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Antipsychotic Drug-Like Effects of the Selective M_4 Muscarinic Acetylcholine Receptor Positive Allosteric Modulator VU0152100

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Accumulating evidence suggests that selective M_4 muscarinic acetylcholine receptor (mAChR) activators may offer a novel strategy for the treatment of psychosis. However, previous efforts to develop selective M_4 activators were unsuccessful because of the lack of M_4 mAChR subtype specificity and off-target muscarinic adverse effects. We recently developed VU0152100, a highly selective M_4 positive allosteric modulator (PAM) that exerts central effects after systemic administration. We now report that VU0152100 dose-dependently reverses amphetamine-induced hyperlocomotion in rats and wild-type mice, but not in M4 KO mice. VU0152100 also blocks amphetamine-induced disruption of the acquisition of contextual fear conditioning and prepulse inhibition of the acoustic startle reflex. These effects were observed at doses that do not produce catalepsy or peripheral adverse effects associated with non-selective mAChR agonists. To further understand the effects of selective potentiation of M_4 on region-specific brain activation, VU0152100 alone and in combination with amphetamine were evaluated using pharmacologic magnetic resonance imaging (phMRI). Key neural substrates of M_4-mediated modulation of the amphetamine response included the nucleus accumbens (NAS), caudate-putamen (CP), hippocampus, and medial thalamus. Functional connectivity analysis of phMRI data, specifically assessing correlations in activation between regions, revealed several brain networks involved in the M_4 modulation of amphetamine-induced brain activation, including the NAS and retrosplenial cortex with motor cortex, hippocampus, and medial thalamus. Using *in vivo* microdialysis, we found that VU0152100 reversed amphetamine-induced increases in extracellular dopamine levels in NAS and CP. The present data are consistent with an antipsychotic drug-like profile of activity for VU0152100. Taken together, these data support the development of selective M_4 PAMs as a new approach to the treatment of psychosis and cognitive impairments associated with psychiatric disorders such as schizophrenia.
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INTRODUCTION

The psychotic symptoms and cognitive impairments observed in neuropsychiatric disorders, including schizophrenia, result in debilitating disruptions to the normal daily activities of patients as well as to their families and caregivers (American Psychiatric Association, 2000; Nuechterlein *et al*, 2004). Unfortunately, clinically available treatments, including typical and atypical antipsychotic

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drugs (APDs), provide only modest relief for some of the psychotic symptoms observed in these patient populations (Swartz *et al*, 2008). Treatment outcomes are further constrained by partial responsiveness, treatment resistance, and dose-limiting adverse effects, including extrapyramidal motor side effects and metabolic syndrome associated with many antipsychotics (Lieberman *et al,* 2003; Parsons *et al,* 2009). Thus, there remains a tremendous unmet need to develop novel treatment strategies to address the complex psychotic symptoms and cognitive deficits associated with various neuropsychiatric disorders.

Recent studies suggest that selective activators of specific subtypes of muscarinic acetylcholine receptors (mAChRs) may provide an alternative approach for the treatment of the psychotic symptoms and cognitive impairments in patients with schizophrenia, as well as the behavioral disturbances observed in neurologic disorders like Alzheimer's disease (AD). mAChRs are members of the family A G protein-coupled receptors (GPCRs) and consist of five subtypes termed M_1–M_5 (Bonner *et al*, 1987; Bonner *et al*, 1988). These different mAChR subtypes modulate multiple functions within the central nervous system (CNS), including motor control, sleep–wake architecture, cognition, and affective responses (Jones *et al*, 2012). Previous clinical studies demonstrated that the M_1/M_4-preferring mAChR agonist xanomeline significantly reduces the behavioral disturbances, including delusions, hallucinations, vocal outbursts and agitation, in AD patients and the psychotic symptoms and some cognitive deficits in individuals with schizophrenia (Bodick *et al,* 1997; Shekhar *et al,* 2008). Although encouraging, there have been only two clinical studies with xanomeline published to date indicating that these initial findings should be viewed with caution until further clinical validation is reported. Xanomeline also showed robust efficacy in animal models predictive of APD-like activity comparable to the effects observed with the atypical antipsychotic clozapine, including reversal of psychostimulant-induced hyperlocomotion and disruption of prepulse inhibition (PPI) of the acoustic startle reflex (Andersen *et al,* 2003; Jones *et al,* 2005; Perry *et al,* 2001; Shannon *et al,* 2000; Stanhope *et al,* 2001). Unfortunately, xanomeline like other muscarinic agonists ultimately failed in clinical development due to a lack of complete receptor subtype selectivity resulting in adverse side effects associated with non-selective activation of peripheral M_2 and M_3 mAChRs (Bodick *et al,* 1997; Shekhar *et al,* 2008).

The lack of selective ligands for M_1 and M_4 mAChRs has made it difficult to develop a clear understanding of the relative contributions of these two mAChR subtypes to the clinical and preclinical effects of xanomeline. Development of allosteric ligands provides a novel approach to address these issues (see Jones *et al*, 2012). Our group and others have identified subtype-selective mAChR ligands that activate a specific receptor subtype at sites that are less highly conserved than the orthosteric binding site of acetylcholine (ACh) and more topographically distinct, termed allosteric sites. These efforts have led to the discovery of highly selective M_4 positive allosteric modulators (PAMs) that do not activate M_4 directly, but markedly potentiate the response of M_4 to ACh (Shirey *et al*, 2008; Chan *et al,* 2008; Brady *et al,* 2008). M_4 PAMs bind to an allosteric site and can increase both the affinity of M_4 for ACh and the efficiency of coupling of M_4 to G proteins (Shirey *et al,* 2008). Consistent with their effects in cell lines, M_4 PAMs potentiate M_4-mediated depression of excitatory synaptic transmission at hippocampal CA1 synapses (Shirey *et al*, 2008). Chemical optimization of early M_4 PAMs resulted in the discovery of VU0152100 as a centrally penetrant highly selective M_4 PAM (Brady *et al,* 2008). Interestingly, VU0152100 produced robust efficacy when administered at a single top dose in a preclinical model of APD-like activity (Brady *et al,* 2008). These studies provided important data supporting the potential for the development of M_4 PAMs for the treatment of psychosis. Conversely, LY2033298, another M_4 PAM, had no effect alone in preclinical models, but did potentiate the effects of a sub-threshold dose of the non-selective mAChR agonist oxotremorine (Chan *et al,* 2008; Leach *et al,* 2010, Suratman *et al,* 2011). The lack of efficacy observed with LY2033298alone when used *in vivo* was likely due to the lower potency of this M_4 PAM at the rat M_4 mAChR and its low cooperativity with the endogenous agonist ACh at the rodent receptor (Suratman *et al,* 2011). However, these studies also raise a potential concern that there may not be sufficient endogenous cholinergic tone to observe robust activity in key limbic and cortical regions when an M_4 PAM is administered alone.

We now report a series of behavioral, neurochemical, and functional imaging studies in which we show that the M_4 PAM VU0152100 has robust efficacy in several rodent models predictive of APD-like activity and reversal of cognitive impairments. In addition, we demonstrate corresponding effects of VU0152100 on specific brain regions and circuits that are thought to have an important role in the underlying psychotic symptoms, behavioral, and cognitive disturbances observed in various psychiatric and neurologic disorders.

MATERIALS AND METHODS

Subjects

Adult male Sprague–Dawley rats, weighing 250–275 g, were purchased from Harlan Laboratories (Indianapolis, IN). C57BL/6NTac wild-type mice and M4 knockout (KO) mice were obtained from Taconic Farms (Hudson, NY) through collaboration with Dr Jürgen Wess (National Institute of Diabetes and Digestive and Kidney Disorders, Bethesda, MD). Animals were group housed in large colony rooms under a 12-h light–dark cycle with food and water available *ad libitum*. All procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health regulations of animal care covered in Principles of Laboratory Animal Care.

Drugs

D-amphetamine hemisulfate, clozapine, haloperidol, and oxotremorine sesquifumarate were obtained from Sigma-Aldrich (St Louis, MO). Xanomeline L-tartrate was synthesized in-house according to the method of Sauerberg *et al* (1992) or from commercially available free base xanomeline.

VU0152100 was synthesized according to our previously described method (Brady *et al,* 2008). Amphetamine, xanomeline, and oxotremorine were dissolved in double deionized water; clozapine and haloperidol were dissolved in acidified double deionized water; and VU0152100 was dissolved in 10% Tween 80 plus double deionized water. All solutions were adjusted to a pH of approximately 6–7 using 1 N sodium hydroxide and administered in a volume of 1 ml/kg, with the exception of VU0152100, which was administered in a volume of 2 ml/kg.

Cell Culture, Transfection, Dopamine Transporter Uptake and Binding Assays

HEK 293T cells were cultured, maintained, and transiently transfected with pcDNA 3-rDAT (rat DAT) as described previously (Mazei-Robison and Blakely, 2005). In short, HEK 293T cells were cultured in media (DMEM (Invitrogen), 10% FBS (Hyclone, Carlsbad, CA), 0.1 U/ml penicillin–0.11 gm/ml streptomycin (pen/strep) (Invitrogen), and 2 mM L-glutamine (Invitrogen)) in a humidified incubator at 37 °C and 5% CO_2. Cells were plated in 24-well plates at a density of 20 000 cells per well. *Trans*-IT LT-1 (Mirus, Madison, WI) in serum-free media was used for transfection of cells approximately 24 h after plating using a 1 : 3 DNA:lipid ratio. Assays were conducted approximately 36 h after transfection. [^{3}H] DA transport assays were performed in triplicate in 24-well plates as described previously (Sakrikar *et al*, 2012). Briefly, cells were washed with Krebs'–Ringer's–HEPES (KRH; 130 mM NaCl, 1.3 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM HEPES, pH 7.4) buffer and incubated in the uptake assay buffer (KRH, 10 mM glucose, 100 μM pargyline, ascorbic acid, and 10 μM tropolone) before incubation with [^{3}H]DA (3,4-[7-^{3}H]-dihydroxyphenylethylamine, ~45 Ci/mmol, NEN-Perkin Elmer, Boston, MA) for 15 min. To determine inhibition, parallel wells were incubated with 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazine hydrochloride (GBR 12909) (Sigma-Aldrich) or VU0152100 10 min before addition of [^{3}H]DA. For each inhibitor—GBR 12909 and VU 0152100—seven concentrations (0.03, 0.1, 0.3, 1, 3, 10, and 30 μM) were tested to determine IC_{50} values. MicroScint 20 (Perkin-Elmer, Boston, MA) scintillation mixture was added to the wells at the end of the assay and DA uptake was measured using a TopCount Scintillation Counter. Values reported from transport studies are derived from three replicate experiments. GraphPad Prism was used to fit the inhibition curves and to determine the IC_{50} values.

In vitro assays on the effects of VU0152100 on human DAT were performed by Eurofins Panlabs (Taipei, Taiwan). Briefly, CHO-K1 cells expressing recombinant human DAT were used (1) to assess if VU0152100 displaces binding of the DAT inhibitor [^{125}I]iometopane ([^{125}I]RTI-55) and (2) to determine if VU0152100 interferes with [^{3}H]dopamine uptake. Detailed assay procedures are available at: https://www.eurofinspanlabs.com/Catalog/Products/ProductDetails.aspx?prodId=wCWTzNIg1Cc%3d (DAT uptake) and https://www.eurofinspanlabs.com/Catalog/Products/ProductDetails.aspx?prodId=umFCt3XtSMg%3d (DAT binding). The IC_{50} values were estimated by a non-linear, least squares regression analysis using MathIQTM (IDBusiness Solutions, UK) and K_i values were calculated using the equation of Cheng and Prusoff (1973).

Amphetamine-Induced Hyperlocomotion

Locomotor activity was assessed in open field chambers as previously described (see Jones *et al,* 2008). Briefly, rats were habituated to the chambers for 30 min, then pre-treated with either vehicle or VU0152100 (3–56.6 mg/kg, interperitoneal (i.p.)). Thirty minutes later, rats were administered either vehicle or amphetamine (1 mg/kg, subcutaneous, s.c.) and monitored for another 60 min.

Locomotor activity in mice was examined using open filed boxes (Med Associates, St Albans, VT). Wild-type and M4 KO mice were habituated in the chambers for 90 min before receiving i.p. injections of vehicle (10% Tween 80) or VU0152100 (30 mg/kg). Amphetamine (1.8 mg/kg, i.p.) was injected 30 minutes later and locomotor activity was monitored for another 120 min.

Amphetamine-Induced Disruption of PPI of the Acoustic Startle Reflex

Studies were conducted using startle chambers with a Plexiglas cylinder mounted on a piezoelectric accelerometer for detecting motion as previously described (see Jones *et al,* 2005). Following a 20-min pre-treatment with vehicle or VU0152100 (3–30 mg/kg, i.p.), rats were injected with amphetamine (3 mg/kg, s.c.), and after an additional 10 min, placed into individual startle chambers for the following paradigm. After a 5-min acclimation period, the rats were presented with five startle stimuli alone, followed by seven randomized presentations of the following trial types: no stimulus, startle pulse alone (120 dB, 40-ms broadband noise burst), prepulse noise alone (82 dB, 40-ms broadband noise burst), and three prepulse (72, 77, or 82 dB; 20 ms) plus startle pulse combinations. The intertrial interval varied pseudorandomly between 15 and 45 s and the interstimulus interval was 100 ms. Background noise of 67 dB was presented continuously. Percent PPI was calculated as $100 \times ((\text{mean ASR}_{\text{startle pulse trials}} - \text{mean ASR}_{\text{prepulse plus startle pulse trials}})/\text{mean ASR}_{\text{startle pulse trials}})$.

Amphetamine-Induced Disruption of Contextual Fear Conditioning

Studies were conducted using conditioning chambers within sound attenuating cubicles as previously described (see Lebois *et al,* 2010). All rats were handled and injected with saline for 2 days before conditioning. On the conditioning day, rats were pre-treated with vehicle or VU0152100 (10–56.6 mg/kg, i.p.) for 15 min, followed by administration of either vehicle or amphetamine (4.8 mg/kg, s.c.), and after an additional 15 min, placed in chambers for conditioning with the following paradigm: 2-min habituation, then four presentations of an unconditioned stimulus (0.5 mA 1-s footshock; 79-s intertrial interval) followed by a 45-s interval without stimuli (7-min total). Approximately 24 h after compound treatment and conditioning, rats were re-exposed to the same conditioning context and freezing behavior, defined as motionless posture, excluding respiratory movements, was measured in the absence of any shock

stimuli for 7 min. The potential effect of VU0152100 on nociceptive response to the footshook stimulus was also separately measured. Rats were pre-treated with vehicle or VU0152100 (56.6 mg/kg, i.p.) for 30 min, then placed in the conditioning chambers and behavioral changes in footshock threshold were assessed using increasing current from 0 to 0.5 mA in increments of 0.05 mA as described by van der Staay *et al* (2011).

Fos Immunochemistry

After a 2-h pre-treatment with either vehicle, VU00152100 (30–100 mg/kg, i.p.), clozapine (30 mg/kg, s.c.), or haloperidol (1 mg/kg, s.c.), rats were perfused with 4% paraformaldehyde. Frozen sections were stained to reveal Fos-like immunoreactivity (Fos-LI) using a goat anti-Fos antibody (see Bubser *et al*, 2005); hypothalamic sections were double-labeled for Fos-LI and orexin A using a mouse anti-orexin A antibody (1 : 1000; R&D Systems, Minneapolis, MN). The density of Fos-like immunoreactive (Fos-li) neurons (number of Fos-li neurons/mm^2) was determined as previously described (Bubser *et al*, 2005).

Pharmacologic Magnetic Resonance Imaging (phMRI)

Isoflurane-anesthetized rats, with preimplanted jugular catheters, underwent endotracheal intubation (14 G catheter), insertion of i.p. catheters (size P50, Braintree Scientific, Braintree, MA), and mechanical ventilation (Kent Scientific, Litchfield, CT; O_2:N_2O 1 : 2; 2% isoflurane). For scanning, isoflurane was set to 0.88% and pancuronium bromide administered (1 mg/kg, i.p.; BoundTree Medical, Dublin, OH). Heart rate, respiration, and rectal temperature were continuously measured and temperature maintained through a thermocoupled heating unit (SAM-PC; SA Instruments, Encinitas, CA). End-tidal CO_2 was continuously monitored (Invivo Research, Orlando, FL). PhMRI data were acquired with a Doty Litz 38-mm transmit-receive radio frequency coil using a 9.4 T Varian magnet controlled by a Varian Inova console (Agilent, Palo Alto, CA). High-resolution fast spin-echo (fse) structural images were collected (repetition time (TR) 2550 ms; effective echo time (TEeff) 40 ms; number of excitations (NEX) 2; 128×128 matrix; $35 \times 35\ mm^2$ field of view; 11 contiguous slices, 1.5 mm thick). Pre-contrast reference images and post-contrast functional images were acquired (fse: TR 2600 ms; TEeff 36 ms; NEX 2; 64×64 matrix). To measure cerebral blood volume (CBV), Molday iron oxide nanoparticles (30 nm; 20 mg/kg, i.v.; BioPAL, Worcester, MA) were injected. For amphetamine interaction experiments, rats were pre-treated for 15 min with vehicle or 56.6 mg/kg VU0152100 (i.p.), then a 15-min baseline was collected, after which rats were administered with vehicle or a 1 mg/kg amphetamine (i.p.) followed by 45 min of continuous acquisition. In a separate study to assess the effects of VU0152100 alone on fractional CBV changes, an initial 15-min baseline was collected, followed by administration of vehicle or VU0152100 (56.6 mg/kg, i.p.), and 45 min of continuous image acquisition.

phMRI data were processed using in-house MATLAB code (MathWorks, Natick, MA) and Analysis of Functional NeuroImages (AFNI; afni.nimh.nih.gov). All masked, motion-corrected (AFNI 2dreg) images were coregistered to the template anatomical images in AFNI. Fractional CBV changes were calculated on a voxel-wise basis for each subject using the equation: $\Delta CBV(t) = (\ln\ S(t) - \ln\ So)/(\ln\ So - \ln\ Spre)$, where $S(t)$ is the measured signal at time t, *So* is the post-contrast baseline signal, and *Spre* is the pre-contrast baseline (Mandeville *et al,* 1998). Regions of interest (ROIs), pre-defined on the template based on a rat brain atlas (Paxinos and Watson, 2007), were applied to all subjects. Mean CBV values (left and right hemispheres averaged) were calculated for each subject at the 15- to 25-min interval after the vehicle or amphetamine challenge or at the 15- to 25-min interval after vehicle or VU0152100 alone. Group-averaged maps were colorized according to the voxel CBV value.

Functional Connectivity Analysis

The strength of functional connections between brain regions was quantified by computing the Pearson linear correlation coefficient between the integral of the CBV response to amphetamine for each ROI pair for the vehicle/amphetamine and VU0152100/amphetamine groups (Schwarz *et al,* 2007b). Correlation coefficients, r, were converted to z-scores using Fisher's transformation ($z = \ln[(1+r)/(1-r)]/[2^*(1/(N-3))^{0.5}]$) and thresholded at $|Z| > 2$ (in-house MATLAB code). Permutation analysis (Holmes *et al,* 1996) was used to identify the correlations that were significantly different (Δz, $p < 0.05$) between vehicle/amphetamine and VU0152100/amphetamine groups (in-house MATLAB code).

Cardiovascular Function

Potential effects of VU0152100 on blood pressure (mm Hg) and heart rate (beats/min) were measured in awake, freely moving rats via a transducer (Biopac Systems, Goleta, CA) connected to a line pre-implanted into the carotid artery (Parasuraman and Raveendran, 2012). VU0152100 (56.6 mg/kg, i.p.) or vehicle were administered after a 30-min baseline recording, and data were collected for 70 additional minutes using Biopac Student Lab Pro Software (Goleta, CA).

Neurochemistry Studies

Rats were pre-treated for 30 min with either vehicle, VU0152100 (10–100 mg/kg, i.p.), or xanomeline (30 mg/kg, i.p.), then killed under isoflurane anesthesia and the medial prefrontal cortex (PFC), nucleus accumbens (NAS), and caudate-putamen (CP) rapidly dissected, frozen on dry ice, and stored at $-80\ ^\circ C$ until dopamine and serotonin levels were analyzed by HPLC-ECD as described by Hackler *et al* (2006). Using the same methods, we assessed whether VU0152100 would potentiate the effects of the non-selective mAChR agonist oxotremorine on monoamine metabolite/parent monoamine ratios, an index of monoamine utilization *in vivo*. Rats were pre-treated for 30 min with vehicle or VU0152100 (56.6 mg/kg) followed by a sub-threshold dose of oxotremorine (0.01 mg/kg, s.c.) or vehicle and killed after another 15 min.

In Vivo Microdialysis and Locomotor Activity

Guide cannulae (BioAnalytical Systems, West Lafayette, IN) were implanted into the NAS (AP +1.5 mm bregma, ML −1.5 mm, DV −7.8 mm) or CP (AP 1.0 mm, ML −2.5 mm, DV −5.5 mm). After recovery for 4–7 days, microdialysis probes (2 mm membrane length) were inserted the night before the experiment using methods previously reported by Perry *et al* (2001). The following day, rats were placed into open field chambers as used for the amphetamine-induced hyperlocomotion studies. Probes were perfused with artificial cerebrospinal fluid (150 mM NaCl, 3 mM KCl, 1.7 mM $CaCl_2$, and 0.9 mM $MgCl_2$) and dialysate samples were collected in 15-min fractions onto a 96-well plate using a refrigerated fraction collector (EFC-82, Eicom). Following a 2-h equilibration, four baseline samples were collected, followed by a 30-min pre-treatment with either vehicle or VU0152100 (56.6 mg/kg, i.p.). Then all rats were injected with either vehicle or amphetamine (1 mg/kg, s.c.) and dialysate samples were collected for another 120 min. At the end of the study, probes were perfused with methyl blue to verify placement. Dialysates were analyzed using an Eicom ECD-700 HPLC system. Dopamine and its two major metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were separated at a flow rate of 400 μl/min using a mobile phase consisting of 0.1 M citrate-acetate buffer/methanol (80 : 20), 220 mg/l sodium octanesulfonic acid, and 5 mg/l EDTA and quantitated by electrochemical detection. Chromatograms were analyzed using PowerChrom (eDAQ, Denistone East, NSW, Australia). Only animals with accurate probe placement that showed three consecutive stable baseline values (within $\leqslant$20%) were included in the statistical analysis.

Catalepsy

Rats were administered either vehicle, VU0152100 (30–100 mg/kg), or haloperidol (1.5 mg/kg), and catalepsy was assessed at 30, 60, 120, and 240-min post-treatment as previously described (Jones *et al*, 2008). In a second study, rats were pre-treated for 60 min with a dose of haloperidol (0.3–1.5 mg/kg, i.p.), followed by administration of a 100 mg/kg i.p. dose of VU0152100 or vehicle; then catalepsy was measured after an additional 30 min.

Modified Irwin Test Battery

Effects of vehicle, VU0152100 (56.6 mg/kg, i.p.), or oxotremorine sesquifumarate (1 mg/kg, s.c.) on autonomic and somatosensory system function were assessed at 5, 15, 60, 120, and 240-min post-injection using the Modified Irwin Neurological Test Battery (Irwin, 1968), as previously described (see Jones *et al*, 2008).

Statistical Analysis

Data were analyzed by one- or two-way ANOVA followed by Dunnett's or Bonferroni's test, or in the case of unequal variance by Kruskal–Wallis test followed by Dunn's test using GraphPad Prism V5.04 (GraphPad Software, La Jolla, CA).

RESULTS

Characterization of VU0152100

Before *in vivo* testing, we verified that the newly synthesized batch of VU0152100 used in the present studies yielded comparable exposure to our previously reported brain and systemic plasma pharmacokinetic profile when dosed at 56.6 mg/kg (i.p.); specifically, brain $AUC_{0-\infty}$ (36.2 μM h) and C_{max} values (7.6 μM) observed in our present study were similar to the previously reported $AUC_{0-\infty}$ (19.2 μM h) and C_{max} (8.8 μM) values (Brady *et al,* 2008). Finally, these exposure values represented C_{max} free brain concentrations of ~340 and ~400 nM, respectively, for the dose of 56.6 mg/kg when the actual amount of unbound or free VU0152100 available in rat brain (rat f_u brain = 0.045) was taken into consideration.

Based on an initial report that VU0152100 displaced binding of a ligand to the dopamine transporter with a 46% inhibition at 10 μM (Brady *et al*, 2008), we also estimated the *in vitro* IC_{50} and K_i binding values for VU0152100 at the human DAT to be 5.98 and 4.75 μM, respectively, using a recombinant CHO-K1 cells expressing human DAT (Eurofins, Lancaster, PA; see Supplementary Figure S1). In addition, we evaluated the effects of VU0152100 at the rat DAT using HEK cells transiently transfected with the rat DAT and found that the IC_{50} value was greater than 10 μM (see Supplementary Figure S1).

VU0152100 Reverses Amphetamine-Induced Hyperlocomotion

The effects of VU0152100 on amphetamine-induced hyperlocomotion were evaluated in rats. When administered alone, amphetamine (1 mg/kg, s.c.) induced a substantial increase in locomotor activity when compared with the vehicle/vehicle group (Figure 1a). VU0152100 pre-treatment produced a robust dose-dependent reversal of amphetamine-induced hyperlocomotion with significant reversal observed at doses of 30 and 56.6 mg/kg (Figure 1a: $F(\text{treatment})_{5,1008} = 11.7$, $p < 0.001$, $F(\text{time})_{23,1008} = 40.14$, $p < 0.001$, and $F(\text{interaction})_{5,115} = 20.12$, $p < 0.001$).

VU0152100-Mediated Reversal of Amphetamine-Induced Hyperlocomotion is Absent in M4 KO Mice

Amphetamine elicited a marked locomotor response in wild-type mice that was blocked significantly by pre-treatment with 30 mg/kg VU0152100 ($F(\text{treatment})_{1,912} = 65.29$, $p < 0.001$, $F(\text{time})_{47,912} = 12.02$, $p < 0.001$, and $F(\text{interaction})_{47,912} = 2.10$, $p < 0.001$; see Figure 1b). In M4 KO mice, amphetamine-induced hyperlocomotion was not reversed at any time point by VU0152100 pre-treatment ($F(\text{treatment})_{1,912} = 3.13$, NS, $F(\text{time})_{47,912} = 7.39$, $p < 0.001$, and $F(\text{interaction})_{47,912} = 0.30$, NS; see Figure 1c). Total locomotor activity induced by amphetamine was significantly reduced by VU0152100 in wild-type animals ($t_{19} = 2.44$, $p < 0.05$); pre-treatment with VU0152100 did not alter amphetamine-induced total locomotor activity in M4 KO mice ($t_{19} = 0.55$, NS).

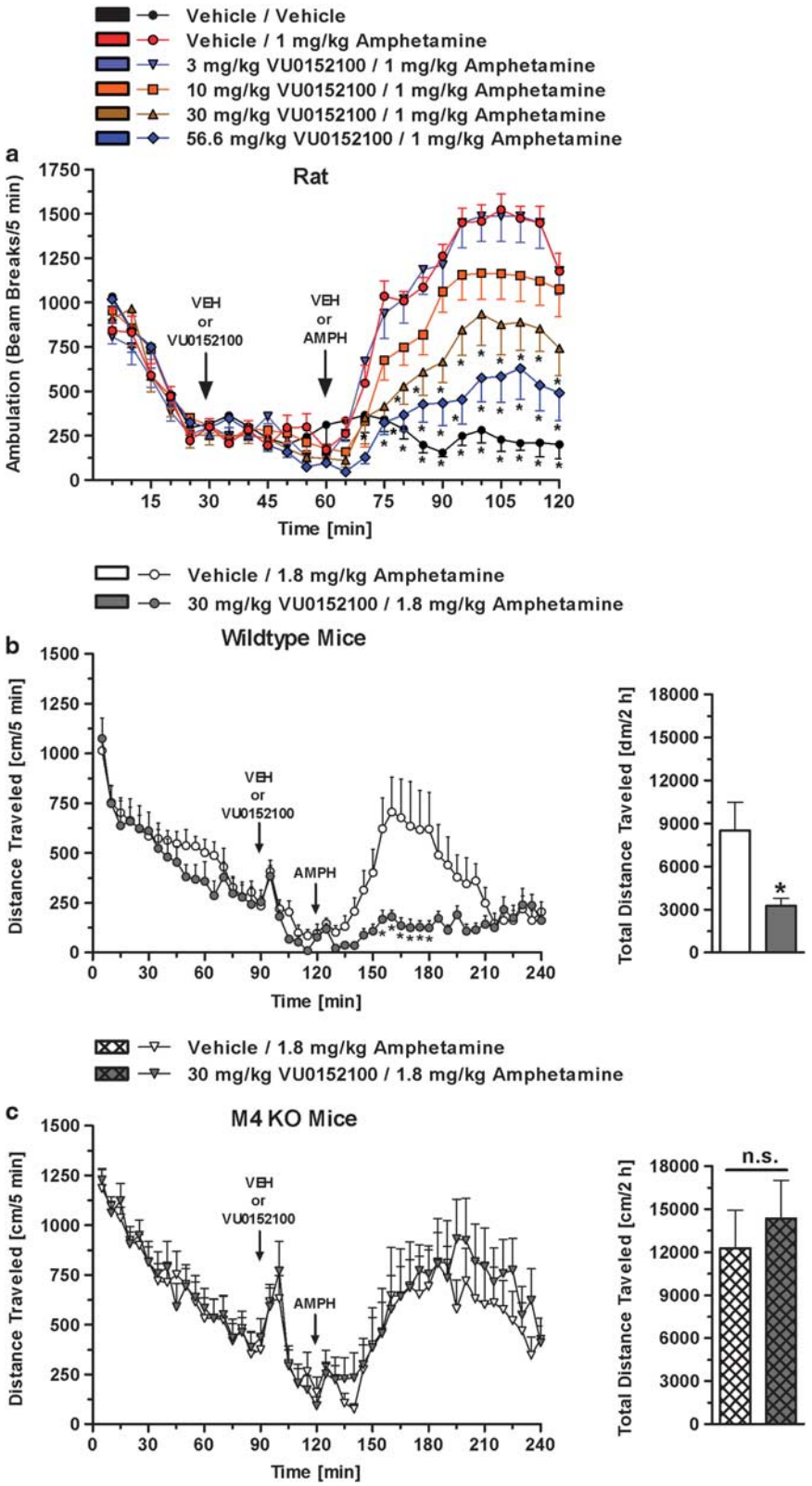

Figure 1 VU0152100 reverses amphetamine-induced hyperlocomotion in rats and wild-type mice, but not in M4 KO mice. (a) Amphetamine-induced (1 mg/kg) hyperlocomotion in rats is dose-dependently reversed by VU0152100 (*$p<0.05$ *vs* vehicle/amphetamine (Dunnett's test)); (b) VU0152100 reverses amphetamine-induced hyperlocomotion in wild-type mice. (c) In M4 KO mice, amphetamine-induced hyperlocomotion is not blocked by VU0152100 (*$p<0.05$, vehicle/amphetamine (Bonferroni's test)). Data are mean ± SEM of 8 rats or 10–11 mice per group.

VU0152100 Reverses Amphetamine-Induced Disruption of PPI

We next evaluated the effects of increasing dose of VU0152100 on amphetamine-induced disruption of PPI of the acoustic startle reflex in rats. As shown in Figure 2a, amphetamine when administered alone produced a robust disruption of PPI as compared with the vehicle/vehicle group across all of the prepulse intensities. However, the disruptive effects of amphetamine were significantly attenuated in the presence of a 30 mg/kg i.p. dose of VU0152100 when compared with the vehicle/amphetamine group at prepulse intensities of 5 and 10 dB with a trend toward significance at 15 dB ($F(\text{treatment})_{4,136} = 18.2$, $p < 0.001$ and $F(\text{prepulse intensity})_{2,136} = 16.0$, $p < 0.001$). As a dose-related reversal of amphetamine-induced disruption of PPI was achieved with VU0152100, significant at the 5 and 10 dB prepulse intensities with a dose of 30 mg/kg, higher doses of VU0152100 were not evaluated in this assay. When administered alone, VU0152100 had no effect on startle amplitude or PPI across the dose range tested (data not shown).

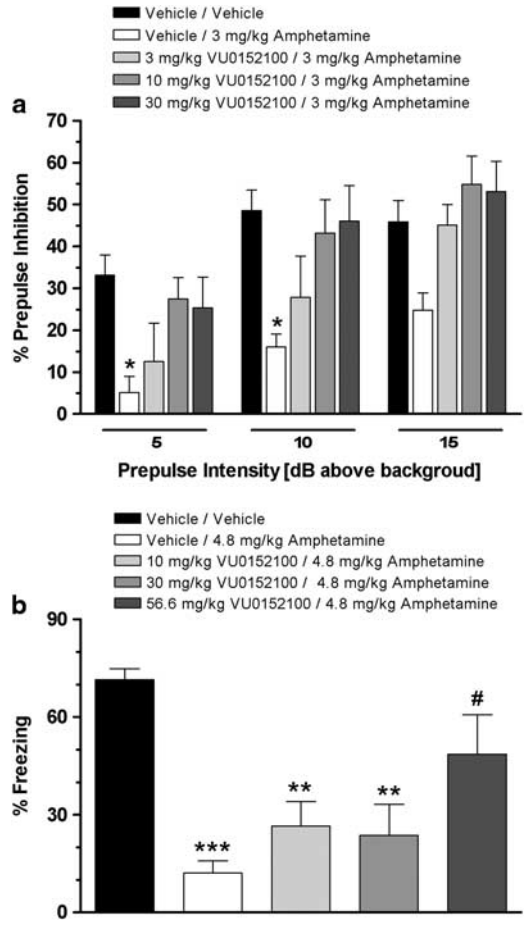

Figure 2 VU0152100 reverses sensorimotor gating and contextual fear conditioning deficits elicited by amphetamine. (a) VU0152100 blocks amphetamine-induced (3 mg/kg) disruption of prepulse inhibition (*$p < 0.05$ *vs* vehicle/vehicle (Dunnett's test)); (b) The disruptive effects of amphetamine (4.8 mg/kg, s.c.) on the acquisition of a context-dependent fear response are dose-dependently reversed by pre-treatment with VU0152100 (**$p < 0.01$, ***$p < 0.001$ *vs* vehicle/vehicle, $^{\#}p < 0.05$ *vs* vehicle/amphetamine (Dunnett's test)). Data are mean ± SEM of 7–11 rats per group.

VU0152100 Reverses Amphetamine-Induced Disruption of Contextual Fear Conditioning

Contextual fear conditioning is a form of Pavlovian conditioning that is mediated by the hippocampus (Phillips and LeDoux, 1992). As shown in Figure 2b, pre-treatment with a 4.8 mg/kg dose of amphetamine disrupted acquisition of contextual fear conditioning as reflected by a significant decrease in percent freezing behavior relative to the vehicle/vehicle-treated rats. VU0152100 produced a dose-related blockade of the amphetamine-induced disruption of the acquisition of contextual fear conditioning; significant at the 56.6 mg/kg dose ($F_{4,43} = 7.66$, $p = 0.001$). In contrast, when VU0152100 was administered alone, it had no effect on the acquisition of contextual fear conditioning across the dose range tested ($F_{3,23} = 0.47$, NS; data not shown). In a separate experiment, pre-treatment with VU0152100 (56.6 mg/kg, i.p.) alone had no effect on the threshold of sensitivity to a footshock stimulus as compared with vehicle-treated rats (data not shown).

VU0152100 does not Induce Fos-LI in Forebrain and Hypothalamus

We next assessed the ability of VU0152100 to induce changes in the expression of Fos-LI in specific cortical and limbic brain regions. In contrast to the increase in Fos-LI expression observed with the typical and atypical antipsychotics, haloperidol and clozapine, respectively, VU0152100 (30 and 100 mg/kg, i.p.) had no effect on Fos-LI expression in the PFC, NAS_{core}, NAS_{shell}, or CP (Supplementary Table S1: PFC ($H = 19.0$, $p < 0.001$), NAS_{shell} ($H = 31.9$, $p < 0.0001$), NAS_{core} ($H = 27.3$, $p < 0.0001$), and dorsolateral CP ($H = 39.6$, $p < 0.0001$). As weight gain liability of certain antipsychotics correlates with their ability to elicit Fos-LI expression in hypothalamic orexin neurons (Fadel *et al*, 2002), we also examined the potential of VU0152100 to induce Fos-LI expression in this brain region (Supplementary Table S2). In the medial and lateral part (with respect to the fornix) of the lateral hypothalamus only haloperidol and clozapine, but not VU0152100, increased the number of Fos-li cells/mm^2 (medial LH ($H = 15.3$, $p < 0.01$); lateral LH ($H = 14.7$, $p < 0.01$)) and the percentage of orexin cells expressing Fos-LI (medial LH ($H = 17.8$, $p < 0.01$); lateral LH ($H = 26.4$, $p < 0.0001$)) (Supplementary Table S1).

phMRI Quantification of VU0152100-Mediated Suppression of Amphetamine-Induced Regional Brain Activation and Functional Connectivity Pattern

In order to more comprehensively assess the specific brain regions underlying the effects of VU0152100 in reversing the amphetamine-induced changes in behavior, we used phMRI to examine the effects of a behaviorally relevant dose of VU0152100 (56.6 mg/kg, i.p.) on amphetamine-evoked (1 mg/kg, i.p.) alterations in CBV. As shown in the group-averaged CBV maps (Figure 3a) and time courses (Figure 3b), amphetamine robustly increased CBV in the NAS and CP, areas of high dopamine transporter expression, as well as in extrastriatal areas, including the thalamus, hippocampus, and retrosplenial cortex. Figure 3b also

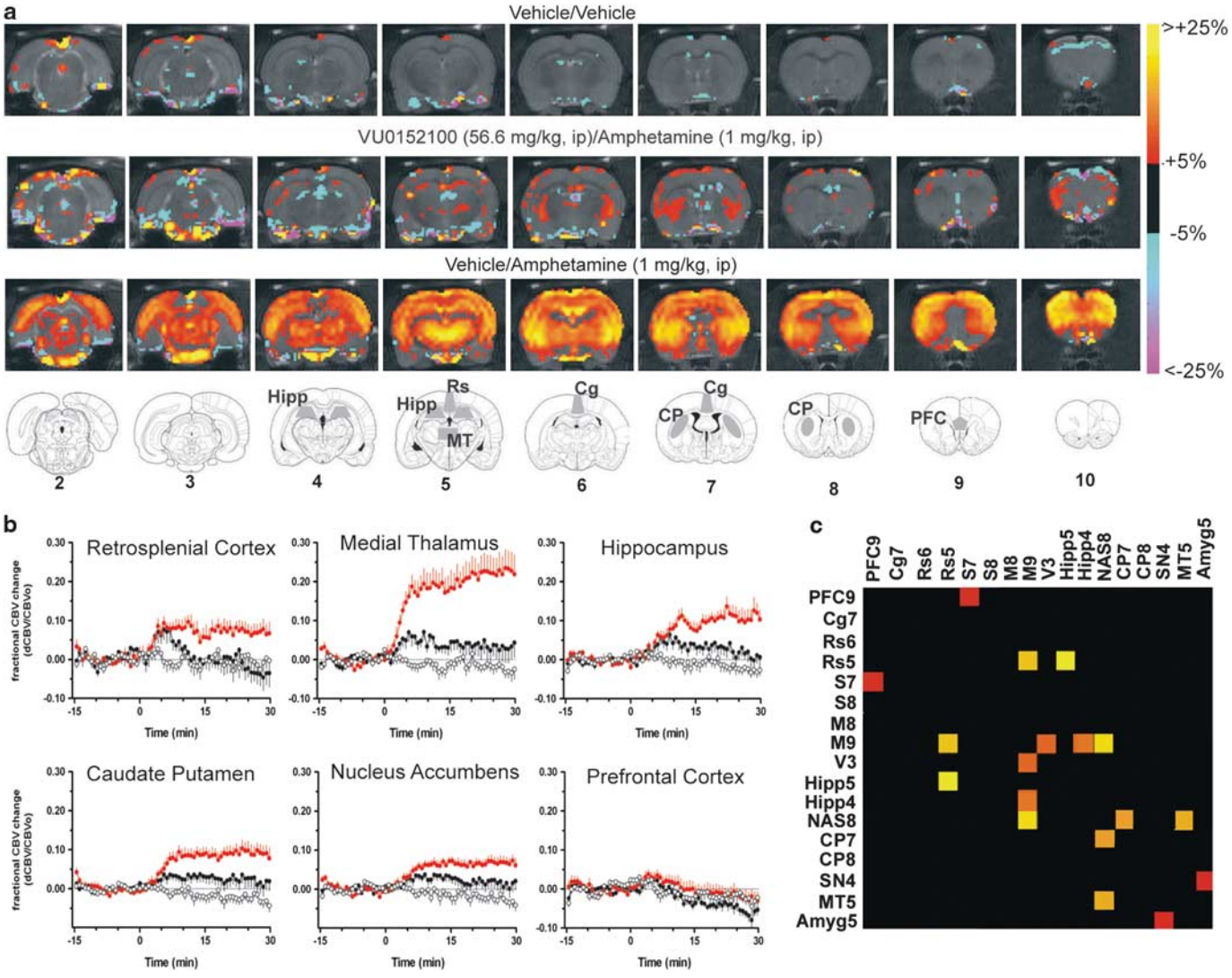

Figure 3 VU0152100 modulates amphetamine-induced cerebral blood volume (CBV) responses. (a) Amphetamine (1 mg/kg, i.p.) elicited CBV increases, reflecting neural activity, while pre-treatment with VU0152100 (56.6 mg/g, i.p.) suppressed this effect in multiple brain areas. (b) Regional CBV time courses for amphetamine and reversal by VU0152100 are shown for the retrosplenial cortex, medial thalamus, hippocampus, NAS, CP, and PFC; note that amphetamine by itself and in combination with VU0152100 did not alter CBV in the PFC. Data are means ± SEM of 10–11 animals per group. Functional connectivity analysis of the phMRI data revealed fewer inter-regional correlations in the VU0152100/amphetamine group and the significant ROI–ROI correlation differences between the vehicle/amphetamine group and the VU0152100/amphetamine group (thresholded at $p<0.05$) are depicted in panel (c). Cells are colored according to the corresponding *z*-statistic. Abbreviations of regions of interest (number indicates slice): Amyg, amygdala; CP, caudate-putamen; Cg, cingulate; Hipp, hippocampus; MT, medial thalamus; M, motor cortex; NAS, nucleus accumbens; PFC, prefrontal cortex; Pir, piriform cortex; Rs, retrosplenial cortex; S, sensory cortex; SN, substantia nigra; V, visual cortex.

shows that a 30-min pre-treatment with VU0152100 (56.6 mg/kg, i.p.) significantly blunted amphetamine-evoked increases in CBV in the medial thalamus ($F_{2,31}=15.7$, $p<0.0001$), hippocampus ($F_{2,31}=11.7$, $p<0.001$), CP ($F_{2,31}=10.3$, $p<0.001$), NAS ($F_{2,31}=9.31$, $p<0.001$), and retrosplenial cortex ($F_{2,31}=3.8$, $p<0.05$). As shown in Figure 3b, in the PFC there were no significant CBV changes in response to amphetamine alone or in combination with VU0152100 ($F_{2,31}=0.94$, NS). However, the lack of observed amphetamine effects may have been due in part to the anesthetized preparation and/or the exclusion of the two most anterior brain slices (1.5 mm in thickness) in the analysis to avoid partial volume effects as these slices also include the frontal pole and olfactory cortex, respectively. There were also no significant CBV responses detected in groups treated with vehicle/vehicle (Figure 3b) or VU0152100 alone (data not shown).

Functional connectivity analysis of the above phMRI data revealed multiple inter-regional correlations in neural activation elicited by amphetamine in rats, while pre-treatment with VU0152100 (56.6 mg/kg, i.p.) modified the amphetamine-induced correlation pattern; fewer significant inter-regional correlations were found in the group pre-treated with VU0152100 before amphetamine (data not shown). As shown in Figure 3c, permutation analysis identified significant differences between the two treatment

groups (the statistical threshold was set at $p<0.05$ and reported as *z*-scores), including retrosplenium-hippocampus ($\Delta z=3.4499$), NAS-motor cortex ($\Delta z=3.3348$), retrosplenium-motor cortex ($\Delta z=3.2268$), and NAS-medial thalamus ($\Delta z=3.1553$). Other ROI pairs with significant differences included the NAS-CP ($\Delta z=3.0555$), motor cortex with hippocampus ($\Delta z=2.8753$), and visual cortex ($\Delta z=2.7884$); PFC-sensory cortex ($\Delta z=2.3990$); and substantia nigra-amygdala ($\Delta z=2.3409$).

We assessed whether VU0152100 produced alterations in peripheral cardiovascular parameters, including blood pressure and heart rate, which might confound hemodynamic readouts of brain function (Ferrari *et al*, 2012). Compared with vehicle treatment, VU0152100 (56.6 mg/kg, i.p.) did not change arterial blood pressure ($F(treatment)_{1,72}=9.28$, $p<0.01$, $F(time)_{7,72}=1.44$, NS, and $F(interaction)_{7,72}=3.83$, NS) or heart rate ($F(treatment)_{1,72}=7.58$, $p<0.05$, $F(time)_{7,72}=7.41$, NS, and $F(interaction)_{7,72}=2.47$, NS) in awake, freely moving animals (Figures 5c and d).

VU0152100 Potentiates the Effects of a Sub-Threshold Dose of Oxotremorine on Dopamine Utilization in the Forebrain

Previous studies demonstrated that xanomeline produced dose-related increases in the dopamine metabolite DOPAC in the rodent striatum (Bymaster *et al*, 1994). Here we assessed whether VU0152100, in comparison with xanomeline, alters the ratio of monoamine metabolites/parent monoamines, an index of monoamine utilization, in post mortem brain tissue. In contrast to xanomeline (30 mg/kg), VU0152100 (10–100 mg/kg) alone did not increase dopamine or serotonin utilization in the PFC, NAS, or CP (Table 1: (PFC) DOPAC/DA ($F_{4,37}=5.79$, $p<0.01$), 5-HIAA/5-HT ($F_{4,39}=0.84$, NS); (NAS) DOPAC/DA ($F_{4,38}=8.08$, $p<0.001$), 5-HIAA/5-HT ($F_{4,39}=0.57$, NS); (CP): DOPAC/DA ($F_{4,38}=0.63$, NS), 5-HIAA/5-HT ($F_{4,38}=0.62$, NS)). However, when VU0152100 (56.6 mg/kg) was administered in combination with an ineffective dose of the non-selective mAChR agonist oxotremorine (0.01 mg/kg), we observed a marked increase in dopamine, but not serotonin, utilization in the PFC (DOPAC/DA ($F_{3,30}=4.01$, $p<0.05$), HVA/DA ($F_{3,28}=5.63$, $p<0.01$), and 5-HIAA/5-HT ($F_{3,30}=0.95$, NS)) and NAS (DOPAC/DA ($F_{3,30}=10.88$, $p<0.0001$), HVA/DA ($F_{3,30}=10.08$, $p<0.0001$), and 5-HIAA/5-HT ($F_{3,30}=0.92$, NS)) as shown in Table 2. In the CP, the combination of VU0152100 (56.6 mg/kg) with oxotremorine (0.01 mg/kg) reduced dopamine and serotonin utilization (DOPAC/DA ($F_{3,31}=11.04$, $p<0.0001$), HVA/DA ($F_{3,31}=7.27$, $p<0.001$), and 5-HIAA/5-HT ($F_{3,31}=5.09$, $p<0.01$)).

VU0152100 Reverses Amphetamine-Induced Increases in Dopamine Release in the NAS and CP

The finding that VU0152100 reverses effects of amphetamine in multiple behavioral models and on CBV in multiple brain regions, raises the possibility that this M_4 PAM may act in part by reducing effects of amphetamine on extracellular dopamine levels. The effects of VU0152100 in reversing amphetamine-induced increases in extracellular dopamine levels in the NAS and CP were assessed using *in vivo* microdialysis in rats at a dose of 56.6 mg/kg (i.p.), which produced maximum efficacy in blocking amphetamine-induced hyperlocomotion and disruption of PPI. As shown in Figures 4a and b, the reversal of amphetamine-induced hyperlocomotion produced by the 56.6 mg/kg dose of VU0152100 was comparable in magnitude to the effects observed in our initial dose response study (Figure 4a: $F(treatment)_{3,312}=30.6$, $p<0.001$, $F(time)_{12,312}=17.5$, $p<0.0001$, and $F(interaction)_{36,312}=34.8$, $p<0.001$; Figure 4b: $F(treatment)_{3,351}=27.1$, $p<0.001$, $F(time)_{12,351}=20.0$, $p<0.0001$, and $F(interaction)_{36,351}=30.7$, $p<0.001$). When administered alone, VU0152100 had no effect on basal dopamine release in either the NAS or CP (Figures 4c and d). However, when given in combination with amphetamine, VU0152100 at the 56.6 mg/kg i.p. dose produced a significant reduction of amphetamine-elicited dopamine release in both the NAS and CP (Figure 4c for NAS: $F(treatment)_{3,297}=19.9$, $p<0.001$, $F(time)_{12,297}=23.3$, $p<0.001$, and $F(interaction)_{36,297}=30.2$, $p<0.001$; Figure 4d for the CP: ($F(treatment)_{3,351}=18.8$, $p<0.001$, $F(time)_{12,351}=27.3$, $p<0.001$, and $F(interaction)_{36,351}=32.4$, $p<0.001$). As shown in Supplementary Figure S2 in the NAS, VU0152100 alone increased extracellular levels of the

Table 1 Effects of VU0152100 (10–100 mg/kg, i.p.) and Xanomeline (30 mg/kg, i.p.) on Dopamine and Serotonin Utilization in the Rat Forebrain

	Dose (mg/kg)	*N*	DOPAC/DA	5-HIAA/5-HT
Prefrontal cortex				
Vehicle		10	0.42 ± 0.04	0.44 ± 0.02
VU0152100	10.0	6	0.52 ± 0.03	0.49 ± 0.03
VU0152100	56.6	11	0.50 ± 0.02	0.47 ± 0.04
VU0152100	100.0	5	0.46 ± 0.04	0.54 ± 0.06
Xanomeline	30.0	6	0.68 ± 0.08[a]	0.50 ± 0.05
Nucleus accumbens				
Vehicle		11	0.21 ± 0.02	0.66 ± 0.07
VU0152100	10.0	6	0.24 ± 0.01	0.66 ± 0.03
VU0152100	56.6	11	0.27 ± 0.02	0.68 ± 0.04
VU0152100	100.0	6	0.26 ± 0.02	0.62 ± 0.05
Xanomeline	30.0	5	0.37 ± 0.01[a]	0.75 ± 0.03
Dorsal striatum				
Vehicle		10	0.13 ± 0.01	1.13 ± 0.06
VU0152100	10.0	6	0.14 ± 0.01	1.20 ± 0.07
VU0152100	56.6	11	0.13 ± 0.01	1.22 ± 0.07
VU0152100	100.0	6	0.14 ± 0.01	1.21 ± 0.02
Xanomeline	30.0	6	0.14 ± 0.01	1.26 ± 0.06

The effects of VU0152100 (10–100 mg/kg, i.p.) or vehicle on monoamine turnover in the medial prefrontal cortex, nucleus accumbens, and dorsolateral caudate-putamen were compared with xanomeline (30 mg/kg, i.p.). Data represent means ± SEM; monoamine and metabolite concentrations in vehicle-treated animals were: (PFC) DOPAC 0.30 ± 0.08, DA 0.84 ± 0.15 5-HIAA 4.67 ± 0.65, and 5-HT 10.3 ± 1.21; (NAS) DOPAC 15.38 ± 2.34, DA 71.27 ± 8.29, 5-HIAA 7.77 ± 0.53, and 5-HT 11.24 ± 1.55; (CP) DOPAC 19.77 ± 3.61, DA 135.30 ± 10.86, 5-HIAA 7.56 ± 0.36, and 5-HT 6.88 ± 0.75.
[a]$p<0.001$ *vs* vehicle (Bonferroni's test).

Table 2 VU0152100 Potentiates Effect of a Sub-Threshold Dose of Oxotremorine on Monoamine Turnover in the Forebrain

Compound 1	Dose	Compound 2	Dose	DOPAC/DA	HVA/DA	5-HIAA/5-HT
Prefrontal cortex						
Vehicle		Vehicle		0.41 ± 0.03	0.65 ± 0.07	0.37 ± 0.01
VU0152100	56.6	Vehicle		0.44 ± 0.03	0.78 ± 0.07	0.39 ± 0.02
Vehicle		Oxotremorine	0.01	0.35 ± 0.02	0.56 ± 0.05$^{\#}$	0.41 ± 0.03
VU0152100	56.6	Oxotremorine	0.01	0.47 ± 0.01$^{\&}$	0.86 ± 0.05$^{\&\&}$	0.39 ± 0.02
Nucleus accumbens						
Vehicle		Vehicle		0.22 ± 0.01	0.07 ± 0.01	0.439 ± 0.060
VU0152100	56.6	Vehicle		0.26 ± 0.01	0.10 ± 0.01	0.428 ± 0.031
Vehicle		Oxotremorine	0.01	0.27 ± 0.01	0.08 ± 0.01	0.503 ± 0.048
VU0152100	56.6	Oxotremorine	0.01	0.34 ± 0.02$^{***,\#\#,\&}$	0.13 ± 0.01$^{***,\&\&}$	0.527 ± 0.060
Dorsal striatum						
Vehicle		Vehicle		0.15 ± 0.01	0.10 ± 0.01	0.88 ± 0.03
VU0152100	56.6	Vehicle		0.16 ± 0.01	0.11 ± 0.01	0.94 ± 0.04
Vehicle		Oxotremorine	0.01	0.16 ± 0.01	0.09 ± 0.01	1.00 ± 0.05
VU0152100	56.6	Oxotremorine	0.01	0.12 ± 0.01$^{***,\#\#\#,\&\&\&}$	0.08 ± 0.01$^{*,\#\#\#}$	0.81 ± 0.02$^{\&}$

Following a 15-min pre-treatment with VU0152100 (56.6 mg/kg, i.p.) or vehicle, rats were injected with oxotremorine sesquifumarate (0.01 mg/kg, s.c.) or vehicle (water) and killed 30 min later for the assessment of tissue monoamines and metabolites. Data represent means ± SEM of 6–8 animals per group; monoamine and metabolite concentrations (ng/mg protein) in vehicle-treated animals were as follows: (PFC) DOPAC 0.0.48 ± 0.06, DA 1.22 ± 0.15, HVA 0.78 ± 0.07, 5-HIAA 2.13 ± 0.18, and 5-HT 5.86 ± 0.49; (NAS) DOPAC 14.97 ± 0.61, DA 69.49 ± 2.36, HVA 4.62 ± 0.38, 5-HIAA 2.64 ± 0.11, and 5-HT), 6.82 ± 1.06; (CP) DOPAC 15.65 ± 0.67, DA 102.5 ± 2.22, HVA 9.76 ± 0.42, 5-HIAA 3.08 ± 0.12, and 5-HT 3.51 ± 0.17.
$^{*}p<0.05$; $^{***}p<0.001$ *vs* vehicle/vehicle; $^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$ *vs* VU0152100/vehicle; $^{\&}p<0.05$; $^{\&\&}p<0.01$; $^{\&\&\&}p<0.001$ *vs* vehicle/oxotremorine (Bonferroni's test).

dopamine metabolites DOPAC (Supplementary Figure S2A: $F((\text{treatment})_{3,296}=16.3$, $p<0.001$, $F(\text{time})_{12,296}=3.87$, NS, and $F(\text{interaction})_{36,296}=21.2$, $p<0.001$) and HVA (Supplementary Figure S2B: $F(\text{treatment})_{3,296}=17.9$, $p<0.001$, $F(\text{time})_{12,296}=2.86$, NS, and $F(\text{interaction})_{36,296}=17.3$, $p<0.001$). In the CP, amphetamine alone or in combination with VU0152100 reduced DOPAC (Supplementary Figure S2C: $F(\text{treatment})_{3,334}=16.4$, $p<0.001$, $F(\text{time})_{12,334}=11.8$, $p<0.001$, and $F(\text{interaction})_{36,334}=17.7$, $p<0.001$) and HVA (Supplementary Figure S2D: $F(\text{treatment})_{3,331}=13.9$, $p<0.001$, $F(\text{time})_{12,331}=9.98$, $p<0.001$, and $F(\text{interaction})_{36,331}=24.0$, $p<0.001$).

VU0152100 does not Induce Catalepsy but Exacerbates Haloperidol-Induced Catalepsy

We assessed the time course for the potential induction of catalepsy, a preclinical model of motor impairments and extrapyramidal motor side effects, by VU0152100 when administered alone in comparison with haloperidol as a positive control in rats. As shown in Figure 5a, haloperidol (1.5 mg/kg, s.c.) produced a robust time-dependent induction of cataleptic behavior, significant at 30, 60, 120, and 240-min post-haloperidol administration, while in contrast VU0152100 (30–100 mg/kg, i.p.) was without effect across the dose range and time course tested ($F(\text{treatment})_{4,125}=9.29$, $p<0.0001$, $F(\text{time})_{1,125}=9.98$, $p<0.0001$, and $F(\text{interaction})_{16,125}=26.2$, $p<0.0001$; Figure 5a). However, when given in combination with submaximal doses of haloperidol (0.3 or 0.5 mg/kg), VU0152100 (100 mg/kg, i.p.) significantly enhanced cataleptic behavior compared with rats treated with haloperidol (0.3 or 0.5 mg/kg) alone ($F(\text{treatment})_{1,50}=28.6$, $p<0.0001$, $F(\text{dose})_{2,50}=20.53$, $p<0.0001$, and $F(\text{interaction})_{2,50}=4.876$, NS; Figure 5b).

VU0152100 does not Produce Side Effects Associated with Activation of Peripheral M_2 and M_3 mAChRs

Although, VU0152100 is highly selective for M_4 relative to the other muscarinic receptor subtypes *in vitro* (Brady *et al*, 2008), it was critical to assess the degree of selectivity of the actions of VU0152100 *in vivo* using the Modified Irwin Neurological Test battery as compared with the non-selective mAChR agonist oxotremorine (Jones *et al*, 2008). As depicted in Supplementary Table S2, oxotremorine (1.0 mg/kg, s.c.) produced a significant time-related induction of salivation ($F(\text{treatment})_{2,48}=96.0$, $p<0.01$, $F(\text{time})_{4,48}=37.7$, $p<0.0001$, and $F(\text{interaction})_{8,48}=37.7$, $p<0.0001$), lacrimation ($F(\text{treatment})_{2,48}=96.0$, $p<0.0001$, $F(\text{time})_{4,48}=37.7$, $p<0.0001$, and $F(\text{interaction})_{8,48}=37.7$, $p<0.0001$), piloerection ($F(\text{treatment})_{2,48}=164.6$, $p<0.0001$, $F(\text{time})_{4,48}=27.7$, $p<0.0001$, and $F(\text{interaction})_{8,48}=27.7$, $p<0.0001$), and decreased respiratory rate ($F(\text{treatment})_{2,48}=12.5$, $p<0.01$, $F(\text{time})_{4,48}=6.00$, $p<0.001$, and $F(\text{interaction})_{8,48}=6.00$, $p<0.0001$) and core body temperature ($F(\text{treatment})_{2,48}=16.1$, $p<0.001$, $F(\text{time})_{4,48}=2.19$, NS, and $F(\text{interaction})_{8,48}=3.41$, $p<0.01$); whereas VU0152100 (56.6 mg/kg, i.p.) produced no changes to any

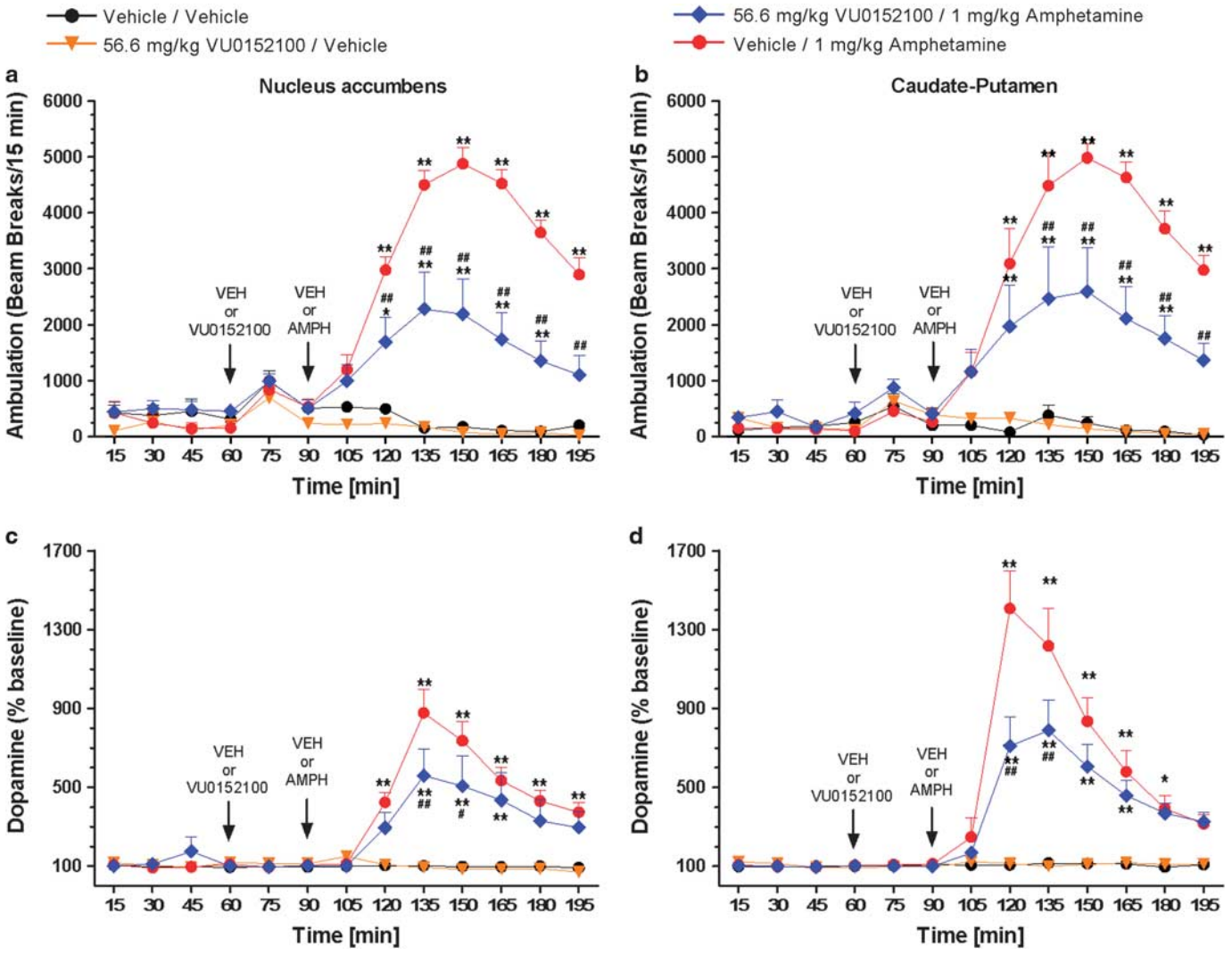

Figure 4 Pre-treatment with VU0152100 (56.6 mg/kg, i.p.) reverses amphetamine-induced (1 mg/kg, s.c.) hyperlocomotion (a, b) and reduces the amphetamine-elicited increase in extracellular dopamine levels in the nucleus accumbens and caudate-putamen (c, d). Data are mean ± SEM of 6–8 rats per group; $^{*}p<0.05$, $^{**}p<0.01$ *vs* vehicle/vehicle; $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* vehicle/amphetamine (Dunnett's test).

of the measurements in the Modified Irwin Neurological Test battery.

DISCUSSION

In recent years, selective activators of mAChRs have emerged as a new approach for the treatment of psychotic symptoms and cognitive disturbances associated with various neuropsychiatric disorders. In this study, the selective M_4 PAM VU0152100 displayed dose-dependent efficacy across multiple behavioral tasks that predict APD-like activity. In contrast to orthosteric mAChR agonists, VU0152100 did not produce adverse effects associated with non-selective activation of peripheral mAChR subtypes or impairments in locomotor activity. phMRI studies also indicated that VU0152100 reduces amphetamine-induced activation of the NAS and CP, as well as certain cortical, thalamic, and hippocampal areas. Functional connectivity analyses of the phMRI data revealed that VU0152100 modulated the activity of neural circuits that included the NAS, retrosplenial and motor cortices, hippocampus and thalamus. Finally, the behavioral effects of VU0152100 were accompanied by reversal of amphetamine-induced increases in extracellular DA levels in the NAS and CP, suggesting that the APD-like activity is mediated in part through modulation of mesolimbic and mesostriatal dopamine function.

The present findings suggest that VU0152100 reverses amphetamine-induced behavioral and neurochemical changes through potentiation of M_4 mAChRs. Although the effects of the M_4 PAM could be due to a drug–drug interaction between VU0152100 and amphetamine, this seems unlikely because only drug-naive rats were used in acute dosing paradigms that do not allow sufficient time for induction of amphetamine metabolism by VU0152100 (Fahmi and Ripp, 2010; FDA, 2012). Moreover, we previously reported that VU0152100 does not interact with a

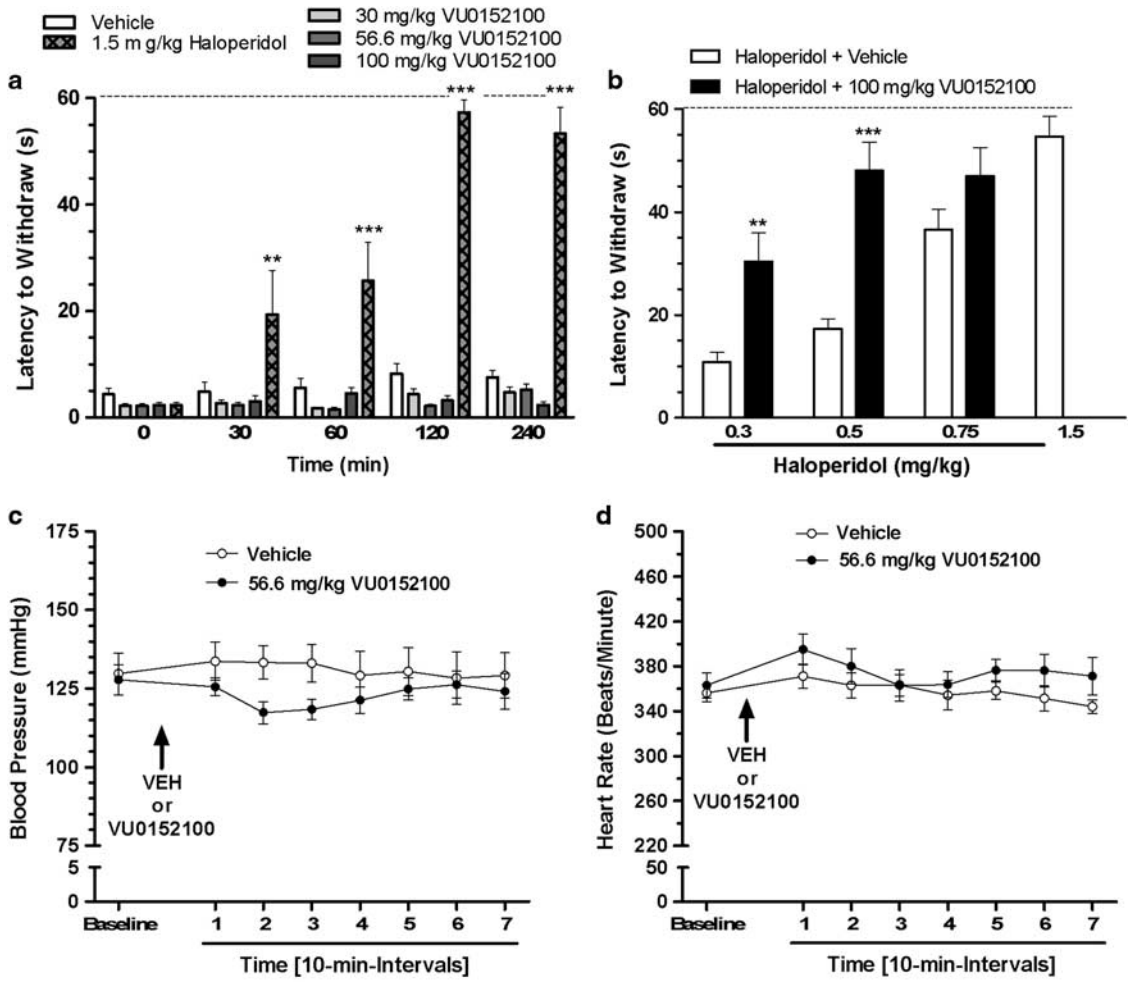

Figure 5 VU0152100 does not cause motor and cardiovascular side effects when given alone, but exacerbates haloperidol-induced catalepsy. (a) VU0152100 (30–100 mg/kg, i.p.) does not induce catalepsy, whereas haloperidol (1.5 mg/kg, s.c.) causes a time-dependent increase in catalepsy (**$p<0.01$, ***$p<0.001$ *vs* vehicle (Bonferroni's test)). (b) VU0152100 (100 mg/kg, i.p.) increases the cataleptogenic effects of submaximal (0.3–0.5 mg/kg, i.p.) doses of haloperidol (**$p<0.01$, ***$p<0.001$ *vs* vehicle/haloperidol (Bonferroni's test)). (c, d) Lack of cardiovascular effects of VU0152100. Rats were injected (arrow) after collecting 30-min baseline data. Following a 15-min post-injection period (acclimation), blood pressure and heart rate data were collected in 10-min intervals. (c) VU0152100 (56.6 mg/kg, i.p.) does not alter blood pressure and (d) heart rate in awake animals. Although two-way ANOVA revealed a significant treatment effect on blood pressure and heart rate there were we no significant differences between vehicle- and VU0152100-treated rats at any time point (Bonferroni test).

wide variety of GPCRs, ion channels, transporters, and enzymes that are present in the CNS and could be relevant for the *in vivo* responses measured (Brady *et al*, 2008). In this initial study, VU0152100 did displace binding of a ligand to the dopamine transporter with a 46% inhibition at 10 μM. As a follow-up, we have now estimated the *in vitro* IC_{50} values for VU0152100 at both the human and rat DAT to be 5.98 μM and >10 μM, respectively. However, at the highest dose of 56.6 mg/kg used in the previous and current studies, VU0152100 achieved only a Cmax free brain concentration of ~340 nM. This unbound brain concentration of VU0152100 is closely aligned with the concentrations required to potentiate M_4 signaling, but well below the concentrations that would be required to impact binding and function of the dopamine transporter. We also performed further studies to definitively confirm a critical role for M4 in the actions of VU0152100. Specifically, we evaluated the effects of VU0152100 in M4 KO mice and demonstrated that the effects of VU0152100 in reversing amphetamine-induced hyperlocomotion are absent in the M4 KO mice. It is, therefore, unlikely that the observed APD-like activity of VU0152100 is because of an off-target-related mechanism. Consistent with this, VU0152100 also reduces cocaine-induced hyperlocomotion, self-administration, and striatal dopamine release, and these effects are abolished in mice with targeted deletion of M_4 mAChRs in striatal neurons expressing DA D1 receptors (D1-M_4 KO mice; Dencker *et al*, 2012). These studies, taken together with a growing body of research discussed below suggest that M_4 PAMs reduce dopaminergic signaling and have a range of APD-like effects in rodent models.

The present findings support and extend evidence that M_4 mAChRs are the likely target involved in the APD-like activity reported with the M_1/M_4-preferring muscarinic agonist xanomeline (see Jones *et al*, 2012). M_4 mAChRs are expressed in multiple cortical and limbic regions thought to be disrupted in neuropsychiatric disorder like schizophrenia, where they function as both pre- and postsynaptic

modulatory receptors (Bubser *et al,* 2012; Tzavara *et al,* 2004). M_4 KO mice display a hyperdopaminergic phenotype with increased basal and psychostimulant-induced locomotor activity coupled with increased basal and psychostimulant-induced extracellular dopamine levels in the NAS (Gomeza *et al,* 1999; Schmidt *et al,* 2011; Tzavara *et al,* 2004; Koshimizu *et al,* 2012). Interestingly, muscarinic agonist-induced reductions in accumbal and striatal DA release are absent in M_4 KO mice, suggesting that M_4 negatively regulates dopaminergic tone in these regions (Threlfell *et al,* 2010). M_4 KO mice also exhibit deficits in PPI and social interaction and enhanced dopamine D1 receptor-mediated effects on locomotor activity, which are recapitulated in D1-M_4 KO mice (Gomeza *et al,* 1999; Jeon *et al,* 2010; Koshimizu *et al,* 2012; Thomsen *et al,* 2010). Importantly, the APD-like effects of xanomeline are also abolished in both M_4 KO and D1-M_4 KO mice (Woolley *et al,* 2009; Dencker *et al,* 2011).

Our data with VU0152100 also validate previously reported effects using the structurally distinct M_4 PAM LY2033298 when given in combination with a sub-threshold dose of the non-selective mAChR agonist oxotremorine (Chan *et al,* 2008; Leach *et al,* 2010; Suratman *et al,* 2011). However, LY2033298 does not provide an optimal tool compound for rodent studies in that it has relatively low potency at the rat M_4 mAChR (Chan *et al*, 2008; Leach *et al*, 2010) and displays only weak cooperativity with ACh, the endogenous agonist of M_4 (Suratman *et al*, 2011), but has high cooperativity with the synthetic mAChR agonist oxotremorine. As LY2033298 induces robust potentiation of oxotremorine, but not ACh effects on M_4, it has no behavioral effects when administered alone unless co-administration with oxotremorine (Suratman *et al*, 2011). Our finding that VU0152100 has robust efficacy in potentiating ACh responses *in vitro* and in multiple *in vivo* models when administered alone supports this interpretation. These findings suggest that M_4 PAMs that display high cooperativity with ACh at the rodent M_4 receptor can potentiate responses to endogenous cholinergic tone to induce robust APD-like activity.

Our finding that VU0152100 attenuates amphetamine-induced increases in extracellular DA in the NAS and CP is consistent with previous studies discussed above and suggests that the *in vivo* APD-like effects of VU0152100 are mediated in part by reducing DA release. Currently, the mechanism by which M_4 activation regulates mesolimbic and mesostriatal dopaminergic transmission is unknown. M_4 has been postulated to act as an inhibitory autoreceptor on terminals of cholinergic neurons originating in the pedunculopontine and laterodorsal tegmental nuclei that excite dopaminergic neurons in the ventral tegmental area (Holmstrand and Sesack, 2011; Yeomans, 2012) and activation of M_4 autoreceptors in the VTA could reduce cholinergic excitation of dopamine neurons (Tzavara *et al,* 2004). In addition, activation of cholinergic interneurons in the NAS elicits dopamine release (Cachope *et al,* 2012). If M_4 acts as an inhibitory autoreceptor in NAS and CP, potentiating M_4 in each of these regions could also reduce cholinergic-mediated dopamine release. However, it is important to note that while M_4-mediated decreases in DA release may contribute to the *in vivo* effects of VU0152100, other mechanisms are also likely contribute to the APD-like actions of M_4 PAMs. Previous studies reported that xanomeline reverses the behavioral effects of both amphetamine and the direct dopamine receptor agonist apomorphine and that these effects are eliminated with genetic deletion of M_4 (Jones *et al*, 2005; Stanhope *et al*, 2001; Dencker *et al*, 2011). The ability of M_4 activation to inhibit responses to a direct acting dopamine receptor agonist suggests that M_4 activation must also act at a level downstream of DA release to induce its overall *in vivo* effects. M_4 receptors are expressed at multiple levels in basal ganglia and forebrain regions and VU0152100 provides an excellent tool for future mechanistic studies aimed at elucidating other actions of M_4 PAMs that may contribute to these overall *in vivo* effects.

Consistent with the behavioral and microdialysis results, VU0152100 suppressed amphetamine-induced increases in CBV in the NAS and CP as well as the medial thalamus, hippocampus, and retrosplenial cortex. Changes in central hemodynamic responses can reflect alterations in neural activity (Kim and Ogawa, 2012) and these alterations can be used to understand spatio-temporal effects of psychoactive drugs (Hackler *et al*, 2010; Chen *et al*, 1997; Schwarz *et al*, 2004). The effects of VU0152100 on brain activity in multiple regions suggest that actions in areas outside of the basal ganglia may also contribute to the *in vivo* effects of VU0152100. These findings are consistent with previous evidence that cortical projections modulate DA function in the striatum (Desole *et al* 1992): specifically, the motor and retrosplenial cortices innervate the lateral and medial striatum, respectively, whereas the PFC innervates the NAS (Ebrahimi *et al* 1992; Sesack *et al* 1989; van Groen and Wyss 1992, 2003). Using functional connectivity analyses (Schwarz *et al*, 2007a, b), we characterized the regional correlation patterns in response amplitude to VU0152100. Interestingly, pre-treatment with VU0152100 attenuated several strong connectivity patterns of the NAS and retrosplenial cortex with other brain areas after amphetamine challenge, including correlated activity between NAS and hippocampus, motor cortex, and CP and the retrosplenial cortex with motor cortex and hippocampus. Importantly, the low isoflurane (0.88%) necessary for proper maintenance anesthesia to prevent movement-related artifacts did not result in negative CBV changes to amphetamine. Also, the observed changes in our phMRI studies were not attributable to changes in blood pressure or heart rate. Although additional studies are needed to further investigate the underlying mechanisms of the observed functional changes by VU0152100, these data suggest a role of M_4 in direct and indirect modulation of dopaminergic signaling in multiple brain regions.

Although previous studies indicate that activation of M_1 mAChRs is critical for learning, memory, and executive functions, the role of M_4 mAChRs in modulating cognitive function remains unclear (Bubser *et al*, 2012; Shirey *et al*, 2009; Digby *et al*, 2012; Ma *et al*, 2009). We found that VU0152100 alone had no effect on PPI or the acquisition of contextual fear conditioning, suggesting that M_4 may not be required for normal hippocampal learning and memory processes in young adult rats. These data are in agreement with recent reports that M_4 KO mice display normal spatial reference, working, and episodic-like hippocampal memory functions in the Morris water maze test (Koshimizu *et al,*

2012). Interestingly, we did observe that VU0152100 reversed amphetamine-induced deficits in PPI and contextual fear conditioning. However, as the higher doses of amphetamine used in the cognitive studies can induce increases in both dopamine and norepinephrine (McKittrick and Abercrombie 2007), it will be important in future studies to confirm that these effects of VU0152100 are mediated predominately through reversal of dopaminergic signaling. Finally, VU0152100 provides an important M_4-selective tool compound that used along with recently reported M_1-selective PAMs (Ma *et al,* 2009; Shirey *et al,* 2009) or allosteric agonists (Lebois *et al,* 2010; Digby *et al,* 2012) will allow a more complete understanding of the relative roles of these two mAChR subtypes in regulating multiple aspects of cognitive function.

Both M_4 KO and D1-M_4 KO mice display attenuated antipsychotic-induced catalepsy (Jeon *et al,* 2010; Fink-Jensen *et al,* 2011). Our data showed that VU0152100 did not induce catalepsy over the dose range that produced APD-like activity, consistent with the lack of induction in Fos-li expression in the CP. These data suggest that M_4 PAMs may not induce the extrapyramidal side effects observed with many APDs. However, VU0152100 did potentiate the induction of catalepsy when given in combination with sub-threshold doses of haloperidol. These findings indicate that the clinical use of VU0152100 may need to be as a stand-alone therapy or restricted to augmentation of atypical APDs.

In summary, these data are consistent with the interpretation that selective modulation of M_4 mAChRs has effects in CNS circuits subserving the actions of APDs. Our results support the development of selective M_4 PAMs as a novel treatment approach for the psychotic symptoms and cognitive impairments observed in psychiatric and neurologic disorders, including schizophrenia.

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