Phosphodiesterase 10A Inhibitor Activity in Preclinical Models of the Positive, Cognitive, and Negative Symptoms of Schizophrenia

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ABSTRACT

Following several recent reports that suggest that dual cAMP and cGMP phosphodiesterase 10A (PDE10A) inhibitors may present a novel mechanism to treat positive symptoms of schizophrenia, we sought to extend the preclinical characterization of two such compounds, papaverine [1-(3,4-dimethoxybenzyl)-6,7-dimethoxysonoquinoline] and MP-10 [2-[[4-(1-methyl-4-pyridin-4-yl-1H-pyrazol-3-yl)phenoxymethyl]methyl]quinoline], in a variety of in vivo and in vitro assays. Both of these compounds were active in a range of antipsychotic models, antagonizing apomorphine-induced climbing in mice, inhibiting conditioned avoidance responding in both rats and mice, and blocking N-methyl-D-aspartate antagonist-induced deficits in prepulse inhibition of acoustic startle response in rats, while improving baseline sensory gating in mice, all of which strengthen previously reported observations. These compounds also demonstrated activity in several assays intended to probe negative symptoms and cognitive deficits, two disease domains that are underserved by current treatments, with both compounds showing an ability to increase sociality in BALB/cJ mice in the social approach/social avoidance assay, enhance social odor recognition in mice and, in the case of papaverine, improve novel object recognition in rats. Biochemical characterization of these compounds has shown that PDE10A inhibitors modulate both the dopamine D1-direct and D2-indirect striatal pathways and regulate the phosphorylation status of a panel of glutamate receptor subunits in the striatum. It is striking that PDE10A inhibition increased the phosphorylation of the ε-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor GluR1 subunit at residue serine 845 at the cell surface. Together, our results suggest that PDE10A inhibitors alleviate both dopaminergic and glutamatergic dysfunction thought to underlie schizophrenia, which may contribute to the broad-spectrum efficacy.

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ABBREVIATIONS: PDE10A, phosphodiesterase 10A; MP-10, 2-[4-(1-methyl-4-pyridin-4-yl-1H-pyrazol-3-yl)-phenoxymethyl]-quinoline; TP-10, 2-[4-(pyridin-4-yl-1-(2,2,2-trifluoroethyl)-1H-pyrazol-3-yl)-phenoxymethyl]-quinoline; MK-801, dizocilpine maleate; CREB, cAMP-response element-binding protein; pCREB, phospho-cAMP-response element-binding protein; DARPP-32, dopamine and cAMP-regulated phosphoprotein of 32-kDa molecular mass; D1ARPP-32, phosphodopamine and cAMP-regulated phosphoprotein of 32-kDa molecular mass; PKA, protein kinase A; cGKII, cGMP-dependent kinase type II; D1, dopamine receptor subtype 1; D2, dopamine receptor subtype 2; GluR1, glutamate receptor subunit 1; pGluR1, phosphoglutamate receptor subunit 1; GluR2/3, glutamate receptor subunit 2/glutamate receptor subunit 3; pGluR2/3, phosphoglutamate receptor subunit 2/phosphoglutamate receptor subunit 3; NMDA, N-methyl-D-aspartate; NR2B, NMDA receptor subunit 2B; pNR2B, phospho-NMDA receptor subunit 2B; AMPA, ε-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; MSNs, medium spiny neurons; EPS, extrapyramidal side effects; ANOVA, analysis of variance; CAR, conditioned avoidance responding; NOR, novel object recognition; SOR, social odor recognition; PFC, prefrontal cortex; PPI, prepulse inhibition; SASA, social approach/social avoidance; PCR, polymerase chain reaction; DMSO, dimethyl sulfoxide; sulfo-NHS-SS-biotin, sulfo-succinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate.

Phosphodiesterase 10A (PDE10A) inhibition has generated much excitement as a potential novel mechanism for the treatment of the positive symptoms of schizophrenia (Menniti et al., 2007; Siuciak, 2008). Current antipsychotic medications are thought to treat these same symptoms suffi-
cien$tly in many patients. Thus, it is other disease domains, namely cognition and negative symptoms, which are linked to functional outcome, that remain underserved and are an opportunity for PDE10A inhibitors (Green et al., 2004; Harvey et al., 2006). PDE10A is a dual specificity of PDE, degrading both cAMP and cGMP ($K_{ic50}$ values of 0.05 and 3.0 μM, respectively), and is remarkable for a highly enriched striatal expression pattern within so-called medium spiny neurons (MSNs) (Menniti et al., 2006, 2007; Siuciak, 2008). This expression pattern stimulated immediate interest in a potential role for this enzyme in treating psychoses, such as those associated with schizophrenia (Coskran et al., 2006). Striatal MSNs are divided into the D1 dopamine receptor expressing “direct” striatal output pathway and the D2 “indirect” output pathway, which regulate behavioral responses upon integration of cortical glutamatergic and midbrain dopaminergic input (Graybiel, 1990, 2000). Marketed antipsychotic drugs are all dopamine D2-receptor antagonists or partial agonists, with a principal site of action on neurons of the indirect pathway. It is intriguing that PDE10A has been shown to be expressed in both the indirect and direct pathways (Seeger et al., 2003; Nishi et al., 2008). These pathways have opposing effects on striatal output, and it has been hypothesized that the additional influence of PDE10A inhibition on the direct pathway could distinguish itself clinically from D2 antagonism (Schmidt et al., 2008).

Preclinical validation for PDE10A inhibition to treat schizophrenia has utilized PDE10A knock-out mice and selective PDE10A inhibitors (Siuciak et al., 2006b; Schmidt et al., 2008). In particular, studies with a PDE10A inhibitor called papaverine and, more recently, a compound called MP-10 (a closely related analog of TP-10), have shown that the PDE10A profile of papaverine and the more potent and specific PDE10A inhibitor, MP-10, is remarkable for a highly enriched striatal role for this enzyme in treating psychoses, such as those associated with schizophrenia (Coskran et al., 2006). Striatal MSNs are divided into the D1 dopamine receptor expressing “direct” striatal output pathway and the D2 “indirect” output pathway, which regulate behavioral responses upon integration of cortical glutamatergic and midbrain dopaminergic input (Graybiel, 1990, 2000). Marketed antipsychotic drugs are all dopamine D2-receptor antagonists or partial agonists, with a principal site of action on neurons of the indirect pathway. It is intriguing that PDE10A has been shown to be expressed in both the indirect and direct pathways (Seeger et al., 2003; Nishi et al., 2008). These pathways have opposing effects on striatal output, and it has been hypothesized that the additional influence of PDE10A inhibition on the direct pathway could distinguish itself clinically from D2 antagonism (Schmidt et al., 2008).

Preclinical validation for PDE10A inhibition to treat schizophrenia has utilized PDE10A knock-out mice and selective PDE10A inhibitors (Siuciak et al., 2006b; Schmidt et al., 2008). In particular, studies with a PDE10A inhibitor called papaverine and, more recently, a compound called TP-10 critical. As an example, TP-10 (PDE10A IC50 = 0.3 nM, selectivity >3000/x other PDEs) has been shown to decrease phencyclidine- and amphetamine-stimulated locomotor activity and inhibit conditioned avoidance responding (CAR) in rats, models that are preclinical predictors of antipsychotic activity (Schmidt et al., 2008). In addition, TP-10 reduced an amphetamine-induced deficit in auditory gating. Deficits in auditory gating, as mimicked by amphetamine in this case, are considered to be a schizophrenia “endophenotype” and thus the reversal observed here provides further encouragement for PDE10A inhibition to treat schizophrenia. In addition, TP-10 produced relatively low levels of catalepsy in the rat, which is a predictor of extrapyramidal side effects (EPS) in man (Wadenberg and Hicks, 1999; Schmidt et al., 2008).

Schizophrenia has additional symptom domains, the so-called “negative symptoms” as exemplified by anhedonia, asociality, and blunted affect and cognitive deficits (Harvey et al., 2004; Keefe et al., 2006; Makinen et al., 2008). Both domains have been linked to functional outcome in patients but are poorly treated by currently available treatments (Green et al., 2004; Harvey et al., 2006). Thus, a major goal in the field is to identify molecules that treat all three symptom domains broadly. In this regard, it is of interest that PDE10A is also expressed at low levels in extrastriatal regions, including the hippocampus and cortex (Seeger et al., 2003; Coskran et al., 2006). Given that these additional domains of schizophrenia are associated with deficits in hippocampal and cortical function, PDE10A is well positioned to modulate these domains (Harrison, 2004; Tan et al., 2007). A core deficit in schizophrenia is seen in executive function and cognitive flexibility that can be picked up by tests such as the Wisconsin Card Sorting Test (Elliott et al., 1995; Haut et al., 1996). It is intriguing that papaverine has been shown to reverse a phencyclidine-induced deficit in the rodent analog of this test, the extradimensional intradimensional attentional set-shifting task (Rodefer et al., 2005). Likewise, there are suggestions that PDE10A inhibition may have the ability to influence negative symptoms, although this is currently purely hypothetical (Schmidt et al., 2008).

In a series of studies, we set out to extend the known profile of papaverine and the more potent and specific PDE10A inhibitor, MP-10 (a closely related analog of TP-10), in a range of biochemical, positive symptom, negative symptom, and cognition assays. Our data show that PDE10A inhibitors are active in models across the symptom domains and clearly modulate dopaminergic as well as glutamatergic signaling, both at the behavioral and biochemical levels.

Materials and Methods

Subjects. Animals were supplied by Charles River Laboratories, Inc. (Wilmington, MA), with the exception of C57BL/6j and BALB/cJ (The Jackson Laboratory, Bar Harbor, ME), and group-housed (unless noted) in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility that was maintained on a 12-h light/dark cycle (lights on 6:00 AM). Food and water were available ad libitum, except where noted. Animals were acclimated to the facility for at least 1 week before the initiation of studies. All studies were approved by the Institutional Animal Care and Use Committee and were performed in accordance with the Principles of Laboratory Animal Care as adopted and promulgated by the National Institutes of Health (Institute of Laboratory Animal Resources, 1996).

Drugs and Reagents. Papaverine (Sigma-Aldrich, St. Louis, MO) and MP-10 (synthesized by Wyeth Research) were dissolved in 2% Tween 80/0.5% hydroxyethylcellulose and administered in a dosing volume of 1 ml/kg in rats and 10 ml/kg in mice. Antibodies to the following proteins were used in the Western blot analysis: pCREB-(Ser133) (Cell Signaling Technology, Inc., Beverly MA); pDARPP-32(Thr34), pGluR2/3(Ser880/Ser891), and NR2B (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); pGluR1(Ser845), GluR1, GluR2/3, pNR2B(Tyr1472) (Millipore Corp., Billerica, MA); and β-actin (Sigma, St. Louis, MO).

In Vitro Phosphodiesterase Assays. Phosphodiesterases 1B, 2A, 3A, 4A, 5A, 7B, 8A, 9A, 10A, and 11A were generated from full-length human recombinant clones. PDE6 was isolated from bovine retina as described previously (Paglia et al., 2002) PDE activity was measured with the preferred substrates. For PDE1B, 2A, 3A, 4A, 7B, 8A, 10A, and 11A, cAMP was used, and for PDE5A, 6, and 9A, cGMP was used at a concentration at or below the Kic50. For measurement of cyclic nucleotides, frozen striatal tissue was homogenized. Samples were stored at −80°C. For measurement of cyclic nucleotides, frozen striatal tissue was

Measurement of Striatal Cyclic Nucleotides and Phosphoproteins. Male CF-1 (20–30 g) mice were dosed intraperitoneally with either papaverine (3–54 mg/kg) or MP-10 (0.1–5 mg/kg), with 2% Tween 80/0.5% hydroxyethylcellulose in water as vehicle (n = 4 or 8). Control groups received vehicle at equal volumes (10 ml/kg). Mice were sacrificed by focused microwave irradiation (TMW-6402C, 4.0 kW, 1.15 s; Muromachi Microwave Applicator; Muromachi Kikai Co., Ltd, Tokyo, Japan) 10 or 30 min after papaverine dosing or 30 min after MP-10 dosing. The striata were removed and immediately frozen on dry ice. Samples were stored at −80°C.
pulverized at dry ice temperature, weighed, and homogenized (Polytron) in 10 volumes of ice-cold 5% trichloroacetic acid in water. The samples were centrifuged at 1500g at 4°C. The supernatants were extracted three times with 5 volumes of water-saturated ether, and the aqueous phase was placed in a 70°C heat block for 5 min to evaporate the residual ether. Samples were then diluted 2- or 3-fold with ether-extracted 5% trichloroacetic acid in water. Cyclic nucleotide concentrations (pmol/mg tissue) were determined for the diluted samples with cAMP and cGMP enzyme immunoassay kits (Cayman Chemical Company, Ann Arbor, MI). Values are expressed as the fold change of the compound treatment divided by the average of the vehicle-treated samples. Data were graphed as the mean ± S.E.M. using Prism software (GraphPad Software, Inc., San Diego, CA).

For analysis of changes in levels of protein phosphorylation, frozen striatal tissue was sonicated in 50 mM sodium fluoride and 1% SDS and boiled for 5 min. After cooling on ice, protein concentrations were determined by the DC protein assay kit (Bio-Rad, Inc., Hercules, CA). The protein (30 μg) of each sample was loaded onto 4 to 12% Bis-Tris NuPAGE polyacrylamide gradient gels (Invitrogen, Carlsbad, CA) and, after electrophoresis, was transferred by Western blotting onto nitrocellulose membranes. The blots were incubated with antibodies to various phosphoproteins and, where available, with antibodies to the associated total proteins. An antibody to β-actin (Sigma-Aldrich) was used as a control for protein loading. Individual protein bands were visualized by incubation of the blots with horseradish peroxidase-conjugated secondary antibodies from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) followed by treatment with ECL Western blotting detection reagents (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and exposure to chemiluminescence film. Individual band densities were determined by a Bio-Rad GS-800 calibrated scanning densitometer. Densities of bands for each sample were normalized to the corresponding β-actin band density to control for variations in the amount of protein loaded onto the gels (Supplemental Fig. 3). These ratios were then expressed as the fold change due to compound treatment divided by the average of the vehicle-treated samples. Data were graphed as the mean ± S.E.M. using GraphPad Prism software.

Biotinylation Analysis of Rat Striatal Slice Preparations. Male Sprague-Dawley rats (200–220 g) were decapitated by guillotine, brains were harvested and cut into 300-μm coronal sections (Vibratome; The Vibratome Company, Maryland Heights, MO) at 4°C in oxygenated Krebs’ buffer (125 mM NaCl, 4 mM KCl, 1.25 mM KH₂PO₄, 10 mM glucose, 1.5 mM MgSO₄, 26 mM NaHCO₃, and 1.5 mM CaCl₂). Striatal sections were separated from surrounding tissue and allowed to recover in Krebs’ buffer for 60 min at 30°C with bubbling (95% O₂/5% CO₂). After recovery, striatal sections were transferred to fresh, oxygenated Krebs’ buffer containing either vehicle (0.1% DMSO) or 1 μM MP-10 (0.1% DMSO) and incubated for 30 min at 30°C with bubbling. For biotinylation of surface proteins, striatal slices were incubated with 1 mg/ml sulfo-NHS-SS-biotin (Thermo Fisher Scientific, Waltham, MA) in oxygenated Krebs’ buffer at 4°C for 45 min. Sections were rinsed with ice-cold Tris-buffered saline and homogenized in radioimmunoprecipitation assay buffer (Millipore, Temecula, CA) containing protease inhibitors (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (Sigma-Aldrich), followed by centrifugation at 20,000g at 4°C to remove insoluble material. Biotinylated proteins were precipitated by incubating detergent extracts containing 100 μg of solubilized protein with NeutrAvidin agarose (Thermo Fisher Scientific) overnight at 4°C. After washing, precipitated material was released from the agarose resin by boiling in Laemmli buffer and analyzed by Western blotting as described.

Quantitative Real-Time Reverse Transcription-PCR. Total RNA from striatum of control and treated mice was isolated using TRIzol reagent according to the manufacturer’s instructions (Invitrogen). The concentration and quality of the total RNA preparations were checked by NanoDrop spectrophotometer and 1% agarose gel electrophoresis. Contaminating genomic DNA was eliminated by treatment with DNase I at 25°C for 15 min. cDNA was synthesized using Superscript II Reverse Transcriptase with random hexamers at 42°C for 50 min. TaqMan real-time PCR primer-probe sets were from ABI (Applied Biosystems Inc.) assay design (assay ID: Mm00436880_m1 for substance-P (FAM probe), assay ID Mm01212875_m1 for enkephalin (FAM probe), and rodent glyceraldehyde-3-phosphate dehydrogenase control reagents (VIC probe)). Real-time reverse transcription-PCR was performed using TaqMan universal PCR Master Mix and ABI 7500 Sequence Detecting System according to the manufacturer’s instructions. Samples containing 50 ng of cDNA from striatum of control and treated mice were amplified in triplicate for 40 PCR cycles (50°C for 2 min, 90°C for 10 min, 95°C for 15 s, extension 60°C for 1 min). At the completion of each PCR reaction, the amount of a target message in each sample was estimated from a threshold cycle number (Ct), which inversely correlated with the abundance of its initial cDNA level. Ct values were converted to relative quantity of mRNA using relative standard curves obtained by 11-fold serial dilutions of 500 ng of cDNA prepared from pooled striatum of untreated mice. The standard curves were constructed to extend above and below the expected abundance of target cDNA. They demonstrated acceptable PCR efficiency (91–99%) and PCR linearity (R² > 0.99). GAPDH, which is not affected by MP-10 treatment, was used as an internal control to normalize the expression of target genes.

Antagonism of Apomorphine Induced Stereotypy and Climbing. Papaverine HCl (10–54 mg/kg i.p.) or MP-10 (0.1–30 mg/kg i.p.) was evaluated in the antagonism of apomorphine-induced stereotypy and climbing assay using a standard 30-min pretreatment interval. Controls were administered to six mice (male CF-1, 23–28 g) per dose level. Control groups, run simultaneously with drug-treated groups, received 2% Tween 80/0.5% hydroxyethylcellulose in water at equal volumes (10 ml/kg). After the pretreatment interval, experimental and control animals were challenged with 1 mg/kg s.c. apomorphine. Five minutes after the apomorphine injection, the sniffing-licking-gnawing (0 = absent, 1 = present) syndrome (e.g., stereotyped behavior) and climbing behavior (0 = all four feet on the ground, 1 = two feet up on the wire cage, 2 = all four feet on the wire cage) induced by apomorphine were scored and recorded for each animal. Readings were repeated every 5 min during a 30-min test session. Scores for each animal were totaled over a 30-min session for each syndrome (stereotyped behavior and climbing). Mean climbing and stereotypy scores were then expressed as a percentage of control values observed in vehicle-treated mice that received apomorphine.

Catalepsy in Mice. Papaverine (10–54 mg/kg), MP-10 (1–30 mg/kg) or vehicle (2% Tween 80 + 0.5% hydroxyethylcellulose) was administered intraperitoneally to six mice (male CF-1, 23–28 g) per treatment group. Every 30 min for 2 h after dosing, the animal’s forelegs were draped over a thin horizontal rod 1 3/4 inches high. The amount of time in (seconds) for which the animal maintained this awkward position was recorded (60 s maximum). Maintenance of this position is considered catalepsy. The mean seconds spent in the catalepsy position for each dose at each time point was expressed as a percentage of maximal catalepsy possible (60 s). The minimal effective dose was defined as the lowest dose producing more than 30% catalepsy.

Conditioned Avoidance Responding in Rats and Mice. Male Sprague-Dawley CD rats (350–450 g) or male CF-1 mice (40–60 g) were individually housed and either maintained (rats) on a food-restricted schedule (15 g of standard rodent feed each day after training/testing) or allowed free access to chow (mice). The testing apparatus consists of a stainless steel and Plexiglas test chamber with two 8 × 6.5 × 8 compartments separated by an arched doorway measuring 3.5 × 4.5 (MED Associates, Burlington, VT). Each of the two floors of the shuttle box comprises a series of stainless steel grid rods and is the presentation of an electric foot shock (0.5 mA). In addition, each side of the chamber is equipped with a stimulus light and tone (Sonalert; Mallory Sonalert, Indianapolis, IN).
and multiple infrared beam source/detectors to locate the subjects within the chamber.

Subjects that are trained to avoid the foot shock were placed in the experimental chambers for a 4-min habituation period followed by 50 trials presented on a 15-s variable interval schedule (range = 7.5–22.5 s). Each trial consisted of a 10 s warning tone and stimulus light (conditioned stimulus) followed by a 10 s shock (unconditioned stimulus), presented through the grid floor on the side where the animal was located, in the presence of the tone and light. If an animal crossed through the archway during the initial 10 s of the trial, the tone and light were terminated, and the response was considered an avoidance response. If an animal crossed through the archway after a foot shock was initiated, the tone, light, and shock were terminated, and the response was considered an escape response. If a response was made during an intertrial interval, the animal was punished with a 0.5-s shock (0.5 mA). A MED Associates computer with MEDSTATE Notation software controlled the test session and counted the number of trials in which the animal avoided shock, escaped shock, and did not respond. Only animals displaying stable performance (approximately 90% avoidance responding on the training session before test day for rats, and 84% avoidance responding for mice) were considered “trained” and included on test day. Training was maintained by a least one nondrug test session each week. Both papaverine and MP-10 were administered via an intraperitoneal route (30 min before testing) for studies using either rats or mice. Eight animals received each dose of the test compounds, as well as a vehicle injection (within subject design).

**Antagonism of MK-801 Induced Prepulse Inhibition Deficits in Rats.** Each testing chamber (SR-LAB system; San Diego Instruments, San Diego, CA) consisted of a Plexiglas cylinder (8.8 cm in diameter) mounted on a frame and held in position by four metal pins to a base unit. Movement of the subjects (male Long Evans rats, 200–300g, n = 8 per treatment group) within the cylinder was detected by a piezoelectric accelerometer attached below the frame. A loudspeaker mounted 24 cm above the cylinder provided background white noise, acoustic noise bursts, and acoustic prepulses. The entire apparatus was housed in a ventilated enclosure (39 × 38 × 56 cm). Presentation of acoustic pulse and prepulse stimuli was controlled by the SR-LAB software and interface system, which also digitized, rectified, and recorded the responses from the accelerometer. Mean startle amplitude was determined by averaging 100 1-ms readings taken from the beginning of the onset of the pulse stimulus. For calibration purposes, sound levels were measured with a Quest sound level meter, scale “A” (Thermo Fisher Scientific), with the microphone placed inside the Plexiglas cylinder.

Test sessions consisted of 61 total trials with a 15-s intertrial interval. After a 5-min acclimation to a 64-dB background noise, four trial types (20-ms, 120-dB pulse or a 69-, 74-, or 79-dB 20-ms prepulse paired with a 120-dB 20-ms pulse occurring 100 ms later, onset to onset) were presented in a pseudorandom order. Papaverine or MP-10 was administered intraperitoneally at 30 and 60 min before testing, respectively, with MK-801 (0.085 mg/kg s.c.) administered 10 min before testing. Prepulse inhibition was defined as 100 − [(startle amplitude on prepulse trials/startle amplitude on pulse alone trials) × 100].

**Prepulse Inhibition of Acoustic Startle in Mice.** Startle responses were measured using the SR-Lab system described above but modified with inserts designed specifically for mice, and the subjects of these studies were male C57BL/6J mice (25–35 g, n = 9–23 per treatment group; The Jackson Laboratory). Test sessions consisted of no stimulus, pulse-only, and prepulse trials. Each “pre-pulse” trial consisted of a 20-ms 69-, 73-, or 81-dB nonstartling prepulse followed 80 ms later by a 40-ms startling pulse of 120 dB. In contrast, “pulse-only” trials consisted of the 120-dB stimulus only, and “no-stimulus” trials contained background noise only. Sessions began with a 5-min acclimation period, during which the background noise was played, and this was followed by a block of five pulse-only trials for habituation purposes. The PPI portion of the session consisted of 10 trials of each prepulse intensity plus pulse, 10 pulse-only trials, and 10 no-stimulus trials, all presented in a random order. Papaverine, MP-10, and rolipram were dissolved in 2% Tween/0.5% methylcellulose and administered intraperitoneally 30, 60, and 20 min, respectively, before the session.

**Novel Object Recognition in Rats.** Novel object recognition (NOR) training and testing was performed in a circular field (diameter ~ 70 cm, 30 cm high) constructed out of plastic and containing bedding. The field was surrounded by black curtains to mask extra-field cues and was located in a dimly lit room (~10 lux at the level of the arena) in the presence of white noise (~65 dB). Animal performance (male Long Evans rats, 290–350 g, n = 10 per treatment group) was tracked by video and monitored by an experimenter located outside of the testing room. Objects, constructed with Duplo blocks (The Lego Group, Billund, Denmark), were placed on the arena floor in one of two locations spaced evenly around the field approximately 10 cm from the field’s edge. To avoid possible olfactory cues, multiple copies of the objects were used throughout the study and cleaned with a 30% ethanol solution between animals. Rats displayed no preference or aversion to either of the objects and spent an equivalent amount of time exploring objects if both were presented simultaneously.

The visual recognition task was divided into three sessions: habituation, a sample trial, and a choice trial. During habituation, the animals were placed into the field containing two identical yellow cubes (~10 × 10 × 10 cm) and allowed to explore the field for 10 min. After habituation, rats were returned to their home cage. One day after habituation, animals were dosed with drug, and after the pretreatment interval, the sample trial was initiated. During the sample trial, rats were allowed to explore the field, now containing two identical objects located at opposing compass points, for 5 min. The amount of time exploring the objects was recorded for the entire trial. Exploration was defined as orientation toward the object with the nose of the rat within < 2 cm of the object. After the sample trial, rats were returned to their home cages for the 48-h retention interval and then tested in the choice trial for recognition memory. The choice trial consisted of a 5-min exploration of the field containing both a familiar, previously explored, object and a novel object with an investigator again recording contact time. The location of the objects, counterbalanced across treatment groups, remained constant for each animal during the habituation, sample, and choice trials. Data were collected using automated Noldus Ethovision XT software.

Significantly more time spent exploring the novel object compared to the familiar one during the choice trial represents intact recognition memory for that treatment group. Vehicle controls show no significant differences between familiar and novel object exploration following the 48-h delay. Papaverine (5.6–30 mg/kg i.p.) or MP-10 (0.3–3, mg/kg i.p.) was administered 30 min before the sample trial.

**NMDA Antagonist-Impaired Social Odor Recognition in Mice.** Male CF-1 mice (18–20 g) were individually housed in plastic cages with corncob bedding and free access to food and water. At the time of individual housing, 1-inch round wooden beads were placed in the animal cages and remained there for 6 days to allow saturation of the beads with the individual animal’s odor. All beads were removed from the cages at least 1 h before training. Training occurred in the animals’ homecages on day 7 after individual housing. Training consisted of three 1-min trials with a 1-min intertrial interval. During these trials, each animal was presented with three of their own homecage beads and one bead from a stranger mouse cage (called donor-1 bead). The training period was intended to allow the animal to become familiar with the odor of the donor-1 bead. Beads used during training were discarded immediately after training. Twenty-four hours after training, the animals received a 1-min test trial in which they were presented with two homecage beads, one donor-1 bead, and one bead from the cage of a new second stranger (called donor-2 bead). The amount of time (in seconds) that mice spent sniffing each bead during the 1-min test trial was recorded. Increased sniffing of the donor-2 bead versus the donor-1 bead dur-
ing the test trial suggests memory of the donor-1 bead. Mice (n = 8–10 per treatment group) received papaverine (3, 10, or 30 mg/kg p.o.), MP-10 (0.3, 1, or 3 mg/kg p.o.), or vehicle administered concurrently with MK-801 (0.1 mg/kg i.p.) immediately after training.

**Social Approach/Social Avoidance in Mice.** The protocol was adapted from Sankoorikal et al. (2006) and Moy et al. (2004). Six-to-eight-week-old male BALB/c test mice and C57BL/6J stranger mice were housed in groups of four in separate rooms. The social testing apparatus was a three-chambered Plexiglas box with white walls and a black floor. The center chamber was 12 × 26 × 50 cm with 10-cm wide openings into the two end chambers, which were 19 × 26 × 50 cm (length/width/height). Three identical three-chambered boxes were located on a single floor (50 × 78 cm), and up to four floors were used simultaneously (i.e., 12 boxes). The two end chambers of each box contained a clear Plexiglas cylinder (8 cm diameter, 13 cm high) in the center. The cylinder had multiple small holes (1.5 cm diameter) over the entire surface to allow visual, olfactory, auditory, and limited tactile contact between the stranger and the test mouse. The activity of the test mice was video-recorded by Ethovision (version 3.1; Noldus Information Technology, Wageningen, The Netherlands) and stored as X-Y coordinates of the subject’s position every 0.1 s. Behavioral testing occurred daily between 10:00 AM and 4:00 PM in a dimly lit testing room (3–5 lux). Test and stranger mice were transported in their home cage to separate ante-rooms of the test room and were identified by tail marks and allowed to acclimate for at least 1 h. On the 1st day, test mice explored the test chamber containing empty cylinders in both ends for 10 min. Stranger mice were habituated for 20 min to the Plexiglas cylinders in their ante-room. All boxes and cylinders were cleaned with Saniplex spray (Saniplex Laboratories, Ridgeland, MS) between subjects. The apparatus was a three-chambered Plexiglas box with white walls and a black floor. The center chamber was 12 × 26 × 50 cm (length/width/height). Three identical three-chambered boxes were located on a single floor (50 × 78 cm), and up to four floors were used simultaneously (i.e., 12 boxes). The two end chambers of each box contained a clear Plexiglas cylinder (8 cm diameter, 13 cm high) in the center. The cylinder had multiple small holes (1.5 cm diameter) over the entire surface to allow visual, olfactory, auditory, and limited tactile contact between the stranger and the test mouse. The activity of the test mice was video-recorded by Ethovision (version 3.1; Noldus Information Technology, Wageningen, The Netherlands) and stored as X-Y coordinates of the subject’s position every 0.1 s. Behavioral testing occurred daily between 10:00 AM and 4:00 PM in a dimly lit testing room (3–5 lux). Test and stranger mice were transported in their home cage to separate ante-rooms of the test room and were identified by tail marks and allowed to acclimate for at least 1 h. On the 1st day, test mice explored the test chamber containing empty cylinders in both ends for 10 min. Stranger mice were habituated for 20 min to the Plexiglas cylinders in their ante-room. All boxes and cylinders were cleaned with Saniplex spray (Saniplex Laboratories, Ridgeland, MS) between subjects. The amount of time the test mice spent in the three chambers during habituation was analyzed to determine any side preference. The least-preferred end was assigned as the social end for mice showing bias >80 s, whereas all other mice had pseudorandom assignment of the social end. The next day, test and stranger mice were returned to their ante-rooms approximately 1 h before testing. Test mice were weighed and treated with vehicle or drug. After the pretreatment period, trial 1 started as a 10-min exploration of the box with clean empty cylinders placed in both end chambers. Trial 2 began immediately afterward with a container containing the stranger mouse in the designated social end and an empty cylinder in the opposite end replacing the previous empty cylinders. The test mice had 4 min to explore in trial 2. Locomotor activity (traveled in centimeters) and a 1.9-fold increase was seen with the 54 mg/kg dose at the 10 and 54 mg/kg doses. These results are in line with results published for the closely related compound TP-10 with the relative increases in cAMP being less than cGMP due to the higher baseline for cAMP (Schmidt et al., 2008).

**Effects of Papaverine and MP-10 upon Key Neuronal Phosphoproteins.** PDE10A inhibition has been previously shown to increase phosphorylation of key cAMP-dependent substrates, such as cAMP-response element-binding protein (CREB), extracellular receptor kinase, and more recently the AMPA-receptor GluR1 subunit (Siuciak et al., 2006a; Nishi et al., 2008; Schmidt et al., 2008). We wanted to confirm and expand on these initial observations to improve our mechanistic understanding of PDE10A inhibition. In our studies, papaverine caused a trend toward a dose-dependent increase in CREB Ser133 (S133) phosphorylation at a 10-min time point, which did not reach a statistically significant difference from vehicle-treated controls. The magnitude of the effect approached a 3-fold increase at the 10 mg/kg dose at the 30-min time point (Fig. 2A). The effects of MP-10 upon CREB phosphorylation were also examined in striatal tissue with 30 min. MP-10 at 0.3, 3, and 5 mg/kg i.p. resulted in robust, statistically significant increases in CREBS133 phospho-

<table>
<thead>
<tr>
<th>Compound</th>
<th>Papaverine IC_{50}</th>
<th>MP-10 IC_{50}</th>
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<tr>
<td>hPDE10</td>
<td>92.3 nM</td>
<td>1.26 nM</td>
</tr>
<tr>
<td>hPDE1B</td>
<td>25,600</td>
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</tr>
<tr>
<td>hPDE2A</td>
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<tr>
<td>hPDE6*</td>
<td>7050</td>
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<tr>
<td>hPDE11A</td>
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* Bovine phosphodiesterase 6.
phorylation of 3-, 4-, and 2.6-fold, respectively, compared with vehicle (Fig. 2B). DARPP-32 is phosphorylated at the Thr34 (T34) residue by both PKA and cGKII upon activation by cAMP and cGMP, respectively (Greengard et al., 1999; Serulle et al., 2007). Previously, papaverine had been shown to increase Thr34 phosphorylation in striatal slices but not in vivo (Nishi et al., 2008). Phosphorylation of DARPP-32T34 at the 54 mg/kg dose of papaverine at 10 min displayed a statistically insignificant 3-fold increase. This effect probably did not reach significance due to the high variable responses from individual animals (Fig. 2C). No effect upon DARPP-32 phosphorylation was seen at the 30-min time point. In contrast, the effect of the data with MP-10 was far more robust, and we are able to report the first in vivo increase in Thr34 phosphorylation after PDE10A inhibition with a 3.5-fold significant increase with the 5 mg/kg dose of MP-10 (Fig. 2D).

Both PKA and cGKII phosphorylate the AMPA receptor subunit GluR1 at the Ser845 (S845) residue (Roche et al., 1996; Serulle et al., 2007). In addition, pDARPP-32 inhibition of the PP-1 phosphatase inhibits dephosphorylation at this site, resulting in an augmented level of GluR1S845 phosphorylation. A recent study, published while this work was in preparation, indicated that papaverine increases the phosphorylation of GluR1S845 in both striatal slices and in striatal tissue in vivo (Nishi et al., 2008). We have expanded the data set with the study of a second compound (MP-10) and also additional glutamate receptor subunit-specific phosphosite results. In addition, we have explored the cellular consequences of these signaling events. It is surprising that the effects of papaverine upon GluR1S845 phosphorylation were far more robust at both the 10- and 30-min time points compared with all of the other phosphoproteins examined (Fig. 3, A–C). The 10 and 54 mg/kg doses of papaverine resulted in statistically significant 2.0- and 3.8-fold increases, respectively, in GluR1S845 phosphorylation and 2.0- and 4.5-fold increases, respectively, in the pGluR1S845/GluR1 ratio at 10 min. The 54 mg/kg dose caused a 6.2-fold increase at 30 min with a 5.6-fold increase in the pGluR1/GluR1 ratio. In line with these findings, MP-10 caused even more striking increases in GluR1 phosphorylation levels of 3-, 5.4-, and 4.1-fold with 0.3, 3, and 5 mg/kg doses, respectively (Fig. 3D). A small significant decrease in GluR1 levels of 0.8-fold was seen with the 0.1 mg/kg dose of MP-10, whereas no change occurred with the other tested doses (Fig. 3E). The increase in phosphorylation levels resulted in an associated increase in the pGluR1/GluR1 ratios of 3-, 6.3-, 4.3-fold for the 0.3, 3, and 5 mg/kg doses, respectively (Fig. 3F).

Small changes were seen in the phosphorylation level of the AMPA receptor subunits GluR2/3S880/S891 and in total levels at the papaverine 10-min time point (Supplemental Fig. 1, A and B). The level of pGluR2/3 decreased with the 3 mg/kg dose by 0.78-fold, but an insignificant decrease in the total GluR2/3 level resulted in no change in the pGluR2/3/GluR2/3 ratio. The 54 mg/kg dose caused statistically significant decreases in both pGluR2/3S880/S891 phosphorylation and total GluR2/3 levels of 0.9-fold at the 10-min time point,
resulting in no change in the pGluR2/3S880/S891/GluR2/3 ratio (Supplemental Fig. 1C). No significant changes in pGluR2/3 or total GluR2/3 were observed with any dose of papaverine tested at the 30-min time point (Supplemental Fig. 1, A–C). No change was seen in GluR2/3 phosphorylation levels or in the pGluR2/3/GluR2/3 ratio at any tested dose of MP-10; however, a small but significant decrease in total GluR2/3 levels was observed (0.87-fold) with the 0.1 mg/kg dose (Supplemental Fig. 1, D–F).

The effect upon the NMDA receptor subunit NR2B-(Tyr1472) phosphorylation levels and total NR2B levels were examined and found to be unchanged by any dose of papaverine tested at the 30-min time point (Supplemental Fig. 1, A–C). No change was seen in GluR2/3 phosphorylation levels or in the pGluR2/3/GluR2/3 ratio at any tested dose of MP-10; however, a small but significant decrease in total GluR2/3 levels was observed (0.87-fold) with the 0.1 mg/kg dose (Supplemental Fig. 1, D–F).

The effect upon the NMDA receptor subunit NR2B-(Tyr1472) phosphorylation levels and total NR2B levels were examined and found to be unchanged by any dose of papaverine tested at the 10- or 30-min time point (data not shown). The 0.3 mg/kg dose of MP-10 caused an increase in the phosphorylation level of NR2B(Tyr1472) by 1.25-fold with an associated small but significant 1.16-fold increase in the pNR2B/NR2B ratio (Supplemental Fig. 2, A and B). No change in phosphorylation levels was seen at the other doses tested. The total NR2B level was decreased with the 0.1 mg/kg dose by 0.88-fold, with no effect upon the pNR2B/NR2B ratio (Supplemental Fig. 2, A and B).

**Fig. 2.** Dose-dependent effects of PDE10 inhibitors upon mouse striatal CREB133 and DARPP-32T34 phosphorylation. A, papaverine effects upon CREBS133 phosphorylation 10 (n = 8; 10 mg/kg, n = 7) and 30 min (n = 4) after dosing. B, MP-10 effects upon CREBS133 phosphorylation 30 min (n = 4) after dosing. C, papaverine effects upon DARPP-32T34 phosphorylation 10 (n = 8; 10 mg/kg, n = 7) and 30 min (n = 4) after dosing. D, MP-10 effects upon DARPP-32T34 phosphorylation 30 min (n = 4) after dosing. V = vehicle-treated. Western blot images are representative of samples treated as shown in the corresponding bar on the graph. The arrow points to the pCREB bands. *, p < 0.05; **, p < 0.01.

**PDE10A Inhibition Increases GluR1S845 Phosphorylation at the Cell Surface but Does Not Change GluR1 Surface Levels.** The effects of MP-10 upon GluR1 cell surface expression and phosphorylation levels were examined in rat striatal slice preparations. As shown in Fig. 4, A and B, total GluR1 levels in whole-cell extracts were unaffected by treatment with 1 μM MP-10 for 30 min. However, GluR1 Ser845 phosphorylation was increased 2.1-fold in these extracts. A similar pattern of GluR1 Ser845 phosphorylation was detected at the cell surface, as determined by biotinylation analysis of surface proteins (Fig. 4, C and D). After treatment with 1 μM MP-10 for 30 min, the level of total GluR1 on the cell surface was unchanged compared with vehicle control, indicating no net increase in total GluR1 surface expression with MP-10 treatment (Fig. 4D). However, the level of GluR1S845 phosphorylation at the cell surface was significantly increased 2-fold with MP-10 treat-
ment compared with vehicle control, indicating that MP-10 enhances phosphorylation of GluR1S845 in receptors at the cell surface without any apparent effect on receptor trafficking.

Activation of the Direct and Indirect Pathways by PDE10A Inhibition. The degree to which haloperidol and MP-10 activate the striatonigral (D1, direct) versus the striatopallidal (D2, indirect) pathways was assessed in striatum of CF-1 mice using TaqMan probes for substance-P and enkephalin, respectively. In comparison to vehicle, haloperidol (0.12–1.2 mg/kg i.p.) increased enkephalin mRNA levels, but not Substance-P levels (Fig. 5). In contrast, MP-10 (3 mg/kg) increased both enkephalin and Substance-P mRNA levels. These results suggest that catalepsy might be a result of the relative balance in activation of the two striatal output pathways by a treatment. In this regard, haloperidol high levels of

Fig. 3. Dose-dependent effects of PDE10 inhibitors upon mouse striatal GluR1S845 phosphorylation. A, pGluR1S845; B, total GluR1; C, pGluR1/total GluR1 ratio 10 (n = 8; 10 mg/kg, n = 7) and 30 min (n = 4) after papaverine dosing. D, pGluR1S845. E, total GluR1. F, pGluR1/total GluR1 ratio 30 min (n = 4) after MP-10 dosing. V = vehicle-treated. Western blot images are representative of samples treated as shown in the corresponding bar on the graph. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
catalepsy would be due to activating the D2-indirect pathway only, whereas MP-10, through activating both D1 and D2 pathways, results in less catalepsy.

**Effect of PDE10A Inhibitors in Models Predictive of Antipsychotic Activity and EPS Liability.** Previously, PDE10A inhibition has been shown to be efficacious in a range of models predictive of antipsychotic activity and to demonstrate minimal predicted EPS liability (Siuciak et al., 2006a; Schmidt et al., 2008). We characterized papaverine and MP-10 in the models that we classically use to detect antipsychotic activity and predict EPS to enable us to compare across active doses in models of cognition and negative symptoms (data summarized in Supplemental Table 1).

**Apomorphine-Induced Climbing.** Inhibition of apomorphine-induced climbing is a preclinical model of antipsychotic efficacy mediated by the mesolimbic dopaminergic

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**Fig. 4.** Acute treatment with MP-10 increases GluR1S845 phosphorylation levels at the cell surface in rat striatal slices. Rat striatal slices were incubated with oxygenated Krebs’ buffer containing either vehicle (0.1% DMSO) or 1 μM MP-10 (in 0.1% DMSO) for 30 min at 30°C. After biotinylation, whole-cell proteins from detergent extracts (A and B) or surface proteins from NeutrAvidin precipitates (C and D) were analyzed by immunoblotting with antibodies to total GluR1 or GluR1 phosphoserine 845 (pS845). B, immunoblots of whole-cell proteins from detergent extracts indicate that total GluR1 levels were unchanged by treatment with MP-10, whereas GluR1 phosphorylation at Ser845 was increased 2.1-fold in these extracts. Likewise, immunoblots of surface proteins indicated that there was no net increase in total GluR1 surface levels with MP-10 treatment, whereas the level of GluR1 Ser845 phosphorylation at the cell surface was significantly increased 2.0-fold with MP-10 treatment (D). The apparent increase in surface GluR1 Ser845 phosphorylation was not a result of nonspecific precipitation of whole-cell proteins because no signal was detected in NeutrAvidin precipitates when sulfo-NHS-SS-biotin was omitted (data not shown), \( p < 0.05 \) for total proteins; \( p < 0.05 \) for surface proteins by paired Student’s \( t \) test.

**Fig. 5.** MP-10 activates both the D1-direct and D2-indirect pathway. TaqMan probes for substance P (D1-direct pathway) and enkephalin (D2-indirect pathway) were used to assess striatal mRNA levels 3 h after intraperitoneal injection with haloperidol (0.12–1.2 mg/kg, i.p.) (A) or MP-10 (3 mg/kg, i.p.; \( n = 8 \) per group) (B). Haloperidol up-regulates only enkephalin mRNA (1.25-fold over vehicle), whereas MP-10 up-regulates both enkephalin (1.25-fold over vehicle) and substance P (1.25-fold over vehicle). \( *, p < 0.05 \) versus vehicle within gene; \( **, p < 0.01 \) versus vehicle across genes.
pathway, whereas inhibition of the accompanying stereotypy is a predictor of EPS mediated by the nigrostriatal dopaminergic pathway (Marquis et al., 2007). An atypical antipsychotic drug will profile a wide separation (>3-fold) between these two measures. Papaverine (10–54 mg/kg i.p.) produced a decrease in apomorphine-induced climbing in mice with an ID$_{50} = 41.5$ mg/kg (34.8–49.5 mg/kg) and decreased apomorphine-induced stereotypy with an ID$_{50} = 53.6$ mg/kg (52.6–54.6 mg/kg), whereas MP-10 (0.1–30 mg/kg i.p.) produced a decrease in apomorphine-induced climbing in mice with an ID$_{50} = 0.375$ mg/kg (0.277–0.507 mg/kg) and decreased apomorphine-induced stereotypy with an ID$_{50} = 25.069$ mg/kg (19.653–31.976 mg/kg) (Fig. 6). These data suggest that PDE10A inhibitors, specifically MP-10, profile like an atypical antipsychotic drug in the apomorphine-induced climbing assay.

**Catalepsy.** To more directly predict the potential for EPS, the two PDE10A inhibitors were assessed in a mouse catalepsy model (Fig. 7). Papaverine produced a main effect of treatment in a repeated measures analysis, $F(4,25) = 13.98; p < 0.0001$, with post hoc analysis revealing that this effect was driven by the 54 mg/kg dose ($p < 0.0001$). There was a significant effect on postadministration time, $F(3,75) = 19.78; p < 0.0001$, with catalepsy only evident at the 30-min
time point compared with all other time points ($p < 0.0001$). There was also a significant treatment by time interaction, $F(12,75) = 11.86; p < 0.0001$. Post hoc analysis shows that this effect was driven by the 30 and 54 mg/kg groups at the 30-min test time ($p < 0.005$) compared with the vehicle control group. The peak cataleptogenic potential observed was $74\%$ of the total possible response at 30-min postadministration of 54 mg/kg.

MP10 did not produce a significant treatment effect in a repeated measures analysis, $F(4,25) = 1.74; p = 0.1718$. There was a significant effect on postadministration time, $F(3,75) = 12.32; p < 0.0001$. There was also significant treatment by time interaction, $F(12,75) = 2.1; p = 0.0270$, with post hoc analysis revealing that this effect was driven by the 3 and 10 mg/kg groups at the 90-min test time ($p < 0.05$) and the 3 mg/kg group at the 120-min test time ($p < 0.05$). The peak cataleptogenic potential observed was $46\%$ of the total possible response at 90 min after dosing in the 3 mg/kg treated group.

**CAR in Rats and Mice.** Disruption of CAR is another preclinical model predictive of antipsychotic activity (Wadenberg and Hicks, 1999). In the mouse CAR model (Fig. 8A), papaverine (30–54 mg/kg i.p.) decreased avoidance responding with a significant treatment effect ($F(2,14) = 122.15, p < 0.0001$), and the post hoc analysis indicated significant reductions at both the 30 and 54 mg/kg doses compared with vehicle. Nonlinear regression analysis calculated an ID$_{50}$ equivalent to 38.047 mg/kg.

**MK-801 Deficit in PPI of Acoustic Startle in Rats.** The PDE10A inhibitors were studied in a MK-801 PPI deficit model in rats (Fig. 9A). Papaverine (3–30 mg/kg i.p.) significantly attenuated an MK-801-induced (0.085 mg/kg s.c.) PPI deficit in male Long Evans rats with repeated measures analysis showing both treatment [$F(4,35) = 18.75, p < 0.0001$] and treatment by prepulse [$F(8,7) = 4.72, p < 0.0001$] effects. The post hoc analysis revealed that papaverine, at either 10 or 30 mg/kg, significantly attenuated the MK-801-induced deficits at both the 10- and 15-dB prepulse intensities, although data are represented as collapsed across prepulse intensities. With respect to startle response, there was a trend toward increased startle response in several of the treatment groups, although this did not reach statistical significance [$F(4,35) = 2.516, p = 0.0589$] (data not shown). MP-10 (1–10 mg/kg i.p.) significantly attenuated an MK-801-induced deficit in PPI of acoustic startle in rats.

**PDE10A Inhibitors and Prepulse Inhibition Paradigms**

**Papaverine and MP-10 disrupt CAR in rats and mice.** Papaverine and MP-10 were evaluated in the conditioned avoidance response in male CF-1 mice (A) and in male Sprague-Dawley rats (B) using a 30-min pretreatment interval and an intraperitoneal route of administration. Data are expressed as average (means ± S.E.M.) avoidance, escape, and response failures observed over 50 trials ($n = 8$ animals/group). *, statistical difference in avoidance response from vehicle-treated controls ($P < 0.05$); #, statistical difference in escape response from vehicle-treated controls ($P < 0.05$); *, statistical difference in response failures from vehicle-treated controls ($P < 0.05$).
induced (0.085 mg/kg s.c.) PPI deficit as well, with repeated measures analysis showing a significant treatment effect [\( F(4,34) = 6.92, p < 0.0003 \)]. The post hoc analysis indicated that these effects were observed at the 3 and 10 mg/kg doses. There were no observed effects on startle response [\( F(3,34) = 1.224, p = 0.3191 \)] (data not shown).

Prepulse Inhibition in C57BL/6J Mice. Previously (Kanes et al., 2007; Kelly et al., 2007, 2008), the phosphodiesterase type 4 (PDE4) inhibitor rolipram was shown to increase the normally low PPI exhibited by C57BL/6J mice, suggesting that PDE4 inhibitors might prove to be a novel class of compounds with antipsychotic-like efficacy. To determine whether PDE10A inhibitors also exhibit similar effects on measures of sensorimotor gating, we tested the ability of papaverine and MP-10 to increase PPI in C57BL/6J mice (Fig. 9B). Consistent with an antipsychotic-like effect, papaverine was shown to significantly increase PPI [\( F(6,98) = 8.42, p < 0.0001 \)]. Post hoc analyses showed that 54 mg/kg papaverine significantly increased PPI relative to vehicle (\( p = 0.005 \)), as did the positive control 1 mg/kg rolipram (\( p < 0.0001 \)). In contrast to rolipram, however, 54 mg/kg papaverine also significantly decreased the startle response in trials where the 120-dB pulse is presented alone (vehicle, 175.25 ± 16.1 startle units; 54 mg/kg papaverine, 55.17 ± 12.9 startle units; rolipram, 148.67 ± 17.0 startle units; \( F(6,98) = 6.04, p < 0.001 \); post hoc vehicle versus papaverine, \( p < 0.001 \)). It is important to note that the ability of 54 mg/kg papaverine to increase PPI is probably independent of its ability to decrease the startle response itself, because there was no statistical correlation between startle magnitude and %PPI in mice treated with papaverine (data not shown).

MP-10 also exhibited antipsychotic-like properties by increasing PPI in C57BL/6J mice [\( F(6,83) = 6.14, p < 0.0001 \)]. Post hoc analyses show that 54 mg/kg MP-10 significantly
increases PPI relative to vehicle ($p = 0.011$), as does rolipram ($p < 0.001$). In contrast to papaverine, however, MP-10 elicited this effect without significantly affecting the startle response itself. Together, these data suggest that high doses of PDE10A inhibitors increase sensorimotor gating.

**Effect of PDE10A Inhibitors in Models of Negative Symptoms and Cognition.** Cognitive deficits and negative symptoms are domains of schizophrenia that are very poorly treated by currently available antipsychotic drugs but are thought to be crucial for functional outcomes of the disease. Papaverine has shown activity in a model of executive function in rats (Rodefer et al., 2005). In addition, PDE10A has shown low levels of expression in brain regions associated with memory and cognition, including hippocampus and cortex (Seeger et al., 2003). Thus, we were compelled to understand the potential of this mechanism in a negative symptom model [social approach/social avoidance (SASA)] and cognition assays [NOR and social odor recognition (SOR)].

**NOR.** The PDE10A inhibitors were studied initially in NOR to find an effect on recognition memory (Young et al., 2009). Treatment with papaverine resulted in significantly greater time spent exploring the novel rather than the familiar object, demonstrating enhanced retention of the previous learning experience (Fig. 10). Doses of 5.6, 10, 17.8, and 30 mg/kg i.p. papaverine were administered 30 min before the sample trial. During the sample trial, there was no overall difference in the total object exploration time among all groups. Evidence of significant retention ($*, p < 0.05$ in post hoc analyses) was observed in the rats treated with 10, 17.8, and 30 mg/kg papaverine [treatment $\times$ time interaction $F(4,45) = 2.56, p = 0.05$]. Vehicle-treated animals displayed no preferential exploration during the test trial of the two objects, suggesting a natural decay in their original memory for the original object.

Treatment with MP-10 did not result in a significant difference in time spent exploring the novel over the familiar object at the doses evaluated (Fig. 10), although there was a strong trend toward a significant effect at the lowest dose tested. Doses of 0.1, 0.3, 1, and 3 mg/kg i.p. MP-10 were administered 30 min before the sample trial. During the sample trial, there was no overall difference in the total object exploration time among all groups. No evidence of significant memory retention was observed in the rats treated with MP-10 [treatment $\times$ time interaction $F(4,45) = 0.32, p = 0.8604$].

**SOR.** As a second model of cognition, we examined the SOR assay with a MK-801 deficit where we were able to measure social odor memory (Spinetta et al., 2008). Papaverine but not MP-10 improves cognition in NOR. Papaverine (5.6–30 mg/kg i.p.) or MP-10 (0.1–3 mg/kg i.p.) was evaluated in the novel object recognition task in male Long Evans rats ($n = 10$ per treatment group), administered 30 min before the sample trial (A) without any effect on total object exploration, averaged (means $\pm$ S.E.M.) across all groups. Amount of time (means $\pm$ S.E.M.) spent exploring the novel and familiar object after a 48-h delay is shown in B. $*$, significantly greater time spent exploring the novel object ($p < 0.05$), indicative of significant retention of a memory for the familiar object.
To understand the preclinical profile of PDE10A inhibition, we have analyzed two PDE10A inhibitors in vitro and in vivo, papaverine and MP-10. In vitro profiles and in vivo biochemical effects on cyclic nucleotides were in accordance with recent reports (Siuciak et al., 2006a; Schmidt et al., 2008). Antipsychotic activity was confirmed and expanded in animal models predictive of antipsychotic activity. This included disrupting the CAR, a classic predictor of antipsychotic efficacy (Wadenberg and Hicks, 1999). It has been suggested that blockade of the D2 dopamine receptor is a probable mechanism underlying CAR disruption (Wadenberg et al., 2000), which fits with the expression of PDE10A in D2-expressing MSNs of the indirect striatal output pathway (Xie et al., 2006) and activation of the indirect striatal pathway as assessed by expression of enkephalin after PDE10A inhibition in our experiments. MP-10 also shows a strong antido-

Discussion

To understand the preclinical profile of PDE10A inhibition, we have analyzed two PDE10A inhibitors in vitro and in vivo, papaverine and MP-10. In vitro profiles and in vivo biochemical effects on cyclic nucleotides were in accordance with recent reports (Siuciak et al., 2006a; Schmidt et al., 2008). Antipsychotic activity was confirmed and expanded in animal models predictive of antipsychotic activity. This included disrupting the CAR, a classic predictor of antipsychotic efficacy (Wadenberg and Hicks, 1999). It has been suggested that blockade of the D2 dopamine receptor is a probable mechanism underlying CAR disruption (Wadenberg et al., 2000), which fits with the expression of PDE10A in D2-expressing MSNs of the indirect striatal output pathway (Xie et al., 2006) and activation of the indirect striatal pathway as assessed by expression of enkephalin after PDE10A inhibition in our experiments. MP-10 also shows a strong antido-

arine significantly reversed a 0.1 mg/kg i.p. MK-801-induced deficit in social odor recognition in mice at 10 and 30 mg/kg p.o. \( F(2,64) = 28.32, \text{post hoc } p < 0.0001 \) (Fig. 11). The repeated measures analysis revealed a significant interaction between treatment × source of bead \( F(6,64) = 4.99, p = 0.0003 \). Post hoc analyses comparing sniffing time of donor-1 versus donor-2 revealed significantly more sniffing of the novel donor-2 bead over the familiar donor-1 bead at doses of 10 and 30 mg/kg. Likewise, MP-10 significantly reversed the MK-801-induced deficit in social odor recognition in mice at 3 mg/kg p.o. \( F(2,66) = 6.84, \text{post hoc } p < 0.001 \) (Fig. 11). The repeated measures analysis revealed a significant interaction between treatment × source of bead \( F(6,68) = 2.25; p = 0.049 \). Post hoc analysis of donor-1 versus donor-2 sniffing revealed a significantly greater sniffing of donor-2 over donor-1 at 3 mg/kg.

SASA in Mice. To determine whether PDE10A inhibition may have activity in a preclinical model of negative symptoms, we used the SASA model (Moy et al., 2004; Sankoorikal et al., 2006). Planned comparisons were used to determine whether time spent in the social and empty sides was different within each treatment group. In trial 2, vehicle treated BALB/cJ mice spent significantly more time in the empty side in the papaverine \( F(1,51) = 7.75, p < 0.05 \) and MP-10 \( F(1,56) = 22.47, p < 0.05 \) experiments shown in Fig. 12, A and B. Papaverine \( (3–30 \text{ mg/kg i.p., } n = 13–14/\text{group}) \) increased time spent in social side so that there was no difference between time spent in the social and empty sides at 3 \( F(1,51) = 0, p > 0.05 \), 10 \( F(1,51) = 2.32, p > 0.05 \), and 30 mg/kg \( F(1,51) = 0.52, p > 0.05 \) (Fig. 12A). Papaverine had no effect on locomotor activity on trial 2 \( F(1,51) = 0.33, p > 0.05 \) (Fig. 12C). MP-10 \( (0.03–0.3 \text{ mg/kg i.p., } n = 15/\text{group}) \) also increased time spent in the social side (Fig. 12B) but only in the 0.1 and 0.3 mg/kg groups \( F(1,56) = 2.84, p > 0.05 \) and \( F(1,56) = 1.69, p > 0.05, \text{ respectively} \). Mice treated with 0.03 mg/kg MP-10 spent more time in the empty than social side \( F(1,56) = 12.12, p < 0.05 \). MP-10 also dose-dependently decreased locomotor activity \( F(3,56) = 6.05, p < 0.05 \) with 0.3 mg/kg reducing activity (see Fig. 12D).
lepsy. We confirmed PDE10A activity in both pathways by measuring increases in expression of substance P (marker of direct striatal pathway) and enkephalin (marker of indirect striatal pathway) (Schmidt et al., 2008).

To understand in greater detail the biochemical pathways engaged in the striatum after PDE10A inhibition, we profiled striatal extracts from dosed animals for phosphorylation changes in a number of key neuronal proteins. These changes may explain behaviors, in particular those linked to positive symptom efficacy. The most robust phospho-change was the increase in phosphorylation of the AMPA-receptor subunit GluR1 at serine 845. Ser845 is a target for both PKA and PKG, which may explain its robust phosphorylation, although recently it has been reported that papaverine works primarily through the cAMP pathway, at least on the DARPP-32T34 site (Nishi et al., 2008). Papaverine caused a maximal increase in pGluR1S845 of 6.2-fold (54 mg/kg, 30 min). During the preparation of this article, a similar data set with papaverine was published that showed a 2.3-fold increase in pGluR1S845 (30 mg/kg, 15 min) (Nishi et al., 2008). In this same assay, we observed that the more potent and specific PDE10A inhibitor (MP-10) caused an even more robust elevation in pGluR1S845. Ser845 phosphorylation is well known to increase GluR1 trafficking to the plasma membrane and is critical for multiple forms of synaptic plasticity including long-term potentiation and long-term depression (Shepherd and Huganir, 2007; Citri and Malenka, 2008). In addition, it directly regulates activity of the channel by potentiating the peak amplitude of glutamate-gated currents and increases the probability of channel opening (Roche et al., 1996). To elucidate the consequences of this phosphorylation event, we analyzed the effects of MP-10 on the trafficking of AMPA receptors in rat striatal slices. It has been previously shown that papaverine increases pGluR1S845 levels in striatal slices from mice (Nishi et al., 2008). We initially confirmed that MP-10 could replicate this effect, and we noted that application of 10 μM MP-10 onto the slice caused a 2.1-fold increase in pGluR1S845. It is critical that we did not measure an increase in GluR1 at the cell surface after MP-10 treatment by using surface protein biotinylation,

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**Fig. 12.** Papaverine and MP-10 increase time spent in the social chamber in SASA. Papaverine (3–30 mg/kg i.p.) and MP-10 (0.03–0.3 mg/kg i.p.) were evaluated in the SASA assay in male BALB/cJ mice (13–15 per treatment group). Thirty minutes after papaverine or MP-10 administration, mice explored the arena with empty cylinders for 10 min, after which the cylinders were replaced by a new empty cylinder and one with a male C57BL/6J mouse. The test mouse spent another 10 min exploring. An increase in amount of time spent in the social chamber suggests an increased interest in social interaction. #, significantly more time spent in empty chamber relative to social chamber.

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# Different from social, * Different from vehicle
but we did measure a similar 2-fold increase in phosphorylation levels of GluR1 present at the cell surface. This suggests that, in the MSNs, PDE10A inhibition may increase the channel opening properties to increase channel activity rather than through altering receptor trafficking. This is currently being confirmed by electrophysiological means but may explain recent suggestions that PDE10A inhibition increases the responsiveness of MSNs to corticostriatal excitatory input (Threlfell et al., 2009).

We also measured the effects of PDE10A on a number of other well characterized phosphosites related to the glutamate synapse. Trends for a decrease in the phosphorylation of AMPA receptor subunits GluR2/3 on residues Ser880/Ser891 were observed. Ser880 phosphorylation is known to play key roles in receptor trafficking and in binding to various intracellular regulatory proteins, such as PICK-1 (protein interacting with C kinase 1) and GRIP (glutamate receptor-interacting protein) (Shepherd and Huganir, 2007). Decreases in pSer880 suggest a possible increase in the recycling of receptor back to the cell surface or decrease in internalization of receptor, which again may increase synaptic efficacy (Shepherd and Huganir, 2007). Certain doses of MP-10 resulted in phosphorylation of the NMDAR subunit NR2B at Tyr1472. This phosphorylation site plays an important role in receptor endocytosis as pY1472 inhibits binding to the clathrin adaptor protein AP-2 and thus inhibits endocytosis (Roche et al., 2001). Together these effects would point toward enhanced glutamate receptor activity and responsibility in MSNs. It is intriguing that there was a considerable overlap in the dose range of the PDE10A inhibitors, which resulted in these biochemical effects and showed efficacy in the psychoses models, in particular the increase in striatal pGluR1S845, suggesting that these biochemical events may play a role in the behavioral effects.

To expand the possible impact of PDE10A inhibition on the cognitive symptoms of schizophrenia, we tested the compounds in two models (NOR and SOR). The NOR and SOR models are thought to depend on the perirhinal cortex, and although there is no direct evidence of PDE10A expression in this region, there is clearly a small amount of protein diffusely expressed in the cortex (Seeger et al., 2003). In addition to inhibition of cortical PDE10A-localized enzyme, there are other explanations for how striatal inhibition could drive a cortical dependent behavior. It is clear that the striatum and prefrontal cortex (PFC) are highly interconnected through neuronal circuitry (Haber, 2003). In the rodent, this has been recently modeled by overexpression of D2-receptors in the striatum, which resulted in altered dopamine metabolism and D1-receptor signaling in the PFC, and caused deficits in PFC-dependent cognition tasks (Kel kondon et al., 2006). Thus, augmented striatal output after PDE10A inhibition could translate into modified cortical activity. It may be also possible to measure such connectivity at the level of neurotransmitters, whereby the release of cognition relevant neurotransmitters in the PFC may be influenced indirectly by the activity of ventral striatal MSNs expressing PDE10A (Brooks et al., 2007). In our studies, papaverine improved recognition memory and social odor memory in the NOR (rat) and SOR (mouse) assays, respectively. It is critical that the active doses in these two models overlap with doses as they appeared to be active in the antipsychotic models and showed activity in the biochemical assays described above. It is interesting that MP-10 was active in the SOR model but not active in the NOR model, although there was a strong trend toward enhanced memory at the lowest dose tested that just missed significance. The more pronounced effects of papaverine in the NOR model could be the result of the less selective nature of this compound, which also inhibits PDE4 activity. Rolipram, a selective PDE4 inhibitor, has demonstrated an ability to improve object recognition in both delay dependent and drug-induced memory deficit models (Rutten et al., 2006). In addition, procedural differences may explain this discrepancy. In the NOR model, the PDE10A inhibitor was dosed before training, whereas dosing occurred after training in SOR, thereby avoiding any drug-induced interference with object exploration. Intriguingly, we have run other PDE10A inhibitors through the NOR model and observed efficacy (Hage et al., manuscript in preparation). We will continue to explore the reasons for the lack of effect of MP-10 in NOR.

There is current controversy whether PDE10A inhibitors are active in models of sensorimotor gating. In a recent study, papaverine was shown to be inactive in a range of PPI assays, which focused on dopaminergic induced PPI deficits in the rat (Weber et al., 2009). In addition, TP-10 could not reverse the natural deficit in PPI in the C57BL/6J line or in CD-1 mice dosed with MK-801 (Schmidt et al., 2008). The fact that we could see both papaverine and MP-10 reversing a MK-801 deficit in Long-Evans rats could be explained by procedural differences in these experiments. Our data are consistent with unpublished meeting reports (T. Hage, S. Grauer, V. Pulito, R. Navarra, M. Kelly, R. Graf, B. Langen, J. Brennan, L. Jiang, U. Egerland, K. Marquis, M. Malamas, T. Comery, and N. Brandon, 2009) and are supportive of the possibility that PDE10A inhibition could improve filtering and processing of sensory information in schizophrenic patients, which might affect cognitive deficits and psychotic symptoms (Swerdlov and Geyer, 1998).

To probe negative symptoms, we ran the compounds in a model that evaluates social behaviors in rodents, known as the SASA model (Moy et al., 2004; Sankoorikal et al., 2006). Because social interaction in rodents can be impaired by NMDA antagonism and PDE10A inhibition normalizes NMDA antagonist effects in other models, we reasoned that these compounds might enhance social behaviors. Moreover, a recent report found increased social interaction in PDE10A2 knockout mice (Sano et al., 2008). In the SASA model, both PDE10A inhibitors increased the amount of time spent by the BALB/c mice on the social side of the test chamber compared with vehicle treatment. This intriguing finding supports further investigation of PDE10A inhibitors in other assays modeling negative symptom domains, such as NMDA antagonist-induced anhedonia. This is an active area of our current research. Together with the efficacy in antipsychotic and cognition models, these data also suggest that PDE10A inhibition has the potential to be a true broad-spectrum agent for the treatment of schizophrenia.

In conclusion, we have provided evidence that suggests that a PDE10A inhibitor could provide antipsychotic, procognitive, and negative symptom efficacy in the same dose range. In addition, we have further elucidated the molecular cascades downstream of this pharmacological inhibition and suggest that activation of the glutamatergic system through effects on AMPA and NMDAR subunits could play a crucial role in striatal-dependent behaviors. It will be important to
understand the molecular basis of this broad efficacy in light of these activated pathways in the striatum and also in other brain regions.

References


