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Nucleus accumbens shell excitability is decreased by methamphetamine self-administration and increased by 5-HT_{2C} receptor inverse agonism and agonism

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Abstract

Methamphetamine profoundly increases brain monoamines and is a widely abused psychostimulant. The effects of methamphetamine self-administration on neuron function are not known for the nucleus accumbens, a brain region involved in addictive behaviors, including drug-seeking. One therapeutic target showing preclinical promise at attenuating psychostimulant-seeking is 5-HT_{2C} receptors; however, the effects of 5-HT_{2C} receptor ligands on neuronal physiology are unclear. 5-HT_{2C} receptor agonism decreases psychostimulant-mediated behaviors, and the putative 5-HT_{2C} receptor inverse agonist, SB 206553, attenuates methamphetamine-seeking in rats. To ascertain the effects of methamphetamine, and 5-HT_{2C} receptor inverse agonism and agonism, on neuronal function in the nucleus accumbens, we evaluated methamphetamine, SB 206553, and the 5-HT_{2C} receptor agonist and Ro 60-0175, on neuronal excitability within the accumbens shell subregion using whole-cell current-clamp recordings in forebrain slices *ex vivo*. We reveal that methamphetamine self-administration decreased generation of evoked action potentials. In contrast, SB 206553 and Ro 60-0175 increased evoked spiking, effects that were prevented by the 5-HT_{2C} receptor antagonist, SB 242084. We also assessed signaling mechanisms engaged by 5-HT_{2C} receptors, and determined that accumbal 5-HT_{2C} receptors stimulated G_q, but not G_{i/o}. These findings demonstrate that methamphetamine-induced decreases in excitability of neurons within the nucleus accumbens shell were abrogated by

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both 5-HT_{2C} inverse agonism and agonism, and this effect likely involved activation of G_q-mediated signaling pathways.

1. Introduction

Psychostimulants increase synaptic concentrations of monoamines and neuronal maladaptations can be a persistent consequence of repeated administration (for review, see (Kauer and Malenka, 2007)). Some maladaptations reflect changes in serotonin (5-HT) systems (Napier and Istre, 2008, Krasnova et al., 2010, Reichel et al., 2012) and 5-HT_{2C} receptors (Rs) are likely involved. For example, chronic methamphetamine (meth) increases neuronal sensitivity in the limbic (ventral) pallidum to systemic administration of a 5-HT_{2A/2C}R agonist (Napier and Istre, 2008). Moreover, agonists at these receptors attenuate cocaine-associated reinstatement (Grottick et al., 2000, Fletcher et al., 2002, Neisewander and Acosta, 2007, Burbassi and Cervo, 2008, Cunningham et al., 2011), self-administration (Grottick et al., 2000, Fletcher et al., 2002, Cunningham et al., 2011), as well as behavioral hyperactivity and sensitization (Grottick et al., 2000, Filip et al., 2004). 5-HT_{2C}Rs are constitutively active in rat brain (De Deurwaerdere et al., 2004), antagonists or inverse agonists with high affinity for 5-HT_{2A/2C}Rs nullify meth-induced neuronal sensitization (McDaid et al., 2007), and the putative 5-HT_{2C}R inverse agonist SB 206553 (SB206) reduces meth-seeking and meth-evoked motor activity (Graves and Napier, 2012).

5-HT_{2C}R inverse agonists and agonists exhibit similar behavioral effects in rats (Graves and Napier, 2012, Navailles et al., 2013). In contrast, neurochemical studies show that inverse agonists *increase* accumbal and striatal dopamine (De Deurwaerdere et al., 2004), whereas agonists have *no effect, or decrease* dopamine (Di Matteo et al., 1998, Willins and Meltzer, 1998, Di Matteo et al., 2000, Gobert et al., 2000). Discord also exists regarding 5-HT_{2C}R-mediated function at the cellular level; inverse agonists *decrease* 5-HT_{2C}R-linked second messengers (e.g., basal phospholipase (PL) PLC, PLA2 and G_{i/o} activity) (Berg et al., 1999, De Deurwaerdere et al., 2004, Berg et al., 2006, Berg et al., 2008a, Berg et al., 2008b, Labasque et al., 2010), but *increase* surface expression of 5-HT_{2C}Rs (Marion et al., 2004, Chanrion et al., 2008). However, after incubation with an inverse agonist, responses to serotonin are *enhanced* (Berg et al., 1998a, Marion et al., 2004, Chanrion et al., 2008), presumably due to the increased surface expression. Pleiotropic signaling further complicates 5-HT_{2C}R function. The canonical pathway involves G_q stimulation (Cussac et al., 2002), yet the receptors are also reported to signal *via* G_{i/o} and G₁₃ proteins as well as non-G protein coupled pathways (Berg et al., 1998b, Cussac et al., 2002, McGrew et al., 2002, De Deurwaerdere et al., 2004, Werry et al., 2005, Labasque et al., 2008, Labasque et al., 2010).

To advance understanding of 5-HT_{2C}Rs on meth-induced neuronal function and to provide insights into signaling mechanisms engaged in medium spiny neurons (MSNs) of the nucleus accumbens by these receptors, we used electrophysiological and biochemical approaches to evaluate 5-HT_{2C}Rs from rats trained to self-administer meth. Based in part on our prior demonstration that SB206 attenuates meth-seeking behavior (Graves and Napier, 2012), we hypothesized that acute 5-HT_{2C}R inverse agonism opposes meth-induced

adaptations in the nucleus accumbens shell and that 5-HT_{2C}Rs in the nucleus accumbens engage the canonical G_q pathway.

2.0 Materials and methods

2.1 Animals

Seventy-seven male Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN). Subjects were housed in pairs, acclimated to the vivarium for 5 days, and handled a minimum of 3 times prior to the surgical procedures required for self-administration protocols. Food and water were provided *ad libitum* throughout the study. Rats were maintained in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) and with approval of the Rush University Institutional Animal Care and Use Committee. All efforts were made to minimize suffering and reduce the number of animals used.

2.2 Test Drugs

(+)-Methamphetamine HCl (meth; Sigma, St. Louis, MO) was dissolved in sterile saline. The stimulant was self-administered at 0.1mg/kg/0.1ml intravenously (iv). Ro 60-0175 (Ro), SB 206553 (SB206), and SB 242084 (SB242) were purchased from Tocris Bioscience (Ellisville, MO), dissolved in ddH₂O as 1.0mM stock solutions, and added at appropriate concentrations to artificial cerebrospinal fluid (aCSF) for electrophysiological studies, or assay buffer for biochemical studies.

2.3 Intravenous catheter implantation

Isoflurane-anesthetized rats (n=77) were instrumented as previously described (Graves and Napier, 2011) with custom built catheters constructed using silastic tubing (0.3mm i.d. x 0.64mm o.d.; Dow Corning Co., Midland, MI) and implanted into the right jugular vein. The distal end of the catheter extended to the mid-scapular region with a metal guide canulae (22 gauge; Plastics One Inc., Roanoke, VA) and anchored to a plastic mesh. Rats were allowed to recover for a minimum of 5 days prior to initiating self-administration procedures.

2.4 Self-Administration

Forty-one rats were trained to self-administer meth for 3hr/day for 14 days in operant chambers enclosed in ventilated, sound-attenuating cabinets (Med-Associates, St. Albans, VT). Each operant chamber contained two levers; the left lever was assigned as the “active” lever and the right lever was assigned as the “inactive” lever. Above each lever was a “cue” light, and located on the opposite wall was an “in-house” light. The cue light above the active lever was illuminated when the infusion pump was activated. The in-house light was subsequently illuminated for 20s, indicating a “time-out” period during which responses had no programmed consequences. Responding on the inactive lever had no programmed consequences. On protocol days 1–7, rats self-administered meth for 3hr/day on a fixed ratio (FR) 1 schedule of reinforcement; on days 8–14 rats self-administered on a FR5 for 3hr/day. The described paradigm was chosen based on our prior studies demonstrating stable and consistent self-administration, as well as the ability of mirtazapine (Graves and Napier, 2011) and SB206 (Graves and Napier, 2012) to attenuate meth-seeking behavior.

Thirty-six saline-yoked rats were used as controls. These subjects were implanted with jugular vein catheters as described for the meth self-administration protocol; 0.1ml infusions of saline were administered with cue and time out lights triggered according to the behavioral pattern of a meth self-administering rat (lever pressing had no programmed consequences). A total of 4 rats died after a behavioral test session (2 saline-yoked and 2 meth self-administering rats).

2.5 Electrophysiological experiments

On protocol days 15–18 (1–4 days after last behavioral test), rats were anesthetized with chloral hydrate (400mg/kg, ip) and transcardially perfused with 60mL of an ice cold, modified aCSF containing (in mM): 248 sucrose, 2.9 KCl, 2.0 MgSO₄, 1.25 NaH₂PO₄, 10.0 glucose, 26.0 NaHCO₃, 0.1 CaCl₂, 3.0 kynurenic acid, and 1.0 ascorbic acid (225–235mOsm, pH=7.4). Forebrain coronal slices (300µm thick) containing the nucleus accumbens shell were cut with a vibrating microtome (Leica VT 1000S; Leica Microsystems Inc., Buffalo Grove, IL), and transferred to a holding chamber containing normal aCSF (in mM: 125 NaCl, 2.5 KCl, 1 MgCl₂, 25 NaHCO₃, 1.25 NaH₂PO₄, 15 glucose, and 2 CaCl₂; 305–315mOsm, pH=7.4) with 1mM ascorbic acid at room temperature. A gas mixture of 95% O₂/5% CO₂ was constantly bubbled for a minimum of 45min prior to beginning patch clamp experiments. The nucleus accumbens shell was selected for study based on evidence for constitutively active 5-HT_{2C}Rs (Navailles et al., 2006) and its involvement in addiction (Di Chiara, 2002, Di Chiara et al., 2004). An early period of withdrawal was selected for study based on the ability of mirtazapine (Graves and Napier, 2011) and SB206 (Graves and Napier, 2012) to attenuate meth-seeking, and presence of psychostimulant-induced plasticity (Kourrich and Thomas, 2009) during this period.

Slices containing the nucleus accumbens shell were moved from the holding to the recording chamber which was perfused at a rate of 2ml/min by normal aCSF and held at 34°C. Neurons were visualized using an Olympus BX51WI upright microscope (Olympus Tokyo, Japan) and targeted under a 40X water-immersion objective, differential interference contrast, and infrared filter. The image from the microscope was enhanced using an IR-1400 DAGE-MIT (Michigan City, IN) camera and displayed on a computer monitor. Electrodes were pulled with a P-97 or P-1000 micropipette puller (Sutter Instruments, Novato, CA) to a resistance of 4–6MΩ and filled with a K⁺/gluconate-based internal solution (in mM: 0.1 EGTA, 120.0 K⁺ gluconate, 10.0 HEPES, 20.0 KCl, 2.0 MgCl₂, 3.0 Na₂ATP, and 0.3 NaGTP; 280–285mOsm, pH=7.3). Whole-cell current clamp recordings were obtained using a Multiclamp 700B (Molecular Devices, Instruments, Sunnyvale, CA). Signals were digitized by a Digidata 1320 A/D converter (Axon Instruments) and stored on-line using pClamp 9 software (Axon Instruments). All neurons included in the analyses were medium spiny neurons (MSNs) that met the criteria of a resting membrane potential more negative than –70mV and action potential peak greater than 60mV under basal conditions. MSNs were readily identifiable by their mid-sized soma and electrophysiological characteristics including hyperpolarized resting membrane potentials, latency before the first action potential at rheobase, and absence of I_h current (Wilson and Groves, 1980, Dong et al., 2006). The recording protocol consisted of 500ms pulses of current beginning at –0.5nA

with increment steps of 50pA. Active and passive membrane properties were measured at the first action potential evoked by the rheobase (i.e., lowest current generating an action potential). Input resistance (R_{in}) was measured at -0.2 nA current injection; data to construct the current-voltage curves were obtained at 400ms from the start of the current pulse.

MSN excitability was tested under one of the following conditions: (i) ascending concentration-response assessment of 0.1, 1.0, and 10.0 μ M of the 5-HT_{2C} inverse agonist SB 206553 (SB206); (ii) 1.0 μ M of the 5-HT_{2C} antagonist SB 242084 (SB242) followed by 1.0 μ M SB242 + 10.0 μ M SB206; (iii) ascending concentration-response assessment of 0.1, 1.0, and 10.0 μ M of the 5-HT_{2C} agonist Ro 60-0175 (Ro); (iv) 1.0 μ M SB242 followed by 1.0 μ M SB242 + 10.0 μ M Ro. Prior to treatment, neurons were recorded while normal aCSF was perfused to establish a baseline (basal). Each drug concentration tested was perfused for at least 5min before collecting concentration-related data. Whole-cell pipette series resistance was less than 20M Ω , and compensation for bridge resistance was monitored throughout experiments.

Pilot studies were conducted with accumbal shell slices from untreated naïve rats to determine the following: (i) Baseline stability. This was accomplished by monitoring the active and passive membrane properties during 30 min of aCSF perfusion. For example, in neurons from naïve rats the resting membrane potential varied by less than 1% over a 30min period with continual aCSF perfusion (data not shown). (ii) Stability of responses to 5-HT_{2C} ligands. This was accomplished by monitoring recordings during continual perfusion of 10.0 μ M of Ro or SB206. For example, in naïve rats the resting membrane potential varied by less than 1% with continual Ro perfusion (data not shown).

2.6 [³⁵S]GTP γ S binding

Ro-induced 5-HT_{2C}R signaling mechanisms were assessed in nucleus accumbens tissue from rats that self-administered meth (n=8). The rats were euthanized one day after the last day of self-administration (i.e., protocol day 15) using rapid decapitation. The nucleus accumbens (including the core and shell subregions) was dissected and flash-frozen on dry ice. Tissue was homogenized in assay buffer (mM: 20.0 HEPES, 100.0 NaCl, 5.0 MgCl₂, and 0.2 EGTA) and membrane preparations were made using a series of four centrifugations for 20min at 15,000rpm with intervening tissue homogenization in assay buffer. Tissue was incubated at 37°C after the second and third centrifugation for 15min to facilitate neurotransmitter dissociation from receptors. Protein concentrations were determined using BCA assay (Thermo Fisher Scientific, Rockford, IL) (Smith et al., 1985), and membranes were stored at -80°C until use. Samples were run in one of two conditions to determine G_q and $G_{i/o}$ stimulation; protocols to determine G_q stimulation were modified from (Adlersberg et al., 2000). For detection of G_q stimulation, 10.0 μ g of tissue was preincubated with 0.1 μ M GDP at 30°C for 30min with either vehicle (basal) or drug after which 0.5nM [³⁵S]GTP γ S was added, and samples were incubated for an additional 60min at 30°C. Samples were passed through a Brandel harvester (Brandel, Gaithersburg, MD) and washed four times using wash buffer (in mM: 20.0 HEPES, 100.0 NaCl, and 5.0 MgCl₂). For detection of $G_{i/o}$ stimulation, the above protocol was altered by incubating with 30.0 μ M GDP and 0.1nM [³⁵S]GTP γ S. DTT was added to assay buffer at a concentration of 0.2mM prior. Tissue

samples were assayed in triplicate. To determine the contribution of G_q vs. $G_{i/o}$ stimulation to receptor agonism, samples were incubated with vehicle (basal), 10.0 μ M Ro, 10.0 μ M Ro + 1.0 μ M SB242, or 10.0 μ M DAMGO (μ -opioid receptor peptide agonist; used as a positive control for activation of $G_{i/o}$). Data are presented as the difference of the average concentration between treatment and basal condition, in pmols [^{35} S]GTP γ S bound/mg of protein.

2.7 Statistics

Number of infusions by rats trained to self-administer meth was analyzed using a one-way repeated measures (rm) ANOVA. To test for differences in the membrane properties between neurons from rats that self-administered meth and saline-yoked controls, Student's *t*-tests were used, except for the current-spike and current-voltage (*I*-*V*) comparisons for which a mixed function two-way ANOVA was used with current as a repeated measure and treatment history as a non-repeated factor. Data assessing 5-HT_{2C}R ligands on neuronal excitability were analyzed using two-way rmANOVA. Biochemical studies were analyzed by one-way rmANOVA. A Newman-Keuls was used *post-hoc* for all of the ANOVAs. Significance was met with $\alpha=0.05$. Statistical outliers were operationally defined as measures outside of two standard deviations from the parameter mean and such outliers were excluded from analysis; less than 5% of collected data met outlier criteria.

3. Results

3.1 Meth self-administration and saline-yoked controls

Outcomes of the meth self-administration protocols are shown in Figure 1. Rats readily acquired the operant task, and when the reinforcement schedule was switched from FR1 to FR5 (day 8), active lever pressing increased to compensate for the more demanding reinforcement schedule. The number of inactive lever presses was consistently minimal, indicating that rats differentiated between the reinforced (active) and non-reinforced (inactive) levers. Finally, the number of infusions by rats self-administering meth was stable across the last 4 days of self-administration, similar to our previous reports (Graves and Napier, 2011, 2012). Average lifetime intake of meth was 17.4 ± 1.7 mg/kg with an average of 1.6 ± 0.2 mg/kg ($n=39$ rats) self-administered on the last day of operant procedures. Lever pressing by saline-yoked rats was minimal throughout behavioral testing with 5.6 ± 1.4 and 4.2 ± 1.0 , active and inactive lever presses, respectively, on the last day of operant procedures (data not shown).

3.2 Consequences of meth self-administration and 5-HT_{2C} receptor ligands on neuronal function in the nucleus accumbens shell

The intrinsic excitability of MSNs in the nucleus accumbens shell (assessed by evoked firing in response to somatic injection of depolarizing currents) was decreased in rats trained to self-administer meth (Figure 2A); no other active or passive membrane properties were altered by meth self-administration (Figure 2B; Table 1).

We have previously shown that the inverse agonist SB206 decreases meth-seeking and meth-induced motor hyperactivity (Graves and Napier, 2012). To determine if SB206

altered MSN function in a manner relevant to meth-induced plasticity, we assessed the effect of this inverse agonist on the intrinsic excitability of nucleus accumbens shell MSNs from rats trained to self-administer meth. SB206 (0.1, 1.0, and 10.0 μ M) increased the number of evoked action potentials (Figure 3A); similar observations were observed in MSNs from saline-yoked rats (Figure 3C). To determine receptor selectivity for the inverse agonist, SB206 was tested in the presence of the 5-HT_{2C}R antagonist SB242. Co-perfusion of SB206 and SB242 prevented the changes in excitability in MSNs from rats trained to self-administer meth (Figure 3B) and in MSNs from saline-yoked controls (Figure 3D).

Agonism at the 5-HT_{2C}R attenuates psychostimulant-mediated behaviors in rats (Grottick et al., 2000, Fletcher et al., 2002, Filip et al., 2004, Frankel and Cunningham, 2004, Neisewander and Acosta, 2007, Burbassi and Cervo, 2008, Cunningham et al., 2011). We therefore assessed the effects of the 5-HT_{2C}R agonist Ro on MSN function in the nucleus accumbens shell. Ro increased the number of evoked action potentials in both MSNs from both rats trained to self-administer meth (Figure 4A) and saline-yoked controls (Figure 4C), similar to findings with the inverse agonist SB206 (Figure 3A&C). To confirm selectivity for the agonist, we tested the ability of the 5-HT_{2C}R antagonist SB242 to occlude Ro-induced changes in MSN excitability using *ex vivo* slices from rats trained to self-administer meth. Comparable to the selectivity seen with SB206, SB242 prevented Ro-induced increased evoked action potential generation (Figure 4B). These data revealed that both agonism and inverse agonism have the same effect on accumbens shell intrinsic excitability and that this effect opposed that induced by meth self-administration. Moreover, 5-HT_{2C}R receptor ligands increased the input resistance in a dose-dependent manner in neurons from both saline-yoked rats and rats trained to self-administer meth (Supplementary Table 1), suggesting altered K⁺ conductances by 5-HT_{2C}R ligands.

3.3 5-HT_{2C} receptor mediated activation of G_q proteins but not G_{i/o} proteins

5-HT_{2C}Rs are reported to signal through a variety of pathways including, but not limited to, G_{i/o} and G_q (De Deurwaerdere et al., 2004). [³⁵S]GTP γ S binding assays were used to evaluate 5-HT_{2C}R-linked coupling to different G proteins in nucleus accumbens tissue harvested one day after the last self-administration session (i.e., protocol day 15). Two experimental conditions were tested, one conducive to detecting G_q and one for detecting G_{i/o} activation (refer to Methods). Incubation of 10.0 μ M Ro (5-HT_{2C}R agonist) stimulated [³⁵S]GTP γ S binding under G_q (Figure 5A) but not G_{i/o} (Figure 5B) conditions. Stimulation under G_q conditions was attenuated by co-incubation of 1.0 μ M of the 5-HT_{2C}R antagonist SB242 (Figure 5A). We tested DAMGO (10.0 μ M), a full agonist at μ -opioid receptors which couple to G_{i/o} but not G_q proteins, as a negative control for G_q activation and positive control for G_{i/o} activation. DAMGO did not increase [³⁵S]GTP γ S binding under G_q conditions (Figure 5A), but did so under G_{i/o} conditions (Figure 5B). These experiments demonstrated that 5-HT_{2C}Rs in the nucleus accumbens from rats trained to self-administer meth activated G_q, but did not stimulate G_{i/o} proteins at detectable levels.

4. Discussion

In the current study, we determined the effects of meth self-administration and 5-HT_{2C}R ligands on the excitability of MSNs in the nucleus accumbens shell. We revealed that: (i)

self-administration of meth (even at doses that were less than 2mg/kg/day) was sufficient to decrease intrinsic excitability. (ii) The 5-HT_{2C}R inverse agonist, SB206, and the agonist, Ro, increased excitability. (iii) Inverse agonist and agonist actions on accumbal excitability and signal transduction were blocked by a 5-HT_{2C}R antagonist. (iv) 5-HT_{2C}R engaged G_q but not G_{i/o} heterotrimeric G proteins.

The decrease in evoked spiking induced by meth in MSNs of the nucleus accumbens shell parallels decreased excitability measured *in vivo* from meth-sensitized rats with more protracted times of forced abstinence (i.e., 17–25 days) (Brady et al., 2003, 2005). Repeated cocaine also produces similar effects. *Ex vivo* studies in cocaine-sensitized mice after short (1–3 days) and protracted (10–14 days) periods of forced abstinence (Kourrich and Thomas, 2009, Kourrich et al., 2012), cocaine-sensitized rats (Zhang et al., 1998) and mice trained to self-administer cocaine (Mu et al., 2010) after short periods of forced abstinence all show decreased excitability. These reports suggest a common and enduring ability of psychostimulants to decrease nucleus accumbens shell excitability. The psychostimulant-mediated effects on evoked firing likely reflect changes in intrinsic excitability that are distinct from changes in synaptic excitability. For example, AMPA receptor trafficking to the cell surface is not changed in the accumbens 1 day following repeated cocaine (Boudreau and Wolf, 2005), acute amphetamine (Nelson et al., 2009), acute meth (Herrold et al., 2013), or 21 days following repeated administration of amphetamine (Nelson et al., 2009), or 14 d following repeated meth (Herrold et al., 2013). However, this interpretation is not definitive, as the biochemical assays for AMPA receptors did not delineate the nucleus accumbens shell and core, and these subregions are differentially altered in rodents chronically exposed to cocaine (Kourrich and Thomas, 2009).

It is unclear which ion channels underlie meth-induced reductions in MSN evoked spiking as none of the measured membrane properties were significantly altered by a history of meth in the current study. Investigations of cocaine-mediated effects indicate several ion channel candidates. For example, during early withdrawal from repeated non-contingent cocaine, rats show reductions in high voltage-voltage activated Ca²⁺ channels (Zhang et al., 2002, Hu et al., 2004), voltage-sensitive Na⁺ channels (Zhang et al., 1998) and enhancements of voltage-gated K⁺ conductance (Hu et al., 2004), all of which diminish excitability.

We have observed that 5-HT_{2C}R inverse agonism reduces meth-seeking behaviors (Graves and Napier, 2012) and several laboratories have shown that 5-HT_{2C}R agonism attenuates cocaine-seeking (Grottick et al., 2000, Fletcher et al., 2002, Neisewander and Acosta, 2007, Burbassi and Cervo, 2008, Cunningham et al., 2011). The current electrophysiological studies complement these behavioral findings by demonstrating that 5-HT_{2C}R agonism and inverse agonism have the opposite effects on accumbens shell excitability than does meth self-administration. These observations support the idea that augmenting 5-HT_{2C}R activity may be a strategy to oppose psychostimulant-induced pathology. However, it is important to note that the effects of 5-HT_{2C}R ligands on intrinsic excitability were similar for MSNs from saline-yoked controls and rats trained to self-administer meth. This suggests that overall 5-HT_{2C}R function in the accumbens shell was not altered by meth self-administration *per se* and that the previously reported behavioral effects of 5-HT_{2C}R agonism and inverse agonism may reflect actions on the downstream consequences of meth-

induced changes. For example, MSNs in the accumbens shell provide GABAergic inputs to the ventral pallidum (Heimer et al., 1987, Zahm and Heimer, 1988, Zahm, 1989, Zahm and Heimer, 1990, Heimer et al., 1991, Zahm and Brog, 1992, Groenewegen et al., 1993), a region known to regulate psychostimulant-mediated behaviors including seeking (McFarland and Kalivas, 2001, Tang et al., 2005, Li et al., 2009). With decreased excitability of MSNs by meth self-administration, the ventral pallidum should be disinhibited, and targeting 5-HT_{2C}R in the accumbens shell could serve to mitigate this disinhibition and help to restore balance in the mesolimbic circuit. While further study is necessary to fully ascertain the engaged circuit, 5-HT_{2C}R pharmacotherapy may still provide palliative care for meth addiction even though the ligands don't directly target the specific cellular maladaptations imposed by meth. This notion is supported by the finding that these 5-HT_{2C}R ligands increased input resistance (Supplementary Table 1), and that this parameter was unchanged by meth self-administration (Table 1). Thus, 5-HT_{2C}R may compensate for meth-induced changes, but do so through a mechanism independent of meth-induced pathology.

We observed that the inverse agonist SB206 exhibited an “agonist-like” effect on accumbal neuronal activity, i.e., SB206 increased excitability similar to that induced by the 5-HT_{2C}R agonist, Ro. Studies with HEK cells and dissociated mouse cortical neurons demonstrate inverse agonist-dependent trafficking of constitutively active 5-HT_{2C}R from intracellular pools to the membrane surface (Marion et al., 2004, Chanrion et al., 2008), and enhanced responses to a subsequent 5-HT challenge (Berg et al., 1998a, Marion et al., 2004, Chanrion et al., 2008). In our study, binding of 5-HT_{2C}R by SB206 may have resulted in a redistribution of intracellular, constitutively active receptors into the neuronal membrane, leading to increased agonist-independent 5-HT_{2C}R signaling and an “agonist-like” action. *In vitro* studies assessing changes in receptor trafficking typically use incubation periods of 30min to 1hr. Marion *et al.* (2004), demonstrated that a two-fold increase in surface expression of constitutively active 5-HT_{2C}R occurs within 30min using HEK 293 cells (shorter time periods were not assessed). This profile contrasts other molecular studies in recombinant systems wherein inverse agonists attenuate basal activity of second messenger systems that are activated by the homotypic agonist (Berg et al., 1999, De Deurwaerdere et al., 2004, Berg et al., 2006, Berg et al., 2008a, Labasque et al., 2010). *In vivo* microdialysis studies in rodents provide a correlate to these oppositional effects wherein striatal and accumbal dopamine levels are increased by SB206 (De Deurwaerdere et al., 2004), and decreased or not changed by 5-HT_{2C}R agonists (Di Matteo et al., 1998, Willins and Meltzer, 1998, Di Matteo et al., 2000, Gobert et al., 2000, Lucas and Spampinato, 2000, De Deurwaerdere et al., 2004). *In vivo* extracellular dopamine concentrations in the striatum and accumbens are dependent on activity of the substantia nigra pars compacta and ventral tegmental area, respectively; whereas our investigations were focused on the intrinsic excitability of MSNs in the nucleus accumbens shell, and these different outcome perspectives complicate direct comparison between the two different experimental approaches. Moreover, the pharmacodynamics of receptors on midbrain dopaminergic neurons may not necessarily translate to other neuronal populations. For example, metabotropic glutamate receptors, muscarinic receptors, and noradrenergic α_1 receptors couple to G_q proteins, which increase excitability of many types of neurons (Valenti et al.,

2002, Mathie, 2007), can inhibit dopaminergic neurons (Fiorillo and Williams, 1998, 2000, Paladini et al., 2001, Paladini and Williams, 2004).

Technical differences between microdialysis studies and the current electrophysiological experiments may also contribute to the divergent outcomes. The microdialysis studies employed halothane anesthesia, which reduces K^+ conductance (Elliott et al., 1992, Mathie, 2007), a parameter consistently altered by 5-HT_{2C}R activation (North and Uchimura, 1989, Stevens et al., 1992, Hu et al., 1998, Di Giovanni et al., 1999, Speake et al., 2004, Xiang et al., 2005, Weber et al., 2008), and a decreased K^+ conductance was likely involved in the current studies given the 5-HT_{2C}R-dependent increased input resistance. Alternatively, SB206 may be acting as an agonist in MSNs (and inverse agonist in dopamine neurons regulating dopamine release). Ligand efficacy is system dependent (for review, see (Kenakin, 2001)) and the pharmacodynamic actions of the tested ligands may be different depending on the neurons (i.e. system) tested. For example, in MSNs 5-HT_{2C}R ligands increased excitability (current study) but the pharmacodynamic actions of these same ligands may have distinct effects in dopamine neurons, potentially contributing to the differences observed between our electrophysiological studies and the previously discussed microdialysis studies. Given the complex pharmacology of SB206, it is important to interpret outcomes *in lieu* of the preparation and neuronal system studied.

Our biochemical assessments demonstrated that 5-HT_{2C}Rs in the nucleus accumbens stimulated G_q but not $G_{i/o}$ and confirmed receptor selectivity for the ability of 5-HT_{2C}Rs to engage G_q proteins. We demonstrated that SB242 (a potent 5HT_{2C}R antagonist in most systems studied (Bromidge et al., 1997, Kennett et al., 1997)) prevented 5-HT_{2C}R inverse agonist and agonist-induced changes in excitability confirming receptor specificity for these parameters. Thus, the observed alterations in neuronal excitability are selective for 5-HT_{2C}Rs and likely a consequence, at least in part, of G_q activation.

In summary, this study revealed that 5-HT_{2C}Rs in the nucleus accumbens engage G_q but not $G_{i/o}$ proteins. This study also demonstrated that contingently-administered meth decreased the intrinsic excitability of MSNs in the nucleus accumbens shell and this effect was acutely opposed by both the inverse agonist SB 206553 and agonist Ro 60-0175. These findings are consistent with behavioral observations where both compounds attenuate psychostimulant-seeking (Grottick et al., 2000, Fletcher et al., 2002, Burbassi and Cervo, 2008, Graves and Napier, 2012). As the accumbal agonist and inverse agonist effects were also seen in saline-yoked controls, our work implicate an involvement of meth-induced changes in brain regions that may be downstream to the nucleus accumbens in the reported behavioral actions of 5-HT_{2C}R agonists on psychostimulant-seeking.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Methamphetamine self-administration decreased excitability of accumbens shell neurons.
- 5-HT_{2C} receptor agonism increased accumbens shell excitability
- 5-HT_{2C} receptor inverse agonism increased accumbens shell excitability.
- 5-HT_{2C} receptors in the accumbens engage G_{i/o} but not G_q proteins.

Figure 1. Behavioral data from rats trained to self-administer methamphetamine and saline-yoked controls

Thirty-nine rats were trained to self-administer methamphetamine for 14 consecutive days at 0.1mg/kg/0.1ml infusion. On days 1–7, rats self-administered on a fixed ratio (FR) 1 schedule of reinforcement (i.e., one lever press per infusion) and on days 8–14, self-administration was switched to an FR5 schedule. Active and inactive lever presses are graphed according to the left Y-axis and infusions are graphed according to the right Y-axis. There was no difference in the number of infusions received over the last four days of self-administration (one-way repeated measures ANOVA; $F_{(3,37)}=1.85$).

Figure 2. Methamphetamine self-administration significantly decreased evoked firing in accumbens shell medium spiny neurons

(A) Medium spiny neurons ($n=17$) from rats ($n=12$) trained to self-administer (SA) methamphetamine (meth) showed a significant decrease in the number of evoked action potentials compared to neurons ($n=16$) from saline-yoked rats ($n=13$). Mixed function two-way ANOVA revealed a significant Treatment History effect ($F_{(1,15)}=5.81$), Current effect ($F_{(7,100)}=120.97$), and Treatment History X Current interaction ($F_{(7,100)}=2.85$); Newman-Keuls *post-hoc* analysis indicated significant differences between neurons from saline-yoked and rats trained to self-administer meth; $*p<0.05$, $**p<0.01$ meth vs. saline-yoked. Sample traces (left panel) from neurons of a saline-yoked (top) rat and a rat trained to SA meth (bottom) illustrate the change in evoked firing at 0.35nA current injection. (B) Meth self-administration did not alter the inward rectification. A mixed function two-way ANOVA revealed no Treatment History effect ($F_{(1,14)}=0.42$), a significant Current effect ($F_{(12,164)}=242.46$), and no Treatment History X Current interaction ($F_{(12,164)}=0.30$); saline-yoked $n=16$ cells from 13 rats; meth self-administration $n=17$ cells from 12 rats.

Figure 3. SB 206553 increased evoked firing in accumbens shell medium spiny neurons
 (A) Medium spiny neurons in the accumbens shell from rats trained to self-administer methamphetamine (meth) were repeatedly recorded while being perfused with aCSF (Basal), 0.1, 1.0, and 10.0 μ M of the 5-HT_{2C}R inverse agonist SB 206553 (SB206) in ascending order. 1.0 and 10.0 μ M SB206 (but not 0.1 μ M) increased the number of evoked action potentials (n=16 neurons from 10 rats); two-way rmANOVA shows significant a Treatment effect ($F_{(3,36)}=11.93$), Current effect ($F_{(7,250)}=56.55$), and a Treatment X Current interaction ($F_{(21,250)}=3.98$). The effect of SB206 on evoked firing was concentration dependent with 0.1 vs. 1.0 μ M significantly different at 0.35 nA, 0.1 vs. 10.0 μ M from 0.20 to 0.40 nA, and 1.0 vs. 10.0 μ M at 0.20 to 0.40 nA (not illustrated). Sample traces (left panel) illustrate differences in

spiking (0.35nA) at basal (top) and after 10.0 μ M SB206 (bottom); step protocol illustrated below. For illustrative purposes, data inset in figure A highlight the differences in evoked action potentials between basal and 10.0 μ M SB206. **(B)** Medium spiny neurons from rats trained to self-administer meth (n=12 neurons from 5 rats) were perfused with aCSF, 1.0 μ M SB 242084 (SB242; 5-HT_{2C}R antagonist), and co-perfusion of 1.0 μ M SB242 + 10.0 μ M SB206 (5-HT_{2C}R inverse agonist). Co-perfusion of the 5-HT_{2C}R antagonist SB242 with SB206 prevented the inverse agonist-induced increases in action potential generation. Two-way rmANOVA revealed a significant Treatment effect ($F_{(2,22)}=3.87$), a Current effect ($F_{(7,154)}=55.72$), and no Treatment X Current interaction ($F_{(14,154)}=0.77$). **(C)** The inverse agonist was also tested in saline-yoked controls; neurons (n=16 from 9 rats) were repeatedly recorded while being perfused with aCSF (Basal), 0.1, 1.0, and 10.0 μ M of the 5-HT_{2C}R inverse agonist SB 206553 (SB206) in ascending order. 1.0 and 10.0 μ M SB 206 (but not 0.1 μ M) increased the number of evoked action potentials; two-way rmANOVA shows significant a Treatment effect ($F_{(3,39)}=3.75$), Current effect ($F_{(7,270)}=54.33$) and a Current X Treatment interaction ($F_{(21,270)}=1.68$). The effect of SB206 on evoked firing did not show concentration-dependence (i.e., 0.1 vs. 1.0 vs. 10.0 μ M were not significantly different at any nA). Sample traces illustrate the differences in spiking (0.35nA) at basal (left) and after 10.0 μ M SB206 (right); step protocol illustrated on the bottom left. For illustrative purposes, data inset in figure A highlight the differences in evoked action potentials between basal and 10.0 μ M SB206. **(D)** The antagonist, SB242, also prevented SB206-induced effects in saline-yoked controls. Neurons (n=12 neurons from 5 rats) were perfused with aCSF, 1.0 μ M SB 242084 (SB242; antagonist), and co-perfusion of 1.0 μ M SB242 + 10.0 μ M SB 206553 (SB206; inverse agonist). Co-perfusion of the 5-HT_{2C}R antagonist SB242 with SB206 prevented the inverse agonist-induced increases in action potential generation. Two-way rmANOVA revealed no significant Treatment effect ($F_{(2,22)}=0.22$), a Current effect ($F_{(7,149)}=18.76$), and no Treatment X Current interaction ($F_{(14,149)}=0.72$). #p<0.05 and ###p<0.001 comparing basal vs. 1.0 μ M; **p<0.01, and ***p<0.001 comparing basal vs. 10.0 μ M.

Figure 4. Ro 60-0175 increased evoked firing in accumbens shell medium spiny neurons
(A) Medium spiny neurons in the nucleus accumbens shell from rats trained to self-administer methamphetamine (meth) were repeatedly recorded while being perfused with aCSF (Basal), 0.1, 1.0, and 10.0 μM of the 5-HT_{2C}R agonist Ro 60-0175 (Ro) in ascending order. 1.0 and 10.0 μM Ro (but not 0.1 μM) increased the number of evoked action potentials (n=14 neurons from 8 rats); the effect of Ro on evoked firing was concentration dependent; 0.1 vs. 1.0 μM was significantly different at 0.2 nA, 0.1 vs. 10.0 μM from 0.15 to 0.3 nA, and 1.0 vs. 10.0 μM at 0.20 nA (not illustrated). Two-way rmANOVA shows a significant Treatment effect ($F_{(3,30)}=6.57$), a Current effect ($F_{(7,210)}=87.14$), and Treatment X Current interaction ($F_{(21,210)}=2.14$). Sample traces (left panel) illustrate the difference in spiking (0.35 nA) at basal (top) and after 10.0 μM Ro (bottom); step protocol illustrated on the bottom left. For illustrative purposes, data inset in figure A highlight the differences in evoked action potentials between basal and 10.0 μM Ro. **(B)** Neurons from rats trained to self-administer meth (n=12 neurons from 4 rats) were perfused with aCSF, 1.0 μM SB 242084 (SB242; antagonist), and co-perfusion of 1.0 μM SB242 + 10.0 μM Ro 60-0175 (Ro). Co-perfusion of the 5-HT_{2C}R antagonist SB242 with Ro prevented the agonist-induced increases in action potential generation in accumbens shell medium spiny neurons from rats trained to self-administer meth. Two-way rmANOVA revealed no Treatment effect ($F_{(2,22)}=0.52$), a Current effect ($F_{(7,148)}=38.068$), and no Treatment X Current interaction

($F_{(14,148)}=0.80$). (C) Medium spiny neurons in the accumbens shell from saline-yoked rats ($n=15$ neurons from 5 rats) were repeatedly recorded while being perfused with aCSF (Basal), 0.1, 1.0, and 10.0 μ M of the 5-HT_{2C}R agonist Ro 60-0175 (Ro) in ascending order. Bath application of Ro (1.0 and 10.0 μ M, but not 0.1 μ M) increased the number of evoked action potentials; two-way rmANOVA shows significant Treatment effect ($F_{(3,36)}=9.74$), Current effect ($F_{(7,248)}=85.75$), and Treatment X Current interaction ($F_{(21,248)}=2.57$). The effect of Ro on evoked firing was concentration-dependent; significant differences between 0.1 and 1.0 μ M occurred from 0.15 to 0.2 and 0.3 to 0.4nA, differences between 0.1 and 10.0 μ M occurred from 0.15 to 0.4nA, and between 1.0 and 10.0 μ M at 0.25nA (for sake of clarity, these points of significance are not illustrated on the graph. Sample traces illustrate the difference in spiking (0.35nA) at basal (left) and after 10.0 μ M Ro (right); step protocol illustrated on the bottom left. For illustrative purposes, data inset in figure C highlight the differences in evoked action potentials between basal and 10.0 μ M Ro. [#] $p<0.05$ and ^{###} $p<0.001$ comparing basal vs. 1.0 μ M; * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ comparing basal vs. 10.0 μ M.

Figure 5. Stimulation of 5-HT_{2C} receptors activated G_q, but not G_{i/o} proteins in rats trained to self-administer meth

Membrane preparations were made from the nucleus accumbens of rats trained to self-administer methamphetamine (n=4). [³⁵S]GTPγS binding was used to detect G_q (A) and G_{i/o} (B) stimulation separately under two distinct assay conditions (refer to methods). (A) The 5-HT_{2C}R agonist Ro 60-0175 (Ro, 10.0μM) increased [³⁵S]GTPγS binding under G_q conditions (i.e., low GDP and high [³⁵S]GTPγS concentrations) in accumbens tissue from meth-exposed rats, indicating that 5-HT_{2C}Rs were coupled to G_q proteins. Co-incubation of 1.0μM SB 242084 (5-HT_{2C} antagonist; SB242) nullified the ability of Ro to stimulate [³⁵S]GTPγS binding. Incubation with 10.0μM DAMGO, an agonist for G_{i/o}-linked μ-receptor, did not stimulate [³⁵S]GTPγS binding under G_q conditions, verifying the absence of measurable G_{i/o} activity under such assay conditions. One-way repeated measures ANOVA ($F_{(3,8)}=7.33$) with Newman-Keuls *post-hoc* reveals significant difference between Ro vs. Basal, Ro vs. Ro+242, and Ro vs. DAMGO (**p<0.01 for each comparison). (B) Under conditions to detect G_{i/o} stimulation (i.e., moderate GDP and low [³⁵S]GTPγS concentrations), Ro (10.0μM) produced no stimulation whereas DAMGO (10.0μM) increased [³⁵S]GTPγS stimulation, which served as a positive control. One-way repeated measures ANOVA ($F_{(2,9)}=40.49$); Newman-Keuls *post-hoc* revealed significant difference between DAMGO vs. Basal and DAMGO vs. Ro (***p<0.001 for each comparison).

Table 1

Methamphetamine self-administration does not alter active or passive membrane properties in the nucleus accumbens shell.

Treatment history:	Saline-Yoked	Meth SA
Passive membrane properties		
RMP (mV)	-79.9±0.9	-79.6±1.0
R _{in} (MΩ)	84.4±6.3	81.7±7.0
Active membrane properties		
Rheobase (nA)	0.18±0.02	0.22±0.02
AP threshold (mV)	-36.8±1.4	-38.5±1.5
AP amplitude (mV)	78.3±2.9	76.5±2.2
AP half duration (msec)	1.54±0.05	1.59±0.05
AHP amplitude (mV)	13.3±0.5	14.2±0.8

Neuronal membrane properties from saline-yoked rats (n=16 neurons from 13 rats) and rats trained to self-administer meth (Meth SA; n=17 neurons from 12) were compared using unpaired *t*-test; no differences were detected.