Phosphodiesterase 9A Regulates Central cGMP and Modulates Responses to Cholinergic and Monoaminergic Perturbation In Vivo


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ABSTRACT

Cyclic nucleotides are critical regulators of synaptic plasticity and participate in requisite signaling cascades implicated across multiple neurotransmitter systems. Phosphodiesterase 9A (PDE9A) is a high-affinity, cGMP-specific enzyme widely expressed in the rodent central nervous system. In the current study, we observed neuronal staining with antibodies raised against PDE9A protein in human cortex, cerebellum, and subiculum. We have also developed several potent, selective, and brain-penetrant PDE9A inhibitors and used them to probe the function of PDE9A in vivo. Administration of these compounds to animals led to dose-dependent accumulation of cGMP in brain tissue and cerebrospinal fluid, producing a range of biological effects that implied functional significance for PDE9A-regulated cGMP in dopaminergic, cholinergic, and serotonergic neurotransmission and were consistent with the widespread distribution of PDE9A. In vivo effects of PDE9A inhibition included reversal of the respective disruptions of working memory by ketamine, episodic and spatial memory by scopolamine, and auditory gating by amphetamine, as well as potentiation of risperidone-induced improvements in sensorimotor gating and reversal of the stereotypic scratching response to the hallucinogenic 5-hydroxytryptamine 2A agonist mesclamine. The results suggested a role for PDE9A in the regulation of monoaminergic circuitry associated with sensory processing and memory. Thus, PDE9A activity regulates neuronal cGMP signaling downstream of multiple neurotransmitter systems, and inhibition of PDE9A may provide therapeutic benefits in psychiatric and neurodegenerative diseases promoted by the dysfunction of these diverse neurotransmitter systems.
deficits in the der Staay et al., 2008; Hutson et al., 2011). Age-related aerts et al., 2002a,b, 2004; Boess et al., 2004; Rutten et al., term potentiation (LTP) and performance in novel object inhibitors, have found that these compounds enhanced long-studies of inhibitors of cGMP hydrolysis, including PDE9A and Garthwaite, 2006; Taqatqeh et al., 2009). Consistent pocampal-mediated tasks (Weitzdoerfer et al., 2004; Hopper pocampal synaptic plasticity or cognitive performance in hip-ruptions of cGMP pathways that result in impaired hip-ponents of cGMP-generating pathways (i.e., NOS1, NO-GC1, cAMP or cGMP concentrations can affect any and all aspects environments depends on proper regulation of cyclic nucleo-tide second messengers. Nowhere is this more important than in the central nervous system, where alterations in cAMP or cGMP concentrations can affect any and all aspects of brain function, including sensory processing, learning, and memory (Kandel, 2001; Prickaerts et al., 2002a). Support for specific actions of cGMP in controlling neurotransmission comes from studies of genetically modified mice lacking compo-cGMP-generating pathways (i.e., NOS1, NO-GC1, and NO-GC2 knockout mice) and from pharmacological dis-ruptions of cGMP pathways that result in impaired hip-pocampal synaptic plasticity or cognitive performance in hip-pocampal-mediated tasks (Weitzdoerfer et al., 2004; Hopper and Garthwaite, 2006; Tagatqeh et al., 2009). Consistent with the hypothesis that elevation of cGMP enhances hip-pocampal plasticity, learning, and memory, pharmacological studies of inhibitors of cGMP hydrolysis, including PDE9A inhibitors, have found that these compounds enhanced long-term potentiation (LTP) and performance in novel object recognition tasks in a time-sensitive fashion and in deficit settings that include tryptophan depletion and aging (Prickaerts et al., 2002a,b, 2004; Boess et al., 2004; Rutten et al., 2007; Menniti et al., 2008; van Donkelaar et al., 2008; van der Staay et al., 2008; Hutson et al., 2011). Age-related deficits in the N-methyl-D-aspartic acid (NMDA)/nitric oxide (NO)εcGMP pathway (Vallebuona and Raiteri, 1995; Chali-moniuk and Strosznajder, 1998) may explain the beneficial effects of cGMP modulation on cognitive impairment during normal aging.

The NO-cGMP signaling cascade also regulates striatal function by virtue of an extensive network of connections between neuronal NO synapse-positive interneurons and the medium spiny neurons (MSNs) that serve as the prin-cipal output neurons of the nucleus. The neuronal NO syn-thase-positive interneurons use NO as a neurotransmitter that stimulates cGMP production within MSNs to subse-quently regulate membrane excitability and the response to excitatory glutamatergic inputs from the cortex (West and Grace, 2004). A principal function of the striatum is to inte-grate sensory input and response options from the cortex with information on salience as encoded by dopaminergic input from the midbrain. One might therefore predict that treatments affecting cGMP signaling within striatum have the potential to alter sensorimotor functions regulated by MSNs.

Here, we characterize brain-penetrant PDE9A inhibitors in preclinical biomarker and efficacy models that reflect a range of neurotransmitter systems and neural circuits. We demonstrate a role for PDE9A-mediated cGMP signaling in the modulation of tasks dependent on hippocampal cholinergic function and sensory gating. We propose that the inhibition of PDE9A may provide therapeutic benefit in patient populations suffering from cognitive deficits and exhibiting disrupted sensory processing such as Alzheimer’s disease (AD), schizophrenia, and/or Huntington’s disease.

Materials and Methods

Chemical Tools. (6-((3S,4S)-4-methyl-1-(pyrimidin-2-ylmethyl) pyrrolidin-3-yl)-1-(tetrahydro-2H-pyran-4-yl)-1H-pyrazolo[3,4-d][pyrimidin-4(5H)-one] (PF-4447943) and ((R)-6-(1-(3-phenoxyazetidin-1-yl)ethyl)-1-(tetrahydro-2H-pyran-4-yl)-1H-pyrazolo[3,4-d][pyrimidin-4(5H)-one] (PF-4449613) were synthesized at Pfizer Worldwide Research and Development, Groton, CT. In all experiments these com-pounds were dissolved in a vehicle consisting of 5% Cremophor (BASF Wyandotte, Wyandotte, MI), 5% dimethyl sulfoxide (DMSO), and 90% saline, except where indicated otherwise.

Antibodies. Anti-PDE9A polyclonal antibodies raised against a “C-terminal protein of 30 kDa” (exons 12–18) from the human PDE9A sequence were purchased from Scottish Biomedical (Glas-gow, UK) and used for immunohistochemistry. This antibody does not cross-react with PDE9A from nonhuman species.

Animals. Male CD rats, male Long-Evans rats, and male CD-1 mice from Charles River Breeding Laboratories (Kingston, NY), male Wistar rats from Shanghai Laboratory Animal Center (Shanghai, China), C57BL/6J mice from The Jackson Laboratory (Bar Harbor, ME), and male and female cynomolgus monkeys from Maccine Pte Ltd. (Singapore) served as subjects in these studies. All animals were housed under standard laboratory conditions on a 12-h light/dark cycle with lights on at 6:00 AM, except where indicated otherwise. Food and water were provided ad libitum except where indicated otherwise, and animals were acclimated to the vivarium for at least 5 to 7 days before experimentation. Animals were handled and cared for according to the Guide for the Care and Use of Laboratory Ani-mals (Institute of Laboratory Animal Resources, 1996), and all pro-cedures performed at Pfizer were approved by the Pfizer Insti-tutional Animal Care and Use Committee. All procedures performed at Maccine Pte Ltd. and HDB Biosciences (Shanghai, China) were approved by their Institutional Animal Care and Use Committees.

Human Brain Tissue Immunocytochemistry. Human post-mortem brain tissue from neurologically normal control cases (n = 6) was obtained from the Sun Health Research Institute (Sun City, AZ). Tissue procurement and acquisition complied with informed consent and local Institutional Review Board guidelines. Average postmor-tem delay interval was 2.8 ± 0.2 h. Both male and female cases were evaluated (six males and one female). All brain tissue slabs were fixed in paraformaldehyde for 24 to 48 h and stored in glycol-formi-lin solution at −20°C. Tissue was sampled from the superior frontal gyrus (cortex), hippocampus, and cerebellum. Blocks of tissue (ap-proximately 4 cm3) were either embedded in paraffin or sectioned on a sliding microtome at 40-μm thickness. Multiple sections were evaluated from individual cases in replicate experiments. Paraffin-embedded cerebellum was sectioned at 5 μm and treated with citra antigen retrieval, followed by 1:100 dilution of Scottish Biomedical antibody incubated for 2 h, and stained by using EnVision rabbit horseradish peroxidase (HRP) detection (rabbit EnVision HRP reagent; Dako North America, Inc., Carpinetra, CA). Immunoperox-o-dase staining of PDE9A in free-floating sections of hippocampus, superior frontal gyrus, and cerebellum was carried out by incubating sections overnight in the Scottish Biomedical antibody (1:2000), staining with EnVision HRP, and developing with 0.03% 3,3’-di-aminobenzidine (Sigma, St. Louis, MO) and 0.0075% H2O2. Immunoadsorption experiments were performed to assess antibody speci-ficity by using the PDE9A C-terminal peptide as described.
previously (Stephenson et al., 2009). Immunoadsorption studies were conducted in parallel with nonadsorbed antibody as well as immunoadsorption with an irrelevant peptide for E-selectin (data not shown).

**Enzyme Assays.** PDE enzyme assays were carried out as described previously (Verhoest et al., 2009). PDE1A-C, PDE2A, PDE3A/B, PDE4A-D, PDE7A/B, PDE8A/B, PDE9A, PDE10A, and PDE11 were generated from full-length recombinant clones. PDE5 was isolated from human platelets, and PDE6 was isolated from bovine retina as (Pentia et al., 2005). PDE activity was measured by using a scintillation proximity assay (SPA). The effects of PDE inhibitors were investigated by assaying a fixed amount of enzyme and varying inhibitor concentrations in the presence of substrate concentrations of 1/3 Kᵋₑ values for each enzyme, so that the IC₅₀ value approximated the Kᵋ value. Test compounds were dissolved in 100% DMSO and diluted to the required concentrations in 15% DMSO water. The enzyme stocks were all thawed slowly and diluted in assay buffer containing 50 mM Tris-HCl (pH 7.5 at room temperature) and 1.3 mM MgCl₂. In addition, the PDE1 assay buffers contained 2.8 mM CaCl₂. The PDE1C assay also required the addition of the activator calmodulin at a final assay concentration of 100 units/mL. Incubations were initiated by the addition of diluted enzyme to 384-well plates containing test drugs and radioligand (50 nM [³H]cGMP for PDE1, PDE2, PDE5, PDE6, PDE9, PDE10, and PDE11 and 20 nM [³H]cAMP for PDE3, PDE4, PDE7, and PDE8). The assays were incubated for 30 min at room temperature (60 min for PDE5 and PDE6). The reactions were stopped by the addition of phosphodiesterase SPA beads (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) at a final assay concentration of 0.2 mg/well. PDE9 required the extra addition of a high concentration (10 μM) of a potent PDE9 inhibitor before beads to stop the reaction completely. Activities of test compounds were assessed by measuring the amount of [⁳H]5'-GMP or [⁳H]5'-AMP produced from [³H]cGMP or [³H]cAMP radioligand, respectively. Levels of [⁳H]5'-GMP or [⁳H]5'-AMP bound to SPA beads were determined by paralax counting of the assay plates in a Microbeta Trilux Counter (PerkinElmer Life and Analytical Sciences, Waltham, MA) 10 h after bead addition. Non-specific binding was determined by radioligand binding in the presence of a saturating concentration of a potent PDE inhibitor. The IC₅₀ value of each test compound (concentration at which 50% inhibition of specific binding occurs) was calculated by nonlinear regression (curve fitting) of the concentration response.

**Mouse Brain cGMP Measurements.** Measurements of cGMP accumulation in brain tissue after drug administration were as described previously (Schmidt et al., 2008). CD-1 mice were euthanized by focused microwave irradiation of the brain 30 min after administration of vehicle (20% hydroxypropyl-β-cyclodextrin, aqueous) or various doses of PF-4447943 (n = 5 animals per group for all groups except the hippocampus vehicle group, which was n = 4). Regions of interest were isolated and homogenates in 0.5 N HCl were centrifuged. Supernatant concentrations of cyclic nucleotides were measured by using enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI). Tissue levels of phosphoCREB-like immunoreactivity (pCREB-LI) were determined by an enzyme-linked immunosorbent assay (BioSource International, Camarillo, CA). Data were analyzed by using a one-way analysis of variance (ANOVA) followed by Dunnett’s post hoc analysis to compare drug doses with vehicle.

**Rat CSF cGMP Sampling.** CSF analysis for cGMP was carried out by treating male CD rats (200–300 g) subcutaneously with vehicle or a PDE9 inhibitor (n = 5 animals per treatment group, except n = 6 for vehicle-treated group). Before CSF sampling, cannula syringes (1 cc) were prepared using PE50 tubing fitted with 22G needles. After CO₂ anesthesia (chamber with tank), the dorsal skull and cervical regions were exposed. CSF was withdrawn by using the assembled cannula to puncture the cisterna magna (5–9 mm caudal of lambda landmark). Average CSF collection for each rat was 20 to 60 μl; samples were excluded that showed signs of blood contamination from the puncture. Using an enzyme immunoassay kit from Cayman Chemical, concentrations of cGMP in diluted CSF were determined. Data were expressed as concentrations (nM) or percentage of average control value. Data were analyzed by using one-way ANOVA followed by Dunnett’s t tests for multiple comparisons.

**Nonhuman Primate CSF Sampling.** These procedures were carried out at Maccine Pte Ltd. Ten naive adult male (one) and female (nine) cynomolgus macaques (Macaca fascicularis) were surgically prepared, each with an indwelling cannula inserted into the cisterna magna and connected to a subcutaneous access port to permit CSF sampling. The animals were individually housed in an air-conditioned unit with a 12-h light/dark cycle (on 7:00 AM, off 7:00 PM). The temperature and relative humidity ranges recorded during the study were 22.0 to 23.0°C and 58 to 69%, respectively. The cages were cleaned at regular intervals throughout the study period to maintain hygiene. Animals were allowed a minimum 10-day recovery period during which the catheter was checked for patency. Animals were fasted overnight before dosing and were fed approximately 4 h after the start of dosing. To facilitate dosing, the animals were restrained in primate chairs during the entire procedure. On the day of dosing, PF-4449613 was administered subcutaneously with a dose volume of 0.5 ml/kg. NHPs (n = 5/dose) were dosed with either 0.3 or 1.0 mg/kg PF-4449613. Blood and CSF samples were obtained at day 1 (24 h; data not shown), predose (0 h), and 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 h relative to the start of dose administration. Whole blood samples of approximately 1 ml were collected from the femoral vein into EDTA-containing tubes on wet ice. Plasma was separated by centrifugation at 4000 rpm for 5 min at 4°C. CSF samples of approximately 300 μl were collected via the cisterna magna catheter by using a sterile needle inserted into the subcutaneous port. For each time point, approximately 100-μl aliquots were snap-frozen in liquid nitrogen and stored for drug exposure analysis. The rest of the samples were placed in a boiling water bath for 5 min, and then snap-frozen in liquid nitrogen. Samples were transferred to −80°C for storage before analysis.

**LC/MS/MS.** An LC/MS/MS assay for cGMP was used to gauge the effects of PF-4447943 in CSF samples after cross-validation with the Cayman chemical immunoassay. Instrumentation consisted of an Agilent 1100 HPLC pump equipped with a HTS PAL autosampler (LEAP Technologies, Carrboro, NC) and interfaced with a PE Sciex API 4000 triple quadrupole mass spectrometer running in negative ion mode and controlled by Analyst 1.4.2 software (Applied Biosystems, Foster City, CA). Chromatography was performed by gradient elution at 0.35 ml/min using an Atlantis dC18 5 μm, 50 × 2.1-mm column (Waters, Milford, MA). Mobile phase A was 0.25% acetic acid in water, and mobile phase B was methanol. After 0.1 min at the initial condition of 95% A, solvent B was increased from 5 to 25% over 0.9 min, maintained at 25% for 1.7 min, then increased to 100% (with flow rate at 0.5 ml/min) for 0.6 min to wash the column. Re-equilibration at initial conditions was then performed for 1.7 min before the next injection, giving a cycle time of 5 min. The autosampler and column were at room temperature. cGMP was detected in multiple reaction monitoring (MRM) mode using a stable-isotope internal standard of [¹³C₁₀,¹⁵N₅]-cGMP prepared as described previously (Zhang et al., 2009). Source conditions were: ionspray voltage −4.0 kV, ion source temperature 450°C, nebulizer gas 45 psi, turbo gas 40 psi, and curtain gas 30 psi. The dwell time for cGMP and the internal standard was 200 ms in each case. Ion transitions used were: cGMP, m/z 344.0 → 150.0; [¹³C₁₀,¹⁵N₅]-cGMP, m/z 358.9 → 160.0. Sensitivity was optimized by manipulating values of entrance potential, declustering potential, collision energy, and collision exit potential. Preferred values of entrance potential, declustering potential, collision energy, and collision exit potential were as follows: −10 V, −80 V, −35 eV, and −5 V, respectively.

Calibration standards were prepared in artificial CSF (Harvard Apparatus Inc., Holliston, MA). Quality-control (QC) samples were prepared in artificial CSF and commercially available CSF (Biorelevant, Westbury, NY). QC CS samples or samples of CSF col-
lected in NHP experiments were treated with sufficient 100 mM 3-isobutyl-1-methylxanthine dissolved in DMSO/methanol (50/50, v/v) to give a final 3-isobutyl-1-methylxanthine concentration of 1 mM.

To initiate the assay, 120 µl of sample (NHP CSF, calibration standard, or QC sample) was pipetted into a 1.2-ml polypropylene tube containing 12 µl of stable-labeled internal standard working solution at 60 ng/ml \((^{13}C_{10}^{15}N_{5}\text{-cGMP})\) in 0.25% acetic acid; 0.25% acetic acid (12 µl) was added instead to tubes not receiving internal standard. Samples were vortexed, and 100 µl of each sample was transferred to the wells of a Whatman (Clifton, NJ) Fast Flow protein precipitation filter plate containing 300 µl of acetone-trile and vortexed for 1 min. The supernatant was collected by vacuum filtration into a 96-well, 1.2-ml polypropylene collection plate. The resulting supernatant was evaporated to dryness under a stream of nitrogen in a plate evaporator at 37°C. Samples were reconstituted with 100 µl of 0.25% acetic acid in water and vortexed for 2 min. Twenty-microliter aliquots of each sample were subsequently analyzed by LC-MS/MS. PE Sciex Analyst software (v. 1.4.2) was used to acquire mass data and determine the peak area ratio (PAR) for cGMP and internal standard. Calibration curves were constructed by using the PARs for artificial CSF calibration samples by applying a 1/concentration\(^2\) weighted linear regression model. All sample concentrations were calculated from their PARs against their respective calibration lines. The assay range for cGMP was 0.1 to 10 ng/ml with precision and accuracy of ≤15%.

CSF cGMP data were analyzed by using Prism 5 software (GraphPad Software Inc., San Diego, CA). A one-way ANOVA with repeated measures was performed, followed by Dunnett's t tests for comparison of treatment groups to the baseline (time = 0 h) CSF cGMP levels.

**Ketamine-Disrupted Radial Arm Maze Task.** The purpose of these experiments was to evaluate the ability of PF-4447943 to antagonize the working memory deficits induced by administration of the NMDA antagonist ketamine. The procedure was adapted from that used in Ward et al. (1990). Male Long-Evans rats served as subjects in these experiments. Body weights ranged between 200 and 225 g upon arrival and between 300 and 500 g during the studies. The eight-arm radial maze (Pathfinder Maze System; Lafayette Instrument Co., Lafayette, IN) consisted of eight equidistantly spaced clear Plexiglas arms (60.9 × 9.8 cm) radiating from a circular central arena (internal diameter 33.9 cm). The maze was mounted on a platform that was attached to a vertical stand and had an overall height of 88.9 cm. At the end of each arm there was a food cup, the contents of which were not visible from the central platform. The doors to the arms of the maze were not used and were always kept open to simulate the “free-foraging” that rodents encounter in the wild. The task required that the animals enter each arm to retrieve the food pellets and use spatial cues in the room to remember which arms of the maze they had previously entered. Several posters mounted on the walls of the experimental room, as well as the permanent items in the room (e.g., sink and experimenter), served as the visual cues and remained in a constant location throughout the experiments. Training and testing were performed under normal lighting conditions in a procedural room located in the vivarium.

To provide motivation to perform the working memory task animals were food-restricted to achieve 80 to 85% of their free-feeding weights. Beginning 7 days before maze habituation animals were weighed weekly and were given 25 to 30 g of rat chow pellets per day with adjustments made as necessary. On the third and fourth days of food restriction, the animals were fed an equivalent weight (approximately 25–30 g) of the vanilla-flavored Noyes pellets that served as reinforcements on the maze, to reduce any potential neophobia when exposed to the pellets during training. During all phases of training/testing the maze was thoroughly cleaned with disposable wipes premoistened with a 70% isopropyl alcohol solution after each animal was removed from the maze.

**Maze Habituation.** On the first day of habituation, reinforcements were placed near the entrance, at the midpoint, and in the food cup at the end of each arm. Each animal was placed on the maze and allowed to explore and consume the reinforcement pellets for a period of 5 min, or until all pellets were consumed. On the second day of habituation, the pellets were placed at the midpoint and in the food cup at the end of each arm. Again, the animals were allowed to explore the maze until all pellets were consumed or until 5 min had elapsed. Training began on the third day of exposure to the maze.

**Maze Training.** During training, one reinforcement pellet was placed in the food cup at the end of each arm. Animals were placed individually on the maze facing away from the experimenter, facing the same arm at the start of each trial. The timer was started, and each arm entry (1–8) was recorded by the experimenter in sequence. An entry was defined as all four paws entering an arm. The animals were allowed to choose arms until all eight arms were entered and pellets were consumed, 30 choices were made, or 5 min had elapsed. Entry into an arm previously chosen was counted as an error. If an animal failed to choose all eight arms in 5 min, the arms not chosen were also counted as errors. Animals were trained once a day 5 days a week (Monday–Friday), and the training criterion was defined as two or fewer errors on 2 consecutive training days, which was typically achieved after 15 days of training (i.e., 3 weeks).

**Maze Testing.** Once the training criterion was met by all subjects (approximately 15 days of training), drug testing was initiated. Testing began with administration of drug treatments followed by placement of the animals individually on the maze after the predetermined drug pretreatment times. Before testing, PF-4447943 was tested in locomotor activity studies to identify doses that may produce locomotor stimulation or sedation. PF-4447943 or vehicle was administered 60 min before testing. The AMPA-positive allosteric modulator (PAL), 4-[4-(1-methyl-2-[methylethyl]-sulfonyl)amino]ethyl)phenylbenzene carboxonitrile (LY451646), was previously demonstrated to produce robust and reliable activity in this test and was therefore included as a positive control in this experiment. LY451646 was dissolved in an aqueous 10% Cremophor EL vehicle and was also administered 60 min before testing. The positive control for cGMP was a 10 mg/kg dose of ketamine diluted from a commercially available prepared stock of 100 mg/ml for a 10 mg/kg dose with saline as the vehicle and was administered 30 min before testing (30 min after PF-4447943). The route of administration for all drugs was subcutaneous, and the dosing volume was 1 ml/kg.

Thus, the experimental design consisted of the following treatment groups: 1) vehicle + vehicle; 2) vehicle + ketamine (10.0 mg/kg); 3) LY451646 (0.032–10.0 mg/kg) + ketamine. After drug treatments, maze testing began. During testing, one reinforcement pellet was placed in the food cup at the end of each arm. Animals were placed on the maze facing away from the experimenter, facing the same arm at the start of each trial. The timer was started, and each arm entry (1–8) was recorded by an experimenter who was blinded to treatment. An entry was defined as all four paws entering an arm. The animals were allowed to choose arms until all eight arms were entered and pellets were consumed, 30 choices were made, or 5 min had elapsed. Entry into an arm previously chosen was counted as an error. If an animal failed to choose all eight arms in 5 min, the arms not chosen were also counted as errors. On Mondays and Thursdays all animals were tested to identify those qualified for drug testing on Tuesdays and Fridays. No testing was performed on Wednesdays. Animals that committed two or fewer errors within the allotted 5 min on qualifying days were randomly assigned to treatment groups and subjected to drug testing. After each qualifying day animals to be tested were again randomly assigned to treatment groups and tested the next day. This procedure was repeated until the number for each group equaled at least nine, which typically took between 3 and 4 test days per experiment.

Dependent measures included number of errors, time (s) to completion, percentage of correct choices, and number of choices to the first error. Data were analyzed by using a one-way ANOVA followed
by Dunnett’s t tests for comparison of treatment groups with the control group (vehicle + ketamine group).

**Auditory Gating.** Experiments were performed as described previously (Krause et al., 2003; Hajès et al., 2005). In brief, male CD rats (250–300 g) were anesthetized with chloral hydrate (400 mg/kg i.p.). The femoral vein was cannulated for administration of additional anesthetic and test agents. Anesthetized rats were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA), and unilateral craniotomies were performed above the level of the hippocampus. Body temperatures of the rats were maintained at 37°C. Field potentials were recorded from the CA3 region of the left hippocampus, 3.8 mm ventral, 3.5 mm posterior, and 3.9 mm lateral from bregma (Paxinos and Watson, 1986), using a monopolar stainless-steel macroelectrode (Rhodes Medical Instruments, Woodland Hills, CA). Data were digitized and stored by using the Spike2 software package (Cambridge Electronic Design, Cambridge, UK). Auditory stimulation consisted of two consecutive tone bursts, 10 ms in duration, at a frequency of 5 kHz, delivered through hollow ear bars. Delay between the first “conditioning stimulus” and second “test stimulus” was 0.5 s, and the time interval between tone pairs was 10 s. Auditory evoked potentials (AEPs) were determined by measuring the potential difference between the positive and negative deflections 20 and 40 ms after stimulation (P20 and N40), respectively. For quantification, 50 sweeps were averaged, amplitudes of AEPs were determined, and the level of sensory gating was expressed as percentage of gating: (1 – test amplitude/conditioning amplitude) × 100. d-Amphetamine sulfate (1 mg/kg i.v.) was administered to disrupt sensory gating. PF-4447943, PF-4449613 (n = 5 per treatment group), or the vehicle (n = 6) was applied subcutaneously, and auditory gating measurements were started 5 min after compound or vehicle administration; efficacy was calculated as the percentage of reversal of the amphetamine-induced gating deficit.

Data were analyzed by using two-tailed paired Student’s t tests.

**Novel Object Recognition Disrupted with Scopolamine.** Novel object recognition is a task that measures nonspatial episodic memory using a rat’s instinct to explore novel objects. The method used was a modification of the procedure developed by Ennaceur and Delacour (1988). A 2-day version of the procedure was used in the current study. On day 1, male CD rats (200–260 g) were placed in an empty test chamber in a dimly lit room and allowed to explore for 3 min. On day 2, PF-4447943 (0.32, 1.0, or 3.2 mg/kg s.c.) or vehicle (2% DMSO dissolved in 20% aqueous hydroxypropyl-β-cyclodextrin) was administered 60 min before testing (n = 5–12 animals per treatment group), followed by scopolamine (0.2 mg/kg s.c.) or vehicle (saline) 30 min later (thus 30 min before testing). The rats were each replaced into the chamber, now containing two identical objects located 2 inches from the chamber sides, and again allowed to explore for 3 min. The amount of time spent exploring each object was measured by using automated videotracking (Cleversys, Reston, VA). Rats with exploration of objects totaling less than 10 s were excluded from further testing. After a delay of 2 h, the rats were again returned to the test chamber, this time containing one object from the exposure trial (familiar) and one new object (novel). A relative increase in the amount of time spent exploring the novel object compared with the familiar object was considered to reflect retention of the memory for the familiar object.

**Morris Water Maze Disrupted with Scopolamine.** These experiments were carried out at HDB Biosciences in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal facility. For each experiment 95 male Wistar rats (160–180 g at arrival) were prescreened, and 60 rats were selected based on that screening to provide five treatment groups with 12 each. While the rats were housed, food and water were offered ad libitum. Thirty minutes before testing on days 1 to 4, rats were injected with scopolamine (0.32 mg/kg s.c. in saline). In addition, rats were pretreated with PF-4447943 (3.2 or 10 mg/kg s.c.) 30 min before scopolamine and pretreated with donepezil (1 mg/kg s.c.) 15 min before scopolamine or vehicle. The total pretreatment time was 60 min for PF-4447943 and 45 min for donepezil. No drugs were given on either the prescreening or the probe trial days.

**Day One Prescreening.** A circular tank was filled with water (24 ± 1°C) to a height of 30 to 35 cm with a visible escape platform (12 cm2) approximately 1 cm above the surface of the water. Each rat was placed in the water facing the pool wall from one of four quadrants (north, south, east, and west) and allowed to swim a maximum of 60 s to find the visible platform. When successful, the rat was allowed a 30-s rest period on the platform. If unsuccessful within the allotted time period, the rat was given a score of 60 s and then hand-guided to the platform and allowed a 30-s rest period. A total of four trials were conducted for each rat, one from each release point (north, west, east, and south). No drugs were given during this prescreening trial. Rats were selected for Morris water maze (MWM) testing based on the criterion of finding the visible platform twice out of four trials within the 60-s cutoff time.

**Place Acquisition with Hidden Platform.** The circular tank was filled with water mixed with white nontoxic paint to reach a level of 1 cm above the platform. Animals were gently placed in the tank facing the pool wall, released from one of four starting points, and allowed to swim in the pool for a maximum of 90 s per trial. When the rat reached the platform, it was allowed to remain on it for 30 s. Animals that failed to find and escape onto the hidden platform within 90 s were gently guided to it by hand. The animals were allowed to remain on the platform for 30 s before being returned to a holding cage, for 1 to 2 min before the next trial. Animals received a total of four trials per day for 4 days. The location of the platform remained the same for all tests except probe trials. Latency or the time elapsed and distance traveled to reach the hidden platform were recorded by using a video monitoring system, and swim speed was calculated. For the hidden platform test, the latencies and distances traveled across the four trials for each rat were averaged for each day. These results were then analyzed across the 4 days of testing. Two-way repeated-measures ANOVAs followed by Dunnett’s t tests for multiple comparisons were used to compare performance between groups for the acquisition trials, and one-way ANOVA was used to compare groups for the probe trial. This experiment was run twice with identical methods.

**Mescaline-Induced Scratching.** CD-1 mice (30–40 g) were administered vehicle (phosphate-buffered saline) or PF-4447943 (1, 3.2, or 10 mg/kg s.c.) 20 min before mescaline (Alltech-Applied Science Labs, State College, PA), 30 mg/kg, p.o. After 15 min, mice were placed in glass beakers for videotaping of the test session, and scoring was performed by blind raters the next day. Animals were viewed for 5 s every 30 s for a total duration of 12 min, yielding a total of 24 observations. The number of animals in each group ranged from four to eight. The occurrence of scratching was scored as present (+) or absent (−) during each 5-s observation period. Data were plotted as percentage of time scratching and analyzed by using one-way ANOVA followed by Dunnett’s t tests for multiple comparisons.

**Prepulse Inhibition of the Acoustic Startle Response.** The ability of PF-4447943 to improve prepulse inhibition (PPI) of the acoustic startle response was evaluated in C57BL6/J mice. These animals have an inherently low level of PPI (Paylor and Crawley, 1997), and it has been shown that antipsychotic-induced increases in PPI are readily observed in mice that exhibit low levels of PPI (Ouagazzal et al., 2001). Compounds were administered subcutaneously, and testing was initiated 30 min after drug administration. To evaluate the potential interaction of PF-4447943 with an atypical antipsychotic, a subthreshold dose of risperidone (0.32 mg/kg s.c.) was administered to mice concurrently with PF-4447943. To evaluate the ability of PF-4447943 to antagonize PPI deficits produced by the NMDA antagonist (+)-5-methyl-10,11-dihydro-5f-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801), relatively normal-gating CD-1 mice were used. The minimal dose of MK-801 that reliably results in a significant disruption of PPI (0.178 mg/kg s.c.; data not shown) was administered to mice concurrently with various doses of...
PF-4447943, and testing was initiated 30 min later. All experiments used eight SR-LAB acoustic startle chambers (San Diego Instruments, San Diego, CA), each consisting of a clear, nonrestrictive Plexiglas cylinder mounted on a platform and housed in ventilated, sound-attenuating external chambers. Placement of mice inside the cylinders allowed the whole-body startle responses induced by acoustic stimuli to be measured via the transduction of movement into analog signals by a piezoelectric unit attached to the platform. A loudspeaker inside each chamber provided continuous background noise and the various acoustic stimuli. Test sessions consisted of placement of individual animals into the startle chambers and initiation of the background noise (68 dB). After a 5-min acclimation period, each subject was presented with 37 trials in quasi-random order and with randomly varied intertrial intervals (10, 15, or 20 s). The trials consisted of the following types: three no-stimulus trials (background only), seven startle trials (pulse alone; 40-ms duration; 120 dB), and prepulse-alone trials (20-ms duration; 3, 7, or 9 dB above background; three at each intensity) presented alone or 80 ms before the startle stimulus (prepulse + pulse; 40-ms duration, 120 dB; six at each prepulse intensity). PPI was calculated as a percentage score for each prepulse trial type, and percentage of PPI was calculated by using the formula: 100 − [(prepulse + pulse)/pulse] × 100.

Startle amplitude was calculated as the average response to the seven pulse-alone trials, excluding the first one. Means were statistically compared by using ANOVA followed by Dunnett’s t tests for multiple comparisons. Data from the no-stimulus and the prepulse-alone trials were not included in the analyses because, as expected, they elicited only negligible startle responses.

**Locomotor Activity Studies in Rats.** Spontaneous locomotor activity was measured after administration of various doses of PF-4449743 to evaluate the effects of PDE9A inhibition on spontaneous locomotion in a novel environment, aid the interpretation of the effects of compounds on hyperactivity induced by the psychomotor stimulants, and aid in dose selection for the other behavioral studies. To evaluate the effects of PDE9A inhibition in assays predictive of antipsychotic-like activity, PF-4449743 was tested for its ability to antagonize the hyperactivity produced by the psychomotor stimulants D-amphetamine and MK-801.

Locomotor activity was measured by using 48 custom-made clear Plexiglas automated activity chambers (30 × 30 cm), which were housed in sound-attenuating cabinets and equipped with photocells and a metal touch plate positioned 7 cm from the floor on all four walls of the chambers. A single 15-W bulb in each cabinet was controlled by a 24-h timer that allowed the behavioral chambers to be maintained on a 4:00 AM/4:00 PM light/dark cycle. Horizontal activity was measured as crossovers from one quadrant of the cage floor to another, whereas vertical locomotor activity (rears) was measured as the number of times an animal stood up on its hind legs against the walls of the chamber, making contact with the metal touch plate. Data were recorded, stored, and analyzed by a computer equipped with LabVIEW software (National Instruments, Austin, TX).

**Spontaneous Locomotor Activity.** To evaluate the effects of PDE9A inhibition on SLA in rats, various doses of PF-4447943 (0.32–10 mg/kg) or its vehicle were administered subcutaneously, and rats were immediately placed into novel activity chambers for overnight habituation to the activity chambers. The next morning each animal was treated with vehicle or various doses of PF-4449743 and immediately returned to the test chambers.

Sixty minutes later, subjects were removed from the test chambers and treated with vehicle, D-amphetamine, or the NMDA antagonist MK-801 (0.178 mg/kg s.c.) and immediately returned to the test chamber for the measurement of locomotor activity over a 3-h test period. Treatment groups were compared with ANOVA followed by Dunnett’s t tests for multiple comparisons.

**Conditioned Avoidance Responding.** Male C57BL/6J mice (30 g at testing) were housed in group cages, four per cage. The conditioned avoidance shuttle chambers consisted of eight individual Plexiglas chambers (Coulbourn Instruments, Allentown PA) each divided by a guillotine door into two compartments, enclosed in sound-attenuating cabinets. The Plexiglas chambers were fitted with metal grid floors, equipped with scrambled/constant current shock generators. Mice were trained to avoid the onset of foot shock (0.6 mA, preceded for 5 s by the activation of house lights, cue lights, and the opening of the guillotine door) by moving to the opposite side of the chamber. Thirty trials were completed per daily session, and the number of avoidance (maximum 30), escapes (maximum 30), escape failures (maximum 30), latency to avoid (maximum 5 s), latency to escape (maximum 10 s), and adaptation crossovers (number of crossovers for a 5-min period before the onset of trials in darkened chambers) were recorded by the computer program WINLINC (Coulbourn Instruments). Intertial intervals were 30 s with the guillotine door closed. Drug treatment began when mice had reached a minimum criterion of 80% avoidances for a session. Testing was performed during the lights-on period of the light/dark cycle, typically between 8:00 AM and noon. Saline vehicle treatment for qualification testing was performed 1 day before drug testing, and statistical analysis compared each drug treatment group versus the test day vehicle treatment group by using one-tailed Student’s t tests. The mean avoidance values represented seven to eight animals per dose group.

**Results**

**PDE9A Is Expressed in Neurons.** To identify cell types expressing PDE9A protein within the central nervous system, several antibodies to PDE9A were evaluated. This resulted in the identification of an antibody capable of producing specific immunohistochemical staining in human brain tissues. This antibody was raised against the human PDE9A sequence and showed no specific immunoreactivity in rodent tissues, suggesting a lack of interspecies cross-reactivity. Nonetheless, the brain regions that exhibited specific PDE9A immunoreactivity were consistent with the regions and cell types previously shown to exhibit PDE9A mRNA expression in rats (Andreeva et al., 2001), including pyramidal cells located in cortex and subiculum and Purkinje cells of the cerebellum (Fig. 1). At the cellular level, PDE9A protein was found primarily in neuronal cell bodies and primary dendrites. No specific staining was observed in glial cells or in sections incubated with PDE9A antibody immunoadsorbed with immunizing peptides (Supplemental Fig. 1). The pattern of PDE9A immunostaining was not altered by preadsorption of the anti-PDE9A antibody with an irrelevant E-selectin peptide.

**Pharmacological Inhibition of PDE9A.** PF-4447943 and PF-4449613 are potent inhibitors of human recombinant PDE9A (IC50 values = 12 and 24 nM, respectively) with >78- and 35-fold selectivity, respectively, over other PDE family members (Fig. 2). X-ray crystallography indicated that both compounds occlude the binding site for cGMP, indicating that they are competitive inhibitors (data not shown). PF-4447943 was more than 1000-fold selective for PDE9A over most of the other 79 non-PDE targets investigated by using a commercial in vitro selectivity panel (Cerep, Le Bois, L’Èveque,.....
France), with the exception of the ML2 (MT3) melatonin receptor ($K_i = 3800$ nM), whereas PF-4449613 was more than 1000-fold selective for PDE9A over most of the other 79 non-PDE targets investigated by using the CEREP panel, with the exception of cytochrome P450 2C19 ($IC_{50} = 1600$ nM), dopamine transporter ($K_i = 110$ nM), $\mu$-opioid receptor ($K_i = 3500$ nM), and sodium channel binding site 2 ($K_i = 470$ nM) (data provided in Supplemental Table 1). PF-4447943 and PF-4449613 exhibited good brain penetration with mouse brain/plasma ratios of 0.39 and 0.8, respectively. The potent, selective, and brain-penetrant properties of these compounds made them well suited for use probing the physiological role of PDE9A in the brain.

**Inhibition of PDE9A Elevates the Level of Central cGMP.** Administration of PF-4447943 significantly increased tissue concentrations of cGMP in the striatum and frontal cortex of CD-1 mice (Fig. 3), with more modest increases that did not reach statistical significance observed in hippocampus and cerebellum. No corresponding change occurred in cAMP or phosphoCREB (data not shown). Administration of either PF-4447943 or PF-4449613 to rats gave dose- and time-dependent increases in CSF concentrations of cGMP (Fig. 4A). Likewise, in NHPs, PF-4449613 administration resulted in significant increases in CSF cGMP from baseline (time 0) levels of $4.13 \pm 0.60$ and $4.01 \pm 1.32$ nM (mean $\pm$ S.E.M.; $n = 5$) for 0.3 and 1.0 mg/kg treatment groups, respectively (Fig. 4B). These data demonstrate that changes in CSF cGMP can serve as a translatable biomarker for clinical studies.

The widespread distribution of PDE9A and global elevation in central cGMP engendered by PDE9A inhibition suggested that interaction might occur with multiple neurotransmitter systems. We therefore evaluated the behavioral response to PDE9A inhibition in a number of animal models, using pharmacological challenges to probe the activity of specific neurotransmitter systems.

**Inhibition of PDE9A Improves Ketamine-Induced Deficits in Working Memory.** The NMDA antagonist ket-
amine produces schizophrenia-like symptoms and cognitive impairment, including working memory deficits, in humans (Javitt, 2007). Because NMDA receptor antagonists are commonly used to model the disrupted glutamate neurotransmission hypothesized to underlie the cognitive deficits in schizophrenia and have been shown to disrupt spatial working memory in rodents, the ability of PF-4447943 to antagonize the disruptive effects of ketamine on spatial working memory was tested in rats. Systemic administration of PF-4447943 dose-dependently reduced working memory errors produced by ketamine on the maze (Fig. 5) at doses of 0.32 and 1.0 mg/kg s.c., with only the 1.0 mg/kg dose achieving statistical significance, and higher doses (3.2 and 10.0 mg/kg) being less effective. This resulted in a U-shaped dose-response curve, which was similar to that obtained in studies with the positive control compound LY451646 (McGinnis et al., 2010), a positive allosteric modulator of the AMPA receptor. The effect obtained at the active dose of PF-4447943 (1.0 mg/kg) was comparable in magnitude to that produced by the positive control and reduced the number of errors from a mean of 7.33 in the vehicle/ketamine group to a mean of 3.7 in the PF-4447943-treated group.

Inhibition of PDE9A Improves Scopolamine-Induced Deficits in Episodic Memory and Spatial Memory. A novel object recognition memory task with a cholinergic deficit was used to evaluate the role of PDE9A in the cholinergic regulation of episodic memory. In this study, cholinergic function was disrupted by pretreatment of rats with the muscarinic cholinergic antagonist scopolamine 30 min before exposure to two identical objects in an open-field arena. Vehicle or PF-4447943 was administered at 0.32, 1.0, or 3.2 mg/kg 30 min before scopolamine (i.e., 60 min before initial object presentation). During the subsequent presentation, one of the original objects (familiar) was replaced with a new object (novel). Vehicle-pretreated animals spent relatively more time exploring the novel object, an effect disrupted by treatment with scopolamine. However, scopolamine-treated animals also receiving the 1.0 or 3.2 mg/kg dose of the PDE9A inhibitor explored the novel object significantly longer (Fig. 6), suggesting enhanced memory for the familiar object and a reversal of the scopolamine-induced memory deficit by PF-4447943.

To further evaluate the consequences of PDE9A inhibition within a context of subchronic dosing, the effects of PDE9A inhibition on scopolamine-disrupted goal acquisition was explored by using the rat MWM test. Because PF-4447943 was shown to be rapidly cleared in rats (t_1/2 = 1.5 h) the use of higher doses than those used in acute assays was used to maintain enzyme inhibition for longer periods of time. On each of 4 consecutive days of testing, rats were injected with scopolamine (0.32 mg/kg s.c. in saline) preceded by either a single administration of vehicle, PF-4447943 (30 min before testing; 3.2 or 10 mg/kg s.c.), or donepezil (15 min before testing; 1 mg/kg s.c.). Repeated training in the MWM resulted in rapid acquisition of memory for the location of the submerged platform, as evidenced by the sequentially shorter latencies to reach it. Pretreatment of animals with scopolamine disrupted the memory of platform location and prevented any improvement in the latency or length of path to escape the water maze on trials carried out on subsequent test days. These effects were partially reversed by daily administration of the acetylcholinesterase inhibitor donepezil.
Pretreatment with either dose of PF-4447943 significantly reduced the effect of scopolamine such that animals improved their escape performance on each of the subsequent days of testing (Fig. 7), reaching a statistically significant benefit after 4 days of dosing, equivalent to that seen with donepezil.

Inhibition of PDE9A Restores Amphetamine-Induced Deficits in Auditory Gating. Recordings of field potentials from the hippocampal CA3 region of anesthetized rats were used to monitor AEPs that demonstrated auditory gating (Fig. 8) consistent with previous observations (Krause et al., 2003; Hajós et al., 2005; Schmidt et al., 2008). Auditory gating was then disrupted by systemic administration of d-amphetamine (1 mg/kg i.v.), as indicated by a significant decrease in the ratio of the test response to the conditioning response. Acute administration of PDE9A inhibitors reversed the effect of amphetamine and restored auditory gating (Fig. 8). The minimum effective dose of PF-4447943 was 0.32 mg/kg s.c. (\(n = 5\) animals/group). The free plasma levels of PF-4447943 associated with the minimum effective doses in the auditory gating model were 180 to 503 nM, whereas free levels in the brain (and CSF) were estimated to be 20% of the free plasma levels. Reversal of amphetamine-induced deficits in auditory gating was also observed with PF-4449613 (1 mg/kg s.c.), whereas administration of vehicle (1

Fig. 4. PDE9A inhibition increases CSF cGMP levels in rat and NHPs. A, dose-dependent elevation of cGMP in rat CSF 1 h after subcutaneous administration of PF-4447943 (●) or PF-4449613 (○) (\(n = 5–6\) animals per treatment group). Open symbols represent concentrations of cGMP in vehicle-treated CSF samples from corresponding drug-treated samples (filled symbols). **, \(p < 0.01\) drug versus control. B, changes in CSF cGMP in nonhuman primates as a function of time after the administration of PF-4449613 are plotted on the left axis (●, 0.3 mg/kg s.c.; ○, 1.0 mg/kg s.c.) for \(n = 5\) animals per group. Corresponding drug levels of PF-4449613 in CSF compartment are plotted on the right axis (●, ○). **, \(p < 0.05\); ***, \(p < 0.001\) for 0.3 mg/kg treatment group, #, \(p < 0.05\); ##, \(p < 0.01\); ###, \(p < 0.001\) for 1.0 mg/kg treatment group.

Fig. 5. Inhibition of PDE9 significantly attenuated ketamine-induced working memory errors on the radial arm maze. Rats trained in a working memory version of the eight-arm radial maze were administered ketamine (10 mg/kg s.c.) 30 min after treatment with vehicle (Veh) or various doses of PF-4447943 (0.032, 0.1, 0.32, 1.0, 3.2, or 10.0 mg/kg) and tested on the maze 30 min later (\(n = 10–20\) animals per treatment group). Ketamine significantly increased the mean number of errors made on the maze. Pretreatment with PF-4447943 dose-dependently reduced working memory errors produced by ketamine on the maze at doses of 0.32 and 10.0 mg/kg s.c., with only the 1.0 mg/kg dose achieving statistical significance and higher doses (3.2 and 10.0 mg/kg) showing decreased efficacy. The AMPA-positive allosteric modulator LY451646 (LY646) showed similar effects to the 1.0 mg/kg dose of PF-4447943 (**, \(p < 0.01\)). Other measures were unaffected (data not shown).

Fig. 6. Inhibition of PDE9A improves scopolamine-induced deficits in novel object recognition. PF-4447943 (0.32, 1.0, or 3.2 mg/kg s.c.) or vehicle was administered 60 min before exposure to two identical objects, followed by scopolamine (0.2 mg/kg s.c.) or saline vehicle 30 min later (\(n = 8–12\) animals per treatment group). Time spent evaluating two identical objects was evaluated 30 min after scopolamine treatment. After a delay of 2 h, the rats were again returned to the test chamber, this time containing one object from the exposure trial (familiar) and one new object (novel). The relative increase in the amount of time spent exploring the novel object compared with the familiar object was measured in seconds. Animals that received vehicle (0) in the absence of scopolamine spent significantly more time investigating the novel object compared with the familiar object was measured in seconds. Animals that received vehicle (0) in the presence of scopolamine spent an equivalent amount of time exploring the novel and familiar objects, whereas pretreatment with 1.0 or 3.2 mg/kg s.c. of PF-4447943 significantly reversed the scopolamine-induced deficit.

Fig. 7. Pretreatment with either dose of PF-4447943 significantly reduced the effect of scopolamine such that animals improved their escape performance on each of the subsequent days of testing (Fig. 7), reaching a statistically significant benefit after 4 days of dosing, equivalent to that seen with donepezil.

Inhibition of PDE9A Restores Amphetamine-Induced Deficits in Auditory Gating. Recordings of field potentials from the hippocampal CA3 region of anesthetized rats were used to monitor AEPs that demonstrated auditory gating (Fig. 8) consistent with previous observations (Krause et al., 2003; Hajós et al., 2005; Schmidt et al., 2008). Auditory gating was then disrupted by systemic administration of d-amphetamine (1 mg/kg i.v.), as indicated by a significant decrease in the ratio of the test response to the conditioning response. Acute administration of PDE9A inhibitors reversed the effect of amphetamine and restored auditory gating (Fig. 8). The minimum effective dose of PF-4447943 was 0.32 mg/kg s.c. (\(n = 5\) animals/group). The free plasma levels of PF-4447943 associated with the minimum effective doses in the auditory gating model were 180 to 503 nM, whereas free levels in the brain (and CSF) were estimated to be 20% of the free plasma levels. Reversal of amphetamine-induced deficits in auditory gating was also observed with PF-4449613 (1 mg/kg s.c.), whereas administration of vehicle (1
Inhibition of PDE9A improves scopolamine-induced deficits in Morris water maze. Rats were pretreated with vehicle or PF-4447943 (3.2 or 10 mg/kg s.c.) 30 min before, or donepezil (1.0 mg/kg) for 15 min, before treatment with vehicle or scopolamine (0.32 mg/kg s.c. in saline) on days 1 to 4 (D1-D4) of water maze testing. The total pretreatment time was 60 min for PF-4447943 and 45 min for donepezil. Baseline performance of all animals on the first trial of day 1 is plotted as T1. The average path length of the escape platform from four trials per day on each day of testing is plotted as D1 to D4. Vehicle/scopolamine (●) treatment prevented learning the position of the submerged platform over the course of 4 days as evidenced by the sustained path length across D1 to D4. Deficits in learning produced by scopolamine were ameliorated by treatment with donepezil (1 mg/kg) (○) or inhibition of PDE9A with PF-4447943 (3.2 and 10 mg/kg) (△) as evidenced by shorter average path lengths on D2 to D4 (n = 12 animals per treatment group). Two-way repeated-measures ANOVAs, followed by Dunnett’s multiple comparisons, were used to compare performance between groups. **p < 0.01 compared with scopolamine + vehicle-treated rats on that day of testing.

ml/kg s.c.; n = 6) produced no improvement in gating over the same period of time (Fig. 8). Furthermore, in a separate set of experiments, a higher dose of PF-4447943 (10 mg/kg s.c.) was evaluated and shown to reverse the amphetamine-induced gating deficit in a similar fashion as the 1 mg/kg s.c. dose (Supplemental Fig. 5).

Inhibition of PDE9A Fails to Improve PPI but Potentiates the Effect of Risperidone. Based on the effect of PDE9A inhibition on auditory gating, the effect of PDE9A inhibition on PPI of the acoustic startle response was investigated as a behavioral measure of sensorimotor gating in mice. Administration of PF-4447943 had no effect on PPI at any dose in the poor-gating C57BL6/J mice, but interacted with a subthreshold dose of risperidone (0.32 mg/kg) to significantly increase PPI at doses of 3.2, 10, and 32 mg/kg (Fig. 9A). PF-4447943, however, was without effect on the disruption of PPI produced by the NMDA receptor antagonist MK-801 (Supplemental Fig. 2).

Inhibition of PDE9A Does Not Affect Spontaneous Locomotor Activity or Conditioned Avoidance Responding. To further characterize the effects of PDE9A inhibition on behavioral endpoints, we evaluated spontaneous locomotor activity of unhabituated rats after systemic administration of 0.32, 1.0, 3.2, or 10 mg/kg s.c. of PF-4447943. A significant (p < 0.05) increase in horizontal (i.e., crossovers) locomotor activity was observed at the lowest dose (0.32 mg/kg) in CD rats (Supplemental Fig. 3A), but this effect did not seem to be dose-dependent and did not replicate in another rat strain (Long-Evans; data not shown). In addition, a similar study in habituated CD rats, which would be expected to more clearly reveal a locomotor stimulant effect, showed no increases in activity at any of the doses tested (data not shown).

There was also no effect of PDE9A inhibitor PF-4447943 at any dose on amphetamine- or MK-801-stimulated locomotor activity (Supplemental Fig. 4). Given that PDE9A inhibition produced a reversal of auditory gating deficits that was comparable with that of clinically efficacious antipsychotic dopamine D2 receptor antagonists (Krause et al., 2003), the ability of PDE9A inhibition to suppress conditioned avoidance responding was examined in C57BL6/J mice. Conditioned avoidance responding is a test widely used to evaluate potential antipsychotic efficacy. Animals were trained to avoid the presentation of a shock upon presentation of a warning signal (conditioned stimulus). Antipsychotic agents produce a dose-related decrease in avoidance behavior at doses that do not significantly affect the ability to escape the shock (Wadenberg and Hicks, 1999). Administration of PF-4447943 before testing had no effect on conditioned avoidance responding in this task at any dose tested (Supplemental Fig. 3B).

Inhibition of PDE9A Prevents Stereotypic Responses to Mescaline. The hallucinogenic 5-hydroxytryptamine 2A (5HT2A) agonist mescaline produces stereotypical scratching behavior in mice. Studies indicate this effect is mediated by excitatory 5HT2A receptors on prefrontal pyramidal neurons (Weisstaub et al., 2006). Published in situ hybridization of PDE9 mRNA suggests high expression levels in layer 5 cortical neurons (Andreeva et al., 2001), which is supported by our immunocytochemistry for PDE9A in human cortex. Given the expression of PDE9A in the cortical output cells implicated in the acute behavioral response to 5HT2A receptor activation, the effects of PDE9A inhibition on mescaline-induced scratching behaviors were evaluated. Mice were treated with PF-4447943 20 min before administration of mescaline (30 mg/kg p.o.), and scratching behaviors were videotaped and subsequently scored during a 30-min period beginning 15 min after mescaline administration. Pretreatment of mice with PF-4447943 dose-dependently reversed the behavioral effects of mescaline in this model, with an ID50 value of 6.2 mg/kg s.c. (Fig. 10).

Discussion

The widespread CNS distribution of PDE9A, as well as its high affinity and specificity for cGMP, suggests that it is a principal regulator of basal concentrations of intracellular cGMP in the brain (Fisher et al., 1998; Andreeva et al., 2001). We showed previously that the brain-penetrant PDE9A inhibitor PF-4181366 increased the accumulation of cGMP in rodent striatum after systemic administration (Verhoest et al., 2009). The current study documents similar effects with the potent, selective, and brain-penetrant PDE9A inhibitor PF-4447943 and extends these results to encompass effects on cGMP accumulation in hippocampus, cortex, and cerebellum. It also demonstrates the accumulation of cGMP in the CSF of rodents and NHPs after systemic administration of the PDE9A inhibitor PF-4449613. The aggregate results demonstrate a pharmacological class effect and establish the CSF concentration of cGMP as a biomarker for inhibition of PDE9A. The widespread central elevation of cGMP in response to PDE9A inhibition suggests the potential for therapeutic utility in disease states where compromised cGMP signaling is a contributing factor.
The striatum is a key structure in the regulation of sensorimotor processing and gating functions (Geyer et al., 2001; Horvitz, 2002; Cromwell et al., 2007). Deficient auditory gating has been documented in patients suffering from multiple diseases, including Alzheimer’s disease (Jessen et al., 2001; Cancelli et al., 2006; Thomas et al., 2010), Huntington’s disease (Uc et al., 2003), and schizophrenia (Siegel et al., 1984; Patterson et al., 2008; Sánchez-Morla et al., 2008). Amphetamine disruption of auditory gating has been used to model primarily the P50-related gating deficits observed in patients with schizophrenia (Krause et al., 2003). Disruptive effects of amphetamine on auditory gating are attributed to an increase in subcortical dopamine release because the amphetamine-induced gating deficit is reversed by dopamine D2 receptor antagonists and inhibitors of the dual-substrate and striatal-enriched phosphodiesterase PDE10A (Schmidt et al., 2008). PF-4447943 produced dose-dependent improvements in amphetamine-disrupted gating without evidence of an inverted U. Efficacy of PF-4447943 was observed across acute electrophysiological and behavioral models at exposures expected to be 3- to 9-fold the in vitro EC_{50} value (12 nM) in the CSF compartment of rodents. This range of values for the ratio of efficacy to EC_{50} matches previous experience with PDE10A inhibitors in acute behavioral models that predict antipsychotic efficacy (Schmidt et al., 2008).

Fig. 8. PDE9A inhibition improves auditory gating deficits induced by d-amphetamine in anesthetized rats. A, typical auditory evoked potentials (summation of 50 consecutive events) recorded from the hippocampus CA3 region; location of the recording electrode is indicated on the left. Amplitude of evoked potentials was measured between the arrows indicated on the traces. B, typical recordings of auditory evoked potentials in response to conditioning and test auditory tones in control conditions (Control) after administration of d-amphetamine (AMP; 1.0 mg/kg i.v.) and after subsequent administration of PF-4447943 (AMP and 943; 1.0 mg/kg s.c.). C, effects of PF-4447943, PF-4449613, or vehicle on an amphetamine-induced gating deficit expressed as percentage of reversal (n = 5–6 animals per treatment group). Error bars represent S.E.M. #, p < 0.05 versus vehicle. *, p < 0.05 versus amphetamine.
PPI. The gating functions regulated by the D2/5HT2A antagonist risperidone may be potentiated by the striatal cGMP signaling, enhanced by either PDE9A or PDE10A inhibition, to improve a range of sensory gating functions subserved by MSNs.

PDE9A inhibitors reversed behavioral responses to mescaline, a hallucinogenic 5HT2A agonist. Reversal of 5HT2A signaling could result from intracellular elevation of cGMP within layer V cortical neurons after PDE9A inhibition, thereby providing a functional antagonism to 5HT2A receptor activation. Alternatively, because layer V pyramidal neurons project to the striatum, it is also possible that elevation of striatal cGMP by PDE9 inhibition accounts for the inhibition of mescaline-induced scratching, which is consistent with the activity of D2 antagonists (Porsolt et al., 2010).

Fig. 9. PDE9A inhibition potentiates the effect of risperidone on prepulse inhibition. A, treatment with the PDE9A inhibitor PF-4447943 alone had no effect on PPI in poor-gating C57BL6J mice (n = 8 animals per treatment group). **, p < 0.01 versus vehicle. B, treatment with PF-4447943 (3.2, 10, or 32 mg/kg s.c.) interacted with a low dose of risperidone (0.32 mg/kg s.c.) to significantly improve PPI *, p < 0.05 versus vehicle; **, p < 0.01 versus vehicle; ***, p < 0.01 versus risperidone.

Therapeutic modulation of cGMP has been proposed as a clinical approach to memory deficits (Prickaerts et al., 2002a, 2004; Boess et al., 2004; Puzzo et al., 2005, 2006, 2008, 2009; de Vente et al., 2006; van der Staay et al., 2008). Early studies used nonselective cGMP PDE inhibitors such as zaprinast, later progressing to selective PDE5 inhibitors such as sildenafil (Puzzo et al., 2008, 2009) and PDE9A inhibitors such as BAY 73–6991 to demonstrate improvements in rodent learning and memory models as well as enhanced LTP in hippocampal slices from aged animals (van der Staay et al., 2008). In contrast to PDE5 (Menniti et al., 2009), PDE9A is widely expressed among neurons across the brain. In situ hybridization for PDE9A mRNA detected prominent expression in presumptive cholinergic neurons located in the diagonal band of the septum in rodents (Andreeva et al., 2001). Consistent with a procholinergic function, we observed significant cognitive benefits after treatment with PF-4447943 in two cholinergic deficit memory models dependent on hippocampal function. The ability of PF-4447943 to acutely reverse the scopolamine-induced deficits produced in a novel object recognition task and the subchronic setting of the Morris water maze task implicates PDE9A in the regulation of hippocampal cholinergic circuits. Reversal of cholinergic deficits could be direct, affecting cGMP within cholinergic input neurons to the hippocampus itself, or indirect via effects on circuitry that participates in episodic memory formation. In addition, PF-4447943 induced improvement in an acute ketamine-disrupted spatial working memory task that exhibited an inverted U with respect to the dose-response relationship. Higher doses of PF-04447943 (3.2 mg/kg) associated with efficacy across behavioral and electrophysiological models (MWM, auditory gating, and mescaline-induced scratching) were ineffective in a ketamine-disrupted radial arm maze task. A recent report (Hutson et al., 2011) confirms that treatment with PF-4447943 improves cognitive perfor-
mance of rodents across a range of acute episodic and working memory tasks including the rat novel object recognition task with a scopolamine deficit. The recent demonstration of efficacy in an attention task disrupted by scopolamine (Vardigan et al., 2011) reinforces the proognitive potential of PF-4447943. These studies reported bell-shaped dose-response curves in several acute cognitive tasks as well as in the acute synaptic plasticity model of LTP (Hutson et al., 2011; Vardigan et al., 2011). We observed a similar bell-shaped dose-response curve in acute ketamine-disrupted cognitive tasks, but not in more subchronic cognitive paradigms (MWM) or for endpoints associated with sensory and auditory gating effects (Supplemental Fig. 4). Thus, the consequences of relatively high levels of drug exposure may vary across tasks and symptom domains.

PF-4447943 produced efficacy after subchronic dosing in the MWM at 3.2 and 10 mg/kg. Given the pharmacokinetic profile of PF-4447943 in rats, it was expected that 3.2 mg/kg results in CSF exposures above the required efficacious concentration (C_{eff}) (as defined by minimum dose needed for efficacy in the auditory gating and novel object recognition models) for at least 2.6 h. The 10 mg/kg dose would cover C_{eff} in the CSF for at least 4 h and sustain enzyme inhibition beyond the acquisition phase of the daily training trials. Daily treatment with PF-4447943 improved performance to the same extent as donepezil in MWM.

PDE9A inhibition has the potential to demonstrate clinical utility in several patient populations. Patients with AD exhibit deficits in cholinergic function and are transiently responsive to acetylcholinesterase inhibitors such as donepezil. Given that PF-4447943 was comparable with donepezil in restoring deficits in cholinergic function in preclinical models, the clinical comparison of these two mechanisms in AD populations may be expected to offer comparable cognitive benefits. Recently, Pfizer disclosed that the placebo-controlled clinical trial for the PDE9A inhibitor PF-4447943 in patients with mild-moderate AD failed to show any significant clinical benefit of active drug over placebo using a single 25-mg dose given to patients twice daily during a 12-week trial (Schwam et al., 2011). There are several possible explanations for the lack of clinical effect. One possibility is poor cross-species translation of scopolamine-induced deficits in rodents to cognitive deficits in patients with AD. Alternately, based on the aggregate preclinical data demonstrating efficacy in a range of acute and subchronic dosing paradigms, the optimal degree of PDE9 inhibition for achieving chronic efficacy in the clinical population may be lower on the dose-response curve than was explored in the single-dose clinical study that achieved significantly higher exposure than required to see efficacy in acute preclinical cognition models. Likewise, based on extrapolation from the subchronic preclinical MWM data, chronic dosing over a longer period of time than the 12-week duration of the clinical trial may be needed to ascertain definitively whether PDE9A inhibition can produce clinical benefit. Finally, it is possible that mild-moderate AD is not the right patient population in which to observe clinical benefits. Chronic dosing studies of PF-4447943 that demonstrated synaptoprotective effects in a Tg2576 mouse model of amyloid-β deposition (Kleiman et al., 2010) suggest that a prodromal AD patient population or age-associated cognitive impairment population could be a better choice for enhancing synaptic function with a PDE9A inhibitor. Alternatively, the present data showing improving models in auditory gating and sensorimotor gating could have additional implications for treatment of disorders that exhibit impaired sensory gating and are improved by antipsychotics, including schizophrenia and Huntington’s disease. Further clinical studies are warranted for this novel class of pharmacological agents.

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