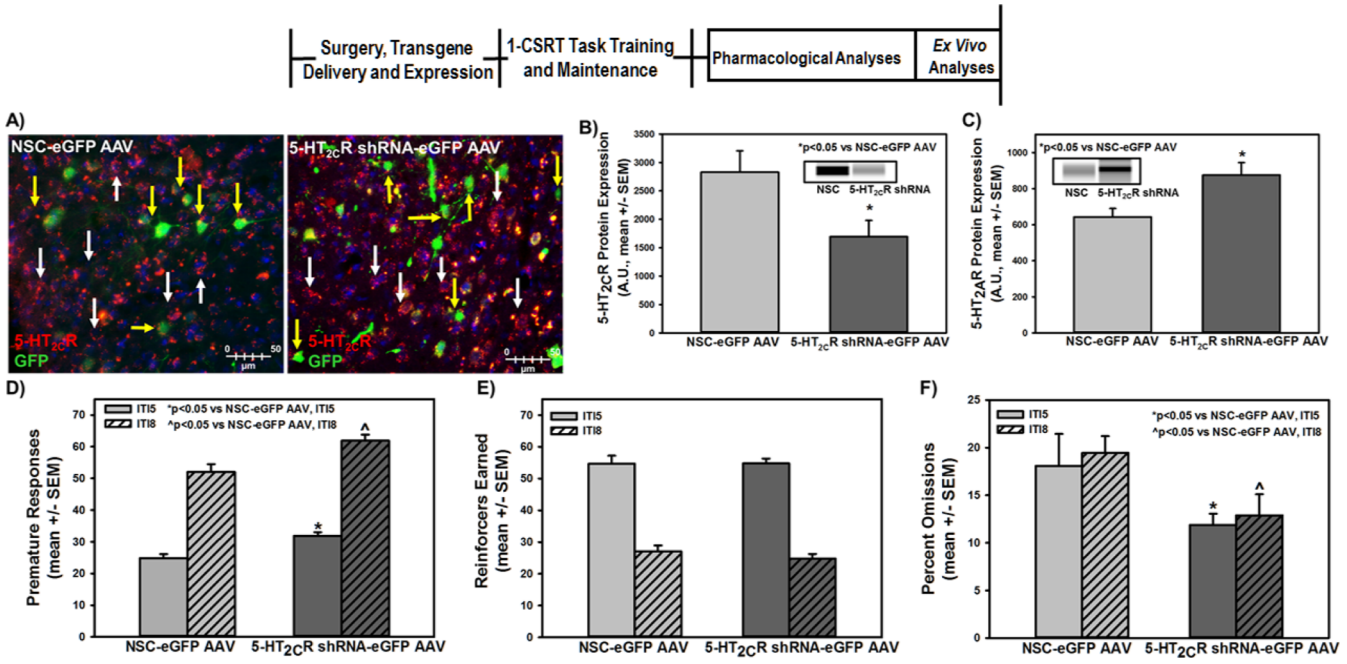

Figure 4. Knockdown of mPFC 5-HT_{2C}R recapitulates high motor impulsivity. Following intra-mPFC transgene delivery and stable viral vector expression, control and 5-HT_{2C}R knockdown rats were subjected to the 1-CSRT task. (A) Neurons infected with the NSC-eGFP AAV (left) or 5-HT_{2C}R shRNA-eGFP AAV (right) demonstrate green immunofluorescence; a subset of infected neurons are denoted by yellow arrows. A subset of noninfected neurons are denoted with white arrows. All infected neurons are not denoted. Red immunofluorescence indicates 5-HT_{2C}R protein expression. 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). The 5-HT_{2C}R shRNA-eGFP AAV (green) induced a knockdown of 5-HT_{2C}R protein (red) in infected neurons (yellow arrows) relative to noninfected neurons (white arrows). Ex vivo biochemical analyses indicate that 5-HT_{2C}R knockdown rats display (B) lower mPFC 5-HT_{2C}R protein levels ($p < 0.05$ vs NSC-eGFP AAV-VEH) and (C) higher mPFC 5-HT_{2A}R protein levels ($p < 0.05$ vs NSC-eGFP AAV-VEH) relative to control rats. The insets are representative electrophoretic bands. Arbitrary units (A.U.) of densitometry are presented. (D) The 5-HT_{2C}R knockdown rats expressed significantly higher premature responses vs control rats on an IT5 maintenance session (open bars) and an ITI8 challenge session (hatched bars) (*$p < 0.05$ vs NSC-eGFP AAV). (E) There was no significant difference between 5-HT_{2C}R knockdown and control rats for the number of reinforcers earned. (F) The 5-HT_{2C}R knockdown rats expressed significantly lower percent omissions vs control rats on an IT5 maintenance session (open bars) and an ITI8 challenge session (hatched bars) (*$p < 0.05$ vs NSC-eGFP AAV).

bound to the target within a tissue lysate (in this case, mPFC crude synaptosomal protein). The coimmunoprecipitation assay was conducted under saturating antibody conditions, but not 100% efficiency, to control for the expression differences in 5-HT_{2A}R and 5-HT_{2C}R in HI vs LI rats. We immunoprecipitated comparable amounts (~5 μg) of 5-HT_{2A}R or 5-HT_{2C}R from mPFC synaptosomal protein extracts of HI and LI rats and then equal amounts of protein (1 μg) were subjected to immunoblot analyses for the receptor (performed in duplicate) (Figure 3). Immunoprecipitation (IP) of

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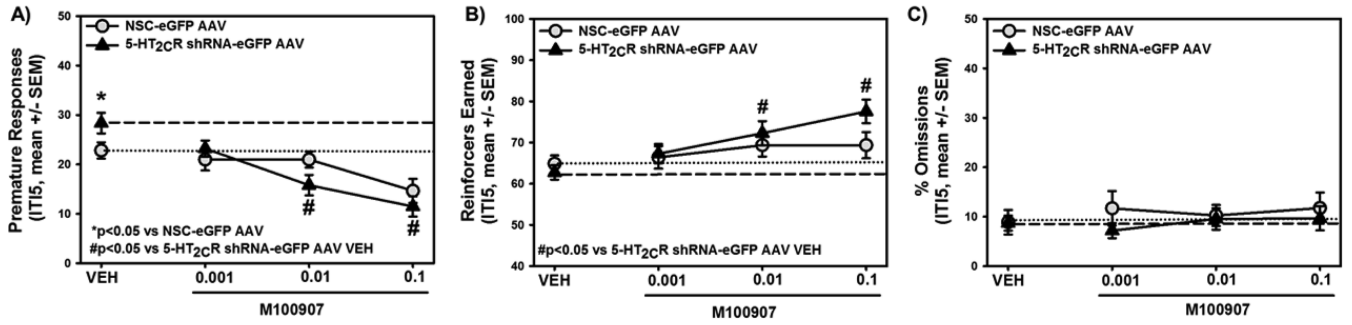

Figure 5. Knockdown of mPFC 5-HT$_{2C}$R recapitulates high motor impulsivity and enhances 5-HT$_{2A}$R regulation of motor impulsivity. Following stable viral vector expression and 1-CSRT task training, the effects of M100907 (0.001, 0.01, and 0.1 mg/kg) were evaluated under ITI5 conditions. (A) Baseline levels of premature responses in 5-HT$_{2C}$R knockdown rats administered vehicle (VEH; ▲, dashed line) were significantly higher than the vehicle baseline in control rats (○, dotted line; *$p < 0.05$ vs NSC-eGFP AAV-VEH). In the 5-HT$_{2C}$R knockdown rats, M100907 significantly suppressed premature responses at 0.01 and 0.1 mg/kg ($^{\#}p < 0.05$ vs 5-HT$_{2C}$R shRNA-eGFP AAV-VEH), below the vehicle baseline of control rats. (B) Baseline levels of reinforcers earned in 5-HT$_{2C}$R knockdown rats administered vehicle (VEH; ▲, dashed line) did not differ from the vehicle baseline in control rats (○, dotted line). M100907 significantly increased the number of reinforcers earned at 0.01 and 0.1 mg/kg ($^{\#}p < 0.05$ vs 5-HT$_{2C}$R shRNA-eGFP AAV-VEH), above the vehicle baseline of control rats. (C) Baseline levels of omissions in 5-HT$_{2C}$R knockdown rats administered vehicle (VEH; ▲, dashed line) did not differ from the vehicle baseline in control rats (○, dotted line). M100907 did not alter the number of percent of omissions in 5-HT$_{2C}$R knockdown or control rats.

synaptosomal protein extracts of the mPFC using the 5-HT$_{2C}$R antibody followed by immunoblot (IB) with the 5-HT$_{2A}$R antibody yielded a band at the expected molecular weight (~55 kDa) for 5-HT$_{2A}$R (Figure 3A); the reciprocal experiment (5-HT$_{2A}$R IP followed by 5-HT$_{2C}$R IB) yielded a band at the expected molecular weight (~46 kDa) for 5-HT$_{2C}$R (Figure 3B). Intriguingly, the synaptosomal 5-HT$_{2A}$R:5-HT$_{2C}$R protein interaction was attenuated in the mPFC of HI relative to LI rats (Figure 3A and 3B, $p < 0.05$), suggesting that a 5-HT$_{2A}$R:5-HT$_{2C}$R complex exists and may be poised to confer regulatory control over the mPFC microcircuitry and output important in inherent motor impulsivity via actions at the single neuron level.

Engineered Loss of 5-HT$_{2A}$R:5-HT$_{2C}$R Balance Confers High Motor Impulsivity. We next tested the hypothesis that, if the 5-HT$_{2A}$R:5-HT$_{2C}$R homeostasis in mPFC is important in the control of motor impulsivity, then loss of the 5-HT$_{2C}$R in this region would tilt the 5-HT$_{2A}$R:5-HT$_{2C}$R balance toward a greater 5-HT$_{2A}$R influence. In the present experiment, we targeted the mPFC comprising the ventral prelimbic and dorsal infralimbic subnuclei[6] and employed adeno-associated viral (AAV) vectors to selectively suppress expression of the 5-HT$_{2C}$R using RNA interference to silence gene expression and to curtail production of the protein. Details of the shRNA design, production and validation of the AAV vectors are published.[6] The *ex vivo* analyses of microinfusion placements in individual rats illustrated that the viral infection was localized within the mPFC along the boundary of the ventral prelimbic/dorsal infralimbic subnuclei (data not shown). The mPFC of a rat infused with the nonsilencing control (NSC)-eGFP AAV exhibited 5-HT$_{2C}$R-immunoreactivity in infected neurons (yellow arrows) and noninfected neurons (white arrows; Figure 4A, left) while that of a rat infused with the 5-HT$_{2C}$R shRNA-eGFP AAV exhibited reduced 5-HT$_{2C}$R-immunoreactivity in infected neurons (yellow arrows) relative to non-infected neurons (white arrows; Figure 4A, right).

Ex vivo analyses indicated that the 5-HT$_{2C}$R shRNA-eGFP AAV significantly attenuated 5-HT$_{2C}$R protein expression (Figure 4B, $p < 0.05$) and augmented 5-HT$_{2A}$R protein expression (Figure 4C, $p < 0.05$) relative to control rats. Rats with a knockdown of the 5-HT$_{2C}$R in the mPFC expressed significantly higher premature responses on both ITI5 maintenance and ITI8 challenge sessions relative to control rats infused with the NSC-eGFP AAV (Figure 4D, $p < 0.05$). The accuracy in control vs 5-HT$_{2C}$R knockdown rats on the ITI5 maintenance (96.3% ± 0.7 vs 97.5% ± 0.2) and ITI8 challenge sessions (97.6% ± 0.8 vs 98.3% ± 0.7) did not differ nor did the latency to start the ITI5 (1.3 ± 0.3 s vs 1.1 ± 0.3 s) or ITI8 (0.7 ± 0.3 s vs 1.3 ± 0.5 s) sessions. Control vs 5-HT$_{2C}$R knockdown rats also did not differ in the number of reinforcers earned (Figure 4E, n.s.). There was a significant decrease in percent omissions between control and knockdown rats on both the ITI5 and ITI8 challenge sessions (Figure 4F, $p < 0.05$). These data indicate a role of the mPFC 5-HT$_{2A}$R:5-HT$_{2C}$R balance to selectively govern premature and motivational responding, key facets underlying motor impulsivity in the 1-CSRT task.[6]

Systemic administration of a selective 5-HT$_{2A}$R antagonist attenuates motor impulsivity in the 1- or 5-CSRT tasks.[11−13,15,24,48] HI rats demonstrated higher levels of mPFC 5-HT$_{2A}$R protein levels (replicated here; Figure 2A) and higher pharmacological sensitivity to the selective 5-HT$_{2A}$R antagonist M100907.[9] Thus, we tested the hypothesis that the engineered imbalance in 5-HT$_{2A}$R:5-HT$_{2C}$R (Figure 4B and 4C) would associate with a leftward shift in the potency of M100907 to suppress motor impulsivity. The dose range of M100907 (0.001−0.1 mg/kg) and time of injection (30 min prior) were chosen based upon the protocols employed to demonstrate that high impulsive outbred Sprague−Dawley rats were more sensitive to the behavioral effects of M100907.[9] Vehicle-treated 5-HT$_{2C}$R shRNA-eGFP AAV rats demonstrated elevated premature responses relative to vehicle-treated NSC-eGFP AAV rats (Figure 5A; $p < 0.05$), as demonstrated previously.[6] M100907 dose-dependently decreased premature responses; there was no main effect of pretreatment (NSC-eGFP AAV, 5-HT$_{2C}$R shRNA-eGFP AAV) on premature responses ($F_{1,68} = 0.05$, n.s.), but a main effect of treatment (M100907 doses) ($F_{3,68} = 13.82$, $p < 0.001$) and a pretreatment × treatment interaction ($F_{3,68} = 3.07$, $p < 0.05$) were observed. In 5-HT$_{2C}$R knockdown rats, a main effect of M100907 treatment was observed ($F_{3,37} = 15.56$, $p < 0.001$); planned comparisons showed that both the 0.01 and 0.1 mg/kg doses of

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M100907 decreased premature responses vs vehicle in knockdown rats. In control rats, a trend toward a main effect of M100907 treatment was observed ($F_{3,30}$ = 2.78, p = 0.06). Hence, the decrement in 5-HT_{2C}R following infusion of the 5-HT_{2C}R-shRNA-AAV into the mPFC enhances 5-HT_{2A}R control over motor impulsivity.

The number of reinforcers earned increased significantly in 5-HT_{2C}R knockdown, but not control, rats administered M100907 (Figure 5B), an effect similarly noted in HI rats.[9] No main effect of pretreatment ($F_{1,68}$ = 0.05, n.s.), a main effect of treatment ($F_{3,68}$ = 9.07, n.s.), but no pretreatment × treatment interaction ($F_{3,68}$ = 2.08, n.s.) on reinforcers earned was observed. In 5-HT_{2C}R knockdown rats, a main effect of M100907 treatment on reinforcers earned was observed ($F_{3,30}$ = 6.56, n.s.); 0.01 and 0.1 mg/kg of M100907 significantly increased reinforcers earned in knockdown rats. In control rats, a main effect of M100907 treatment on reinforcers earned was not observed ($F_{3,30}$ = 0.42, n.s.). No main effect of pretreatment ($F_{1,68}$ = 0.64, n.s.), no main effect of treatment ($F_{3,68}$ = 0.61, n.s.), and no pretreatment × treatment interaction ($F_{3,68}$ = 1.20, n.s.) on percent omissions were observed (Figure 5C). These finding suggests that M100907 may selectively enhance cognition under conditions in which the impulse control system is taxed.[9,49,50]

Components of motor impulsivity as a cognitive construct include, but are not limited to, inhibitory control, failure to consider the consequences of behavior, a sense of urgency prior to or during task performance, and the attraction to approach and attain rewards (incentive motivation; "wanting").[1,51,52] At both molecular and systems levels, the brain utilizes 5-HT within cellular cascades and webs comprised of transcription factors, growth factors and other neurotransmitters, as a mediator of integral physiological and psychological functions, including the processing of incentive-motivational stimuli and impulse control.[51,53−56] Acute tryptophan depletion in humans, which temporarily lowers brain 5-HT levels, elevates motor impulsivity[57] and disrupts motivated actions selectively in individuals with high inherent impulsivity.[58] Depletion of 5-HT in the dorsal raphe nucleus causes a robust increase in impulsive action in rodents,[59,60] while elevated 5-HT release in the mPFC positively correlates with motor impulsivity.[61,62] The conflicting outcomes from global 5-HT manipulations highlight not only the multidimensionality of impulsivity, but also that the ultimate impact of 5-HT is governed by its actions at 5-HT receptor proteins, including the 5-HT_2R family.

The engagement of 5-HT_{2A}R and 5-HT_{2C}R mechanisms in the control of motor impulsivity (for review)[31] is further supported by the results of the present study. We discovered that high impulsive rats (both inherent and engineered) displayed enhanced prepotent responding and motivationally driven behavior (as evidenced by greater premature responses and fewer omissions made, respectively). Further, motor impulsivity (premature responses) and motivation to respond (percent omissions) associated with the ratio of 5-HT_{2A}R to 5-HT_{2C}R protein expression in the mPFC. The mechanisms that generate a specific receptor protein profile that associates with an expressed behavioral phenotype are currently unknown. Given that the expression of 5-HT receptors in human PFC appears to remain balanced across development into adulthood[63] and that early life events are known to trigger plasticity of 5-HT systems,[64−66] it is possible that differential developmental experience[67] could have durable ramifications for 5-HT receptor control of the mPFC microcircuitry. This hypothesis remains to be tested; however, a functional 5-HT_{2A}R:5-HT_{2C}R rheostat in mPFC may play a pivotal role in spontaneously occurring individual differences in impulsivity.

Phenotypic variance in motor impulsivity has been linked to several candidate neurotransmitter mechanisms within limbic-corticostriatal circuitry.[68] We propose that vulnerability to motor impulsivity associates with expression patterns of 5-HT_{2A}R and 5-HT_{2C}R (present study),[6,7,9] while multiple studies implicate dopamine,[5,7,69] γ-aminobutyric acid (GABA),[8,10] and glutamate system involvement.[37,70−73] Our observation that knockdown of the mPFC 5-HT_{2C}R does not fully recapitulate the motor impulsivity phenotype characterized in outbred rats suggests an interplay between serotonergic and other neurochemical circuits may be differentially recruited in the expression of distinct components of impulsive behavior. One candidate mechanism is the interaction between 5-HT and glutamate systems in the mPFC. Pretreatment with a glutamate mGlu2/3 receptor agonist has been shown to suppress motor impulsivity generated by excessive 5-HT_{2A}R stimulation locally in the mPFC,[37] presumably due to the actions of mGlu2/3 agonists to suppress 5-HT_{2A}R-mediated glutamate release from neurons that terminate onto mPFC pyramidal cells.[74] Thus, the differential recruitment of a "functional crosstalk" between the 5-HT_{2A}R and 5-HT_{2C}R (present results), the mGlu2/3 and 5-HT_{2A}R,[37] or other combinations of receptors may be critical drivers of the phenotypic profile of impulsivity.

A higher ratio of the 5-HT_{2A}R to 5-HT_{2C}R protein density in frontal cortex has been noted for the mouse, rat, and human,[75] and here we report that the ratio of 5-HT_{2A}R to 5-HT_{2C}R protein expression in the rat mPFC predicts the inherent level of motor impulsivity in individual rats. That the net consequence of 5-HT_{2A}R and 5-HT_{2C}R function in mPFC may be reciprocal or interactive is supported by the present finding that knockdown of 5-HT_{2C}R in the mPFC increased motor impulsivity and resulted in a compensatory upregulation of 5-HT_{2A}R protein expression and a leftward shift in the potency of M100907 to suppress impulsive behavior. These findings are consistent with a study which suggested that the constitutive knockout of the 5-HT_{2A}R upregulated 5-HT_{2C}R control over the excitability of mPFC pyramidal neurons.[39] Further, signal transduction through the 5-HT_{2A}R and 5-HT_{2C}R locally controls the intrinsic microcircuitry of the PFC through postsynaptic modulation of synaptic input to pyramidal neurons and subpopulations of interneurons (for review)[76] and potentially via regulation of neurotransmitter release (e.g., acetylcholine, dopamine) by presynaptic 5-HT_{2A}R and/or 5-HT_{2C}R heteroreceptors (for review).[77]

Our finding that these two GPCRs reside in the same protein complex and that this complex is enriched in the mPFC of low impulsive rats suggests that the behavioral output of the mPFC is dependent in part upon a 5-HT_{2A}R:5-HT_{2C}R protein:protein interaction. Our unpublished data corroborates a recent publication[36] which demonstrated that the majority of GABA neurons in the prelimbic mPFC expressed 5-HT_{2C}R immunoreactivity and most coexpressed 5-HT_{2A}R immunoreactivity; a small population of cells with a pyramidal profile also expressed both receptors. The colocalization of the receptors in the same neuron suggests that the 5-HT_{2A}R:5-HT_{2C}R protein complex may occur at the single cell level. Serotonin actions at the 5-HT_{2A}R are excitatory in both interneurons and pyramidal neurons of the neocortex (for review),[76] with these actions noted to be activity-dependent[78] and projection-specific.[79] There is much less known, and some debate, concerning the

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modulatory actions of 5-HT at the 5-HT_{2C}R in cortical neurons.[80] Activation of 5-HT_{2C}R depolarized pyramidal neurons in piriform cortex;[81] however, the observed 5-HT-induced depolarization of mPFC pyramidal neurons is not blocked by the selective 5-HT_{2C}R antagonist SB242084.[39,82] The complexity of the mPFC functional microcircuitry modulated by 5-HT via the 5-HT_2R may be founded in the different subpopulations of interneurons which express the receptors (e.g., parvalbumin, calbindin, calretinin).[46,83] Further, the impact of a 5-HT_{2A}R:5-HT_{2C}R protein complex on neuronal firing is not known and it is wholly possible that the biochemical, signaling, and pharmacological properties of the protein complex diverge from that of the single receptor, although this is a controversial and active research topic.[84−87] Thus, while future research is required to tease apart the relative role of these two GPCRs within the same neurons versus interacting neurons in the mPFC, altered firing of GABA neurons which colocalize the 5-HT_{2A}R and 5-HT_{2C}R may dysregulate pyramidal outflow to drive motor impulsivity. The fact that systemic administration of 5-HT_{2A}R and the 5-HT_{2C}R ligands oppositionally control motor impulsivity[9,11−20,22−26] (and other behaviors)[88] adds further impetus to disentangling the mechanisms through which these receptors control neuronal output to mediate behaviors that contribute to chronic health disorders (e.g., drug addiction, attention deficit disorder, autism, and obesity/binge eating disorder).

METHODS

General Methods. *Animals.* Male, outbred Sprague−Dawley rats (n = 120); Harlan, Houston, TX) weighing 250−275 g upon arrival were housed two/cage under a 12 h light−dark cycle with controlled temperature (21−23 °C) and humidity (40−50%). Animals were acclimated for 7 days to the colony room prior to the start of handling and experimental procedures. During the 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. All experiments were conducted 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.

Drugs. M100907 [*R*-(1)-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidine-methanol] was synthesized by Kenner Rice (National Institute on Drug Abuse, Bethesda, MD) and dissolved in 1% Tween 80 in 0.9% NaCl.

1-Choice Serial Reaction Time (1-CSRT) Task. Procedures occurred in standard five-hole nose-poke 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 ventilated and sound-attenuated chambers (MedAssociates, St. Albans, VT). The 1-CSRT task methodology has been described in detail previously.[6,9,13,15,17] Briefly, rats were habituated to the test chamber; a nose-poke into the singly illuminated center hole resulted in the delivery of one food pellet into the magazine on the opposite wall of the chamber and simultaneous illumination of the magazine light. 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 composed of daily sessions of 100 trials to be completed in a maximum of 30 min; each training stage (9 total) involved incrementally lowering the stimulus duration with a 5 s limited hold and an intertrial interval (ITI) of 5 s. A maximum of 100 correct responses in a session resulted in a maximum of 100 reinforcers earned; incorrect, premature responses or omissions resulted in a 5 s time-out period and a reduction in reinforcers obtained. Advancement to the next training stage required rats to meet acquisition criteria: ≥50 correct responses, >80% accuracy [correct responses/(correct + incorrect) × 100], and <20% omissions (omitted responses/trials completed × 100).[13] Premature responses were employed as the primary indication of motor impulsivity. The number of reinforcers earned provides a measure of task competency and a secondary assessment of motor impulsivity, while percent accuracy was a general indication of attentional capacity. Percent omissions indicated failures of detection of the visual stimuli in the center hole as well as motivation to perform the task.

Identification of Motor Impulsivity Phenotype. After meeting stability criteria for the final training stage over three consecutive ITI5 sessions (with <20% variability, approximately days 25−30), an ITI8 challenge session was conducted in which the ITI was 8 s for the session.[6,9,61] High and low impulsive rats were defined as the upper and lower quartile of premature responses assessed on the ITI8 challenge, respectively.

Research Design. *Inherent Motor Impulsivity Predicts Expression Patterns of 5-HT_{2A}R to 5-HT_{2C}R Protein in mPFC in Individual Rats (See Experimental Timeline; Figure 2).* HI and LI rats were identified from three independent cohorts of outbred rats (Cohort 1; 5-HT_{2A}R and 5-HT_{2C}R expression and ratio; Cohorts 2 and 3; 5-HT_{2A}R:5-HT_{2C}R protein complex). At 2 to 3 days following behavioral testing, rats were anesthetized [chloral hydrate solution (400 mg/kg)] and decapitated, and brains were extracted. The mPFC (containing infralimbic, prelimbic, and anterior cingulate cortex) was microdissected immediately over ice, flash frozen in liquid nitrogen, and stored at −80 °C for subsequent protein extraction.

A crude synaptosomal protein fraction enriched for pre- and postsynaptic proteins (i.e., presynaptic terminals, postsynaptic membranes, postsynaptic density, and synaptic protein complexes) was prepared as described previously.[40,83] Tissue from the mPFC was homogenized in 10 times w/v ice cold Krebs buffer (125 mM NaCl, 1.2 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 22 mM Na_2CO_3, 1 mM NaH_2PO_4, 10 mM glucose) containing 0.32 M sucrose plus protease inhibitor cocktail and phosphatase inhibitor 2 and 3 cocktails (10 μL/mL; 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 collected and centrifuged at 16 000*g* for 20 min at 4 °C to pellet the crude synaptosome. The pellet was resuspended in Krebs buffer with 1% dodecyl maltoside.

Equal amounts of crude synaptosomal protein prepared from the mPFC[6,9,40] were separated by SDS-PAGE and transferred to a PVDF membrane for blotting with 5-HT_{2A}R antibody (AB16028, 1:1000; Abcam, Cambridge, MA), 5-HT_{2C}R antibody (D12, 1:100: Santa Cruz Biotechnology, Dallas, TX) or pan-cadherin antibody (AB6528, 1:10000; Abcam). Membranes were incubated with mouse IgG IRDye 800 (1:10000) or rabbit IgG IRDye 680 (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 5-HT_{2A}R and 5-HT_{2C}R immunoreactivity normalized to pan-cadherin immunoreactivity.

Co-immunoprecipitation methodology was employed to assess the 5-HT_{2C}R protein complex with 5-HT_{2A}R in the mPFC of HI and LI rats (n = 4−5/phenotype). The 5-HT_{2C}R antibody (D12, 45 μg, Santa Cruz) or 5-HT_{2A}R antibody (AB16028, 10 μg, Abcam) was covalently cross-linked onto protein A/G resin as previously described with minor modifications.[9,40] Synaptosomal protein was incubated with the antibody-cross-linked resin for 48 h at 4 °C with constant shaking. The eluted protein was resuspended in resuspension buffer containing 1% SDS and 0.5% NP40 and subjected to the Wes automated Western blotting system (ProteinSimple, San Jose, CA), which utilizes capillary electrophoresis-based immunodetection for higher resolution, sensitivity, and reproducibility (even at low sample concentrations) relative to traditional immunoblotting techniques.[9,89] Wes reagents (biotinylated molecular weight marker, streptavidin-HRP fluorescent standards, luminol-S, hydrogen peroxide, sample buffer, DTT, stacking matrix, separation matrix, running buffer, wash buffer, matrix removal buffer, secondary antibodies, antibody diluent, and capillaries) were obtained from the manufacturer (ProteinSimple) and used according to the manufacturer's recommendations. The 5-HT_{2A}R antibody

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(AB16028, 1:250) or 5-HT_{2C}R antibody (D12, 1:50; Santa Cruz) was diluted with ProteinSimple antibody diluent.

Equal amounts of protein (1 μg) were combined with 0.1× sample buffer and 5× master mix (200 mM DTT, 5× sample buffer, 5× fluorescent standards), gently mixed, and then denatured at 95 °C for 5 min. The denatured samples, biotinylated ladder, antibody diluent, primary antibodies, HRP-conjugated secondary antibodies, chemiluminescent substrate, and wash buffer were dispensed to designated wells in a prefilled microplate (ProteinSimple). Separation electrophoresis (375 V, 31 min, 25 °C) and immunodetection in the capillaries were fully automated using the following settings: separation matrix load for 200 s, stacking matrix load for 14 s, sample load for 7 s, antibody diluent for 30 min, primary antibody incubation for 60 min, secondary antibody incubation for 30 min, and chemiluminescent signal exposure for 30, 120, 240, and 480 s. Data analyses were performed using the Compass Software (ProteinSimple).

Genetic Loss of mPFC 5-HT_{2C}R Confers High Motor Impulsivity and an Imbalance in the 5-HT_2R System (See Experimental Timeline, Figure 4). A 24-nucleotide sequence within the coding region of the *Htr2c* was identified employing methods we have previously reported.[90] Two sets of oligonucleotides (Integrated DNA Technology, Coralville, IA) for cloning were synthesized [*Htr2c* shRNA (top, 5′-TTGAATCCAGACGGGGCACAAATATCCTTC-CTGTCAGATATTTGTGCCCCGTCTGGATTATTTTT-3′; bottom, 5′-CTAGAAAAATAATCCAGACGGGGCACAAATATCTGA-CAGGAAGGATATT TGTGCCCCGTCTGGATTC-3′); Nonsilencing control (NSC) shRNA (top, 5′-TTTGTGGAGCCGAGTTTCTAAATTCCGCTTCCTGTCACGG-AATTTAGAAACCCGGCTCCAATTTTT-3′; bottom, 5′- CTAGA-AAAATTGGAGCCGGGTTTCTAAATTCCGTGACAGGAAGCG-GAATTTAGAAACTCGGCTCCAC3′)]. 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. 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 $(1-2) \times 10^{11}$ transducing units/ml.

Rats (n = 24) 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 midsagittal plane relative to bregma[91] to target the mPFC encompassing the ventral prelimbic and dorsal infralimbic subnuclei;[6] 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 allow for stable transgene expression prior to behavioral assessment. 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 postinfection.[92,93]

Following intra-mPFC transgene delivery and stable viral vector expression,[6] control (n = 12) and 5-HT_{2C}R knockdown (n = 12) rats were trained to criteria on the 1-CSRT task. Pharmacological test sessions commenced after animals met the stable training criteria >80% accuracy and <20% omissions for five consecutive training sessions on the final training stage with less than 15% variability across sessions;[9,15,17] vehicle (1% Tween80; i.p.) or M100907 (0.001, 0.01, 0.1 mg/kg; i.p.) was injected 30 min prior to commencement of 1-CSRT task sessions under ITI5 conditions. Each rat received all doses of M100907 in a balanced, pseudorandomized order. Rats underwent five daily 1-CSRT task sessions per week; rats were treated with vehicle the day before drug treatments and received only one drug treatment per week.

At the termination of the pharmacological assessments (∼1 week), rats were anesthetized (chloral hydrate; 400 mg/kg, i.p.), decapitated and tissue extracted for visualization and 5-HT_2R capillary electrophoretic immunoblot analyses.[6] A 1 mm coronal section containing the mPFC was placed on 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).[6,94] Photomicrographs of coronal sections were taken with a DSLR camera equipped with a macro lens and yellow filter.[6,94] Fluorescent and nonfluorescent regions from the mPFC were microdissected and assayed for immunoblotting to assess 5-HT_{2C}R knockdown and 5-HT_{2A}R protein levels *ex vivo*.[6,94] The S2 protein fraction (i.e., soluble protein; see above) was modified by the addition of 0.5% NP40. The 5-HT_{2A}R and 5-HT_{2C}R protein levels were assessed using the Wes automated Western blotting system (ProteinSimple) as described above. Data analyses were performed using the Compass Software (ProteinSimple).

A subset of rats (n = 6) was anesthetized (sodium pentobarbital; 100 mg/kg, i.p.) and perfused transcardially with 3% paraformaldehyde for immunohistochemical analyses.[6,95] Brains were removed, postfixed (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) prior to incubation with 5-HT_{2C}R antibody (D12; 1:100; Santa Cruz; 2 h 25 °C, 18 h 4 °C) followed by AlexaFluor 555 to mouse IgG (A21424, 1:2000; Life Technologies; 1 h 25 °C). Slides were coverslipped with Vectashield fluorescent mounting medium with DAPI (Vector Laboratories, Burlingame, CA).

Statistical Analyses. Student's t test was employed to analyze outcome measures of 1-CSRT task performance between cohorts, phenotypes or pretreatment groups. The 5-HT_{2A}R and 5-HT_{2C}R protein expression data and the ratio were assessed by Student's t test and Pearson's correlation. The effects of M100907 on 1-CSRT task performance in control and knockdown rats were analyzed by two-way repeated-measures ANOVA for the factors of pretreatment (control or knockdown) and treatment (vehicle or M100907); the effects of treatment were assessed by one-way repeated-measures ANOVA followed by Dunnett's procedure (for comparisons of treatment means vs vehicle). The experimenter was blinded to the group allocation (e.g., HI vs LI; control vs knockdown) throughout the duration of the study. Analyses were performed in SAS (version 9.4; Cary, NC) with an experiment-wise error rate of α = 0.05.

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Author Contributions

N.C.A. and K.A.C. conceptualized the experiments, designed and directed the behavioral, biochemical, and pharmacological research, interpreted the results, and wrote the manuscript. S.J.S. carried out behavioral evaluations. R.M.S. and R.J.D. created the 5-HT_{2C}R shRNA AAV. L.H.L.F., S.E.S-J., and F.G.M. were involved in the conception and design of the experiments and edited the manuscript. K.C.R. synthesized M100907 which was provided under a Material Transfer Agreement.

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Notes

The authors declare the following competing financial interest(s): Dr. Cunningham is a consultant for Arena Pharmaceuticals. Dr. Moeller is a consultant for Boehringer-Ingelheim. All other authors declare no conflicts of interest.

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■ ABBREVIATIONS

1-CSRT, one-choice serial reaction time task; 5-HT, serotonin (5-hydroxytryptamine); 5-HT_XR, 5-HT_X receptor; GPCR, G-protein coupled receptor; AAV, adenoassociated virus

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