

Selective Activation of M₄ Muscarinic Acetylcholine Receptors Reverses MK-801-Induced Behavioral Impairments and Enhances Associative Learning in Rodents

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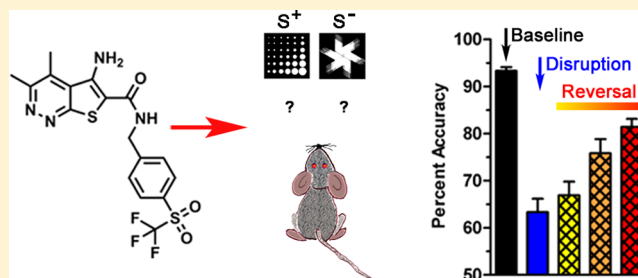
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ABSTRACT: Positive allosteric modulators (PAMs) of the M₄ muscarinic acetylcholine receptor (mAChR) represent a novel approach for the treatment of psychotic symptoms associated with schizophrenia and other neuropsychiatric disorders. We recently reported that the selective M₄ PAM VU0152100 produced an antipsychotic drug-like profile in rodents after amphetamine challenge. Previous studies suggest that enhanced cholinergic activity may also improve cognitive function and reverse deficits observed with reduced signaling through the N-methyl-D-aspartate subtype of the glutamate receptor (NMDAR) in the central nervous system. Prior to this study, the M₁ mAChR subtype was viewed as the primary candidate for these actions relative to the other mAChR subtypes. Here we describe the discovery of a novel M₄ PAM, VU0467154, with enhanced *in vitro* potency and improved pharmacokinetic properties relative to other M₄ PAMs, enabling a more extensive characterization of M₄ actions in rodent models. We used VU0467154 to test the hypothesis that selective potentiation of M₄ receptor signaling could ameliorate the behavioral, cognitive, and neurochemical impairments induced by the noncompetitive NMDAR antagonist MK-801. VU0467154 produced a robust dose-dependent reversal of MK-801-induced hyperlocomotion and deficits in preclinical models of associative learning and memory functions, including the touchscreen pairwise visual discrimination task in wild-type mice, but failed to reverse these stimulant-induced deficits in M₄ KO mice. VU0467154 also enhanced the acquisition of both contextual and cue-mediated fear conditioning when administered alone in wild-type mice. These novel findings suggest that M₄ PAMs may provide a strategy for addressing the more complex affective and cognitive disruptions associated with schizophrenia and other neuropsychiatric disorders.

KEYWORDS: Allosteric modulator, antipsychotic, cognitive enhancement, M₄ muscarinic, MK-801, VU0467154



Normal signaling of the N-methyl-D-aspartate subtype of the glutamate receptor (NMDAR) is critical for the acquisition and consolidation of associative learning and memory functions, and its disruption is thought to underlie many of the symptoms associated with schizophrenia and other neuropsychiatric disorders.^{1–4} Previous studies suggest that enhancement of central cholinergic neurotransmission can improve

cognitive performance and restore impairments associated with reduced NMDAR signaling.^{5,6} Of the five muscarinic acetylcholine receptor (mAChR) subtypes (M₁–M₅^{7,8}), M₁ has

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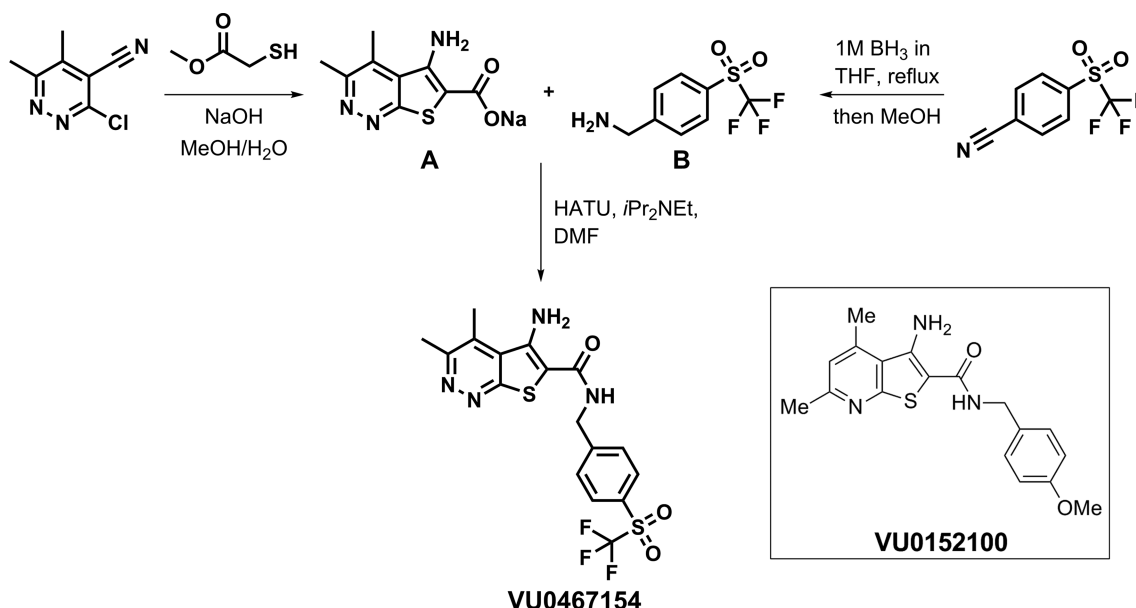


Figure 1. Synthesis and structure of VU0467154. For comparison, the structure of the M_4 PAM VU0152100 is shown as an inset. Reagents and conditions for each step are as follows: (a) methyl 2-mercaptoacetate, sodium hydroxide, methanol, microwave at 150 °C for 30 min; (b) BH_3 , THF (borane-tetrahydrofuran), tetrahydrofuran, reflux, ca. 18 h; (c) A + B, HATU, *N,N*-diisopropyl-ethylamine at room temperature.

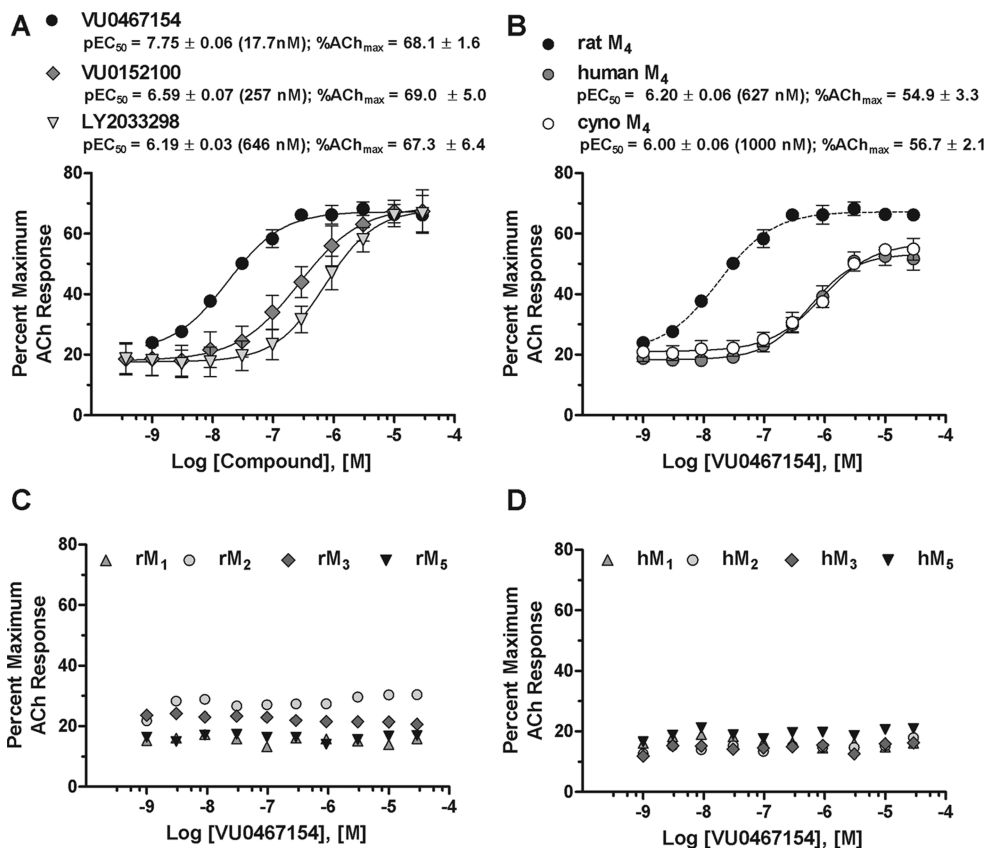


Figure 2. VU0467154 potentiates acetylcholine (ACh) responses in M_4 -expressing cell lines. (A) VU0467154 is more potent at rat M_4 than VU0152100 and LY2033298. (B) Robust potentiation of an ACh EC₂₀ is seen across species at rat, human, and cynomolgus monkey (cyno) M_4 . VU0467154 does not potentiate the ACh response at rat (C) and human (D) M_1 , M_2 , M_3 , or M_5 in calcium mobilization assays. Data were normalized as a percentage of the maximum response to 10 μ M ACh and are shown as means ± SEM of at least three independent experiments.

historically been regarded as the primary candidate for these actions. Previous studies have reported that activation of M_1 potentiates NMDAR currents in hippocampal pyramidal cells, increases excitatory postsynaptic currents in medial prefrontal

cortical neurons, improves fear conditioning, and reverses deficits in discrimination reversal learning observed in a mouse model of Alzheimer's disease.^{9–14} In contrast, M_1 knockout mice display decreased hippocampal long-term potentiation and

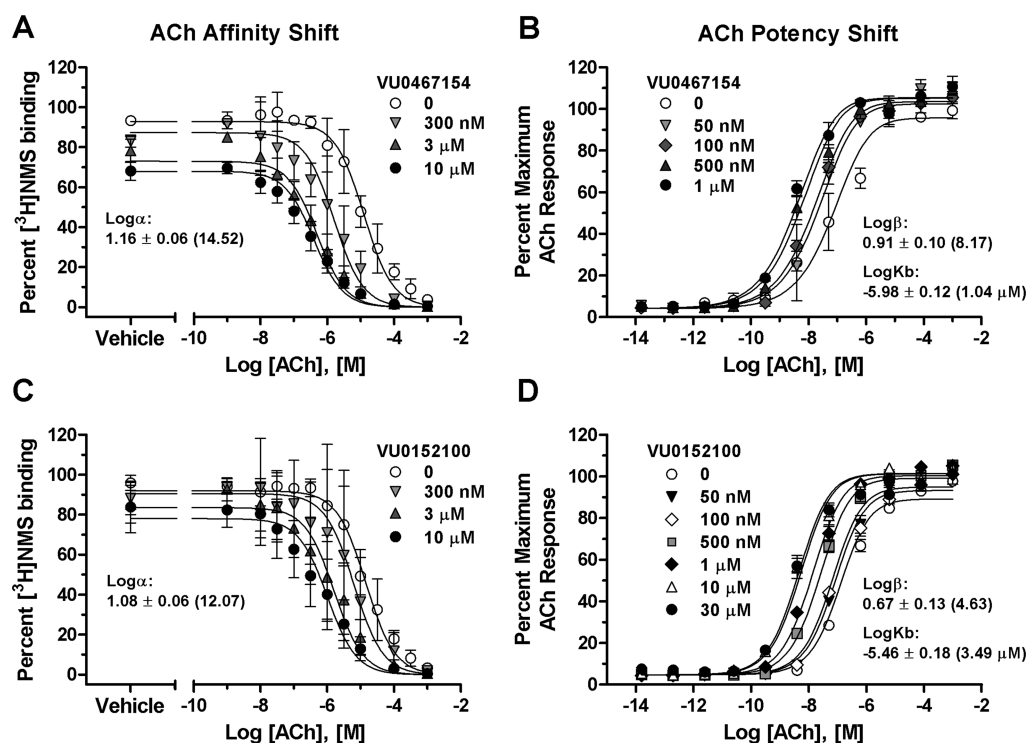


Figure 3. Increasing fixed concentrations of VU0467154 (A) and VU0152100 (C) increase the acetylcholine (ACh) affinity at rat M_4 as measured by ACh-induced displacement of [3 H]*N*-methylscopolamine ([3 H]NMS) binding. VU0467154 (B) and VU0152100 (D) cause a progressive leftward shift of an ACh concentration–response curve at rat M_4 . Data are shown as means \pm SEM of at least three independent experiments.

impaired performance in medial prefrontal cortex (PFC)-dependent cognitive tasks.¹⁵ While the involvement of M_1 in these processes has been well characterized, the contribution of the other mAChR subtypes has not been adequately investigated due to the lack of highly subtype-selective mAChR ligands.

Over the past decade, we and others have developed subtype-selective mAChR ligands that do not target the orthosteric binding site of acetylcholine (ACh), which is highly conserved across the mAChR subtypes, but instead act at more topographically distinct allosteric sites. This strategy has led to the discovery of several highly selective M_4 positive allosteric modulators (PAMs). These modulators do not activate the receptor directly, but substantially potentiate the M_4 response to ACh by increasing the affinity of M_4 for ACh and/or the efficiency of M_4 coupling to signaling partners, such as G proteins.^{16–20} Recently, we reported that the M_4 PAM VU0152100 exhibits an antipsychotic drug (APD)-like profile in rodents comparable to the M_1/M_4 -preferring agonist xanomeline.^{6,18}

In the present study, we describe the discovery of 5-amino-3,4-dimethyl-*N*-(4-((trifluoromethyl)sulfonyl)benzyl)thieno[2,3-*c*]pyridazine-6-carboxamide (VU0467154), a novel M_4 PAM with enhanced *in vitro* potency and improved pharmacokinetic properties relative to other published M_4 PAMs, which allows for extensive characterization of the role of M_4 *in vivo*. Using VU0467154, we demonstrated that selective potentiation of M_4 signaling could ameliorate the behavioral and associative learning impairments induced by the noncompetitive NMDAR antagonist MK-801 in wild-type but not in M_4 knock-out (KO) mice. Moreover, we showed that VU0467154 enhances the acquisition of contextual and cue-dependent fear conditioning when administered alone in wild-type mice. Collectively, these findings

provide new evidence to support a role of M_4 in the modulation of cognitive function mediated by NMDAR activation.

RESULTS AND DISCUSSION

VU0467154 Is a Potent M_4 Positive Allosteric Modulator in Recombinant Systems. The novel M_4 PAM 5-amino-3,4-dimethyl-*N*-(4-((trifluoromethyl)sulfonyl)benzyl)thieno[2,3-*c*]pyridazine-6-carboxamide (VU0467154) was synthesized using the synthetic scheme illustrated in Figure 1 and detailed in the Methods.

Calcium mobilization assays were conducted to determine the potency of VU0467154 at the rat M_4 receptor for potentiating an EC₂₀ concentration of ACh. VU0467154 robustly potentiated the response to ACh with a pEC₅₀ of 7.75 ± 0.06 (17.7 nM) and 68% of the E_{max} of ACh (ACh_{max}) (see Figure 2A). In comparison, the potencies of previously identified M_4 PAMs VU0152100 and LY2033298 were considerably lower than that of VU0467154 with pEC₅₀ values of 6.59 ± 0.07 (257 nM) and 6.19 ± 0.03 (646 nM), respectively. Both compounds produced comparable maximum responses to VU0467154 at 69% and 67% of the ACh_{max}, respectively. To assess potential species-specific differences in the effects of VU0467154 at M_4 , calcium mobilization assays were also conducted in cell lines expressing the human or cynomolgus monkey (cyno) M_4 receptors (Figure 2B). VU0467154 was more potent at rat M_4 compared with either the human M_4 (pEC₅₀ = 6.20 ± 0.06 [627 nM]) or cyno M_4 (pEC₅₀ = 6.00 ± 0.09 [1000 nM]) receptors. The efficacy of VU0467154 was also greater at the rat M_4 than at the human M_4 (55% ACh_{max}) or cyno M_4 (57% ACh_{max}) receptors. The ability of VU0467154 to potentiate ACh responses was M_4 -selective, as demonstrated by the lack of a calcium response at the rat and human M_1 , M_2 , M_3 , or M_5 receptors, respectively (Figure 2C,D).

Table 1. Lack of Off-Target Binding of VU0467154 at CNS-Relevant Receptors, Transmitter Transporters, and Cation Channels

target	species	tissue/cell line	radioligand	% inhibition ^a
Acetylcholine Receptors				
M ₁ muscarinic	human	CHO-K1 cells	[³ H]N-methylscopolamine	3
nicotinic	human	IMR-32 cells	[¹²⁵ I]epibatidine	−4
α1 nicotinic	human	RD cells	[¹²⁵ I]α-bungarotoxin	1
Dopamine Receptors				
D ₁	human	CHO cells	[³ H]SCH-23390	9
D _{2S}	human	CHO cells	[³ H]spiperone	6
D ₃	human	CHO cells	[³ H]spiperone	10
D _{4,2}	human	CHO-K1 cells	[³ H]spiperone	15
Serotonin Receptors				
5-HT _{1A}	human	CHO-K1 cells	[³ H]8-OH-DPAT	16
5-HT _{2B}	human	CHO-K1 cells	[³ H]LSD	8
5-HT ₃	human	HEK-293 cells	[³ H]GR-65630	12
γ-Aminobutyric Acid (GABA) Receptors				
GABA _A	rat	brain (without cerebellum)	[³ H]flunitrazepam	28
GABA _A	rat	brain (without cerebellum)	[³ H]muscimol	3
GABA _{B1A}	human	CHO cells	[³ H]CGP-54626	14
Glutamate Receptors				
kainate	rat	brain (without cerebellum)	[³ H]kainic acid	3
NMDA (glycine)	rat	cerebral cortex	[³ H]MDL 105,519	14
NMDA (phencyclidine)	rat	cerebral cortex	[³ H]TCP	15
Adenosine Receptors				
A ₁	human	CHO cells	[³ H]DPCPX	20
A _{2A}	human	HEK-293 cells	[³ H]CGS-21680	10
A ₃	human	CHO-K1 cells	[¹²⁵ I]AB-MECA	12
Adrenergic Receptors				
α _{1A}	rat	submaxillary gland	[³ H]prazosin	17
α _{1B}	rat	liver	[³ H]prazosin	11
α _{1D}	human	HEK-293 cells	[³ H]prazosin	5
α _{2A}	human	insect Sf9 cells	[³ H]MK-912	13
β ₁	human	CHO-K1 cells	[¹²⁵ I]cyanopindolol	3
β ₂	human	CHO cells	[³ H]CGP-12177	9
Opiate Receptors				
δ ₁	human	HEK-293 cells	[³ H]naltrindole	−3
κ	human	HEK-293 cells	[³ H]diprenorphine	−2
μ	human	CHO-K1 cells	[³ H]diprenorphine	16
Histamine Receptors				
H ₁	human	CHO-K1 cells	[³ H]pyrilamine	5
H ₂	human	CHO-K1 cells	[¹²⁵ I]aminopotentidine	0
H ₃	human	CHO-K1 cells	[³ H]N-α-methylhistamine	0
Purinergic Receptors				
P _{2X}	rabbit	urinary bladder	[³ H]α,β-methylene-ATP	22
P _{2Y}	rat	brain	[³⁵ S]ATP-αS	21
Melatonin Receptors				
MT ₁	human	CHO-K1 cells	[¹²⁵ I]2-iodomelatonin	18
Bradykinin Receptors				
B ₁	human	IMR-90 cells	[³ H](des-Arg ¹⁰)-kallidin	23
B ₂	human	Chem-1 cells	[³ H]bradykinin	3
Neuropeptide Y Receptors				
Y ₂	human	KAN-TS cells	[¹²⁵ I]peptide YY	4
Sigma Receptors				
σ ₁	human	Jurkat cells	[³ H]haloperidol	13
Imidazoline Receptors				
I ₂ (central)	rat	cerebral cortex	[³ H]idazoxan	−13
glucocorticoid receptor	human	insect cells	[³ H]dexamethasone	15
phorbol ester receptor	mouse	brain	[³ H]PDBu	21
Transmitter Transporters				
DAT ^b	human	CHO-K1	[¹²⁵ I]RTI-55	12
NET ^c	human	MDCK cells	[¹²⁵ I]RTI-55	8
SERT ^d	human	HEK-293 cells	[³ H]paroxetine	5

Table 1. continued

target	species	tissue/cell line	radioligand	% inhibition ^a
Transmitter Transporters				
GABA-T ^e	rat	cerebral cortex	[³ H]GABA	5
Calcium Channels				
L-type (benzothiazepine)	rat	brain	[³ H]diltiazem	30
L-type (dihydropyridine)	rat	cerebral cortex	[³ H]nitrendipine	26
N-type	rat	frontal brain	[¹²⁵ I] ω -conotoxin GVIA	-11
Potassium Channels				
K _{ATP}	hamster	pancreatic HIT-T15 β cells	[³ H]glyburide	19

^aDisplacement of radioligand at 10 μ M VU0467154. ^bDopamine transporter. ^cNorepinephrine transporter. ^dSerotonin transporter. ^eGABA transporter.

Radioligand binding assays and progressive fold-shift experiments were conducted followed by application of the operational model of allosterism²¹ to determine the affinity and efficacy of modulation of VU0467154 at the rat M₄ receptor. For these experiments, both VU0467154 and VU0152100 were utilized since they possessed different potencies in the calcium functional assay. To determine the ability of VU0467154 or VU0152100 to alter the affinity of ACh, radioligand binding studies were performed with [³H]N-methylscopolamine ([³H]NMS) in the presence of fixed concentrations of M₄ modulator (300 nM to 10 μ M) and increasing concentrations of ACh (1 nM to 1 mM). As shown in Figure 3A, VU0467154 produced a leftward shift in the ACh concentration–response curve. The increased ability of ACh to displace [³H]NMS binding resulted from a 14.5-fold increase in M₄ affinity for ACh (log α = 1.16 \pm 0.06). VU0152100 produced a similar leftward shift in the ACh affinity of 12.1-fold (log α = 1.08 \pm 0.06). Progressive fold-shift experiments were performed using the calcium mobilization assay with fixed concentrations of VU0467154 (50 nM to 1 μ M) or VU0152100 (50 nM to 30 μ M) and increasing concentrations of ACh (1.5 fM to 1 mM). Increasing concentrations of VU0467154 resulted in a leftward shift of the ACh concentration–response curve with an approximate 62-fold shift at 1 μ M VU0467154 (Figure 3B). Similarly, VU0152100 induced an approximate 60-fold leftward shift in the ACh concentration–response curve at 30 μ M (Figure 3D). The fold-shift for VU0152100 at 1 μ M (the maximum concentration used for VU0467154) was approximately 23-fold. The operational model of allosterism²¹ was applied utilizing the ACh affinity determined from the radioligand binding assay. This resulted in a predicted affinity (log K_i) of -5.98 (1.0 μ M) and an efficacy cooperativity factor (log β) between ACh and VU0467154 of 0.91 (8.17). Both the predicted M₄ affinity of VU0152100 (-5.46 [3.5 μ M]) and its efficacy factor 0.67 (4.63) were slightly lower compared with VU0467154.

VU0467154 Exhibits Little to No Ancillary off-Target Pharmacology When Assessed *in Vitro*. To assess the selectivity of VU0467154 for M₄ relative to other potential central nervous system (CNS) targets, the binding of this compound was evaluated in the Ricerca lead profiling screen, a radioligand binding panel consisting of 57 GPCRs, ion channels, and transporters. Included in the panel were receptors commonly engaged by clinically used antipsychotic drugs, such as D₂ dopamine (DA), serotonin 5-HT_{2A} and 5-HT_{2C}, and H₁ histamine receptors^{22,23} as well as the DA, norepinephrine, and serotonin transporters, and calcium and potassium channels. At all targets included in this panel, VU0467154 (10 μ M) caused \leq 30% displacement of the radioligand used for the assessment; for the majority of targets, VU0467154 exhibited \leq 20%

displacement (see Table 1). The only significant off-target activity of VU0467154 was observed at the adenosine transporter following a larger, secondary screen (Cerep ancillary pharmacology radioligand binding panel). In this screen, VU0467154 exhibited K_i values >30 μ M at all molecular targets (data not shown), with the exception of a 98 nM K_i for displacement of [³H]NTBI binding to the guinea pig adenosine transporter. In a subsequent functional assay employing cells expressing the human adenosine transporter, VU0467154 inhibited [³H]-adenosine uptake with an IC₅₀ of 240 nM.

VU0467154 Exhibits a Favorable Pharmacokinetic Profile for Studies in Rodents. In rats, VU0467154 exhibited low clearance from plasma (CL_p = 7.8 mL/min/kg) with a moderate volume of distribution at steady-state (V_{ss} = 3.1 L/kg), a long half-life (t_{1/2} = 5.7 h), and a long mean residence time (MRT = 6.8 h) following a single IV administration of 1 mg/kg (Table 2 and Figure 4A,C).

Negligible biliary and urinary excretion of the parent compound was observed, suggesting metabolism is the predominant mechanism of clearance (data not shown). A single PO administration of VU0467154 (3 mg/kg) revealed high bioavailability (%F = 61) and a maximum concentration

Table 2. Rat Pharmacokinetic Properties of VU0467154

route (dose, n)	rat PK parameter ^a	value
IV (1 mg/kg, 2)	CL _p (mL/min/kg)	7.8
	V _{ss} (L/kg)	3.1
	elimination t _{1/2} (h)	5.7
	MRT (h)	6.8
	F (%)	61
PO (3 mg/kg, 2)	C _{max,p} (μ M)	0.7
	T _{max,p} (h)	3
	AUC _{0-∞} (μ M·h)	9.0
	F (%)	33
PO (10 mg/kg, 2)	C _{max,p} (μ M)	1.3
	T _{max,p} (h)	2
	AUC _{0-∞} (μ M·h)	17
	C _{max,p} (μ M)	0.3
	T _{max,p} (h)	2
IP (10 mg/kg, 2)	AUC _{0-∞} (μ M·h)	3.4
	brain/plasma K _p ^b	0.49
	brain/plasma K _{p,uu} ^c	1.1

^aAUC, area-under-the curve; CL_p, plasma clearance; C_{max,p}, maximum plasma concentration; %F, bioavailability (%); K_p, partition coefficient; MRT, mean residence time; T_{1/2}, half-life; T_{max,p}, time to reach maximum plasma concentration; V_{ss}, volume of distribution at steady-state ^bC_{plasma} and C_{brain} at 0.5 h postadministration; ^c*In vitro* rat fu_{plasma} (0.031), fu_{brain} (0.067).

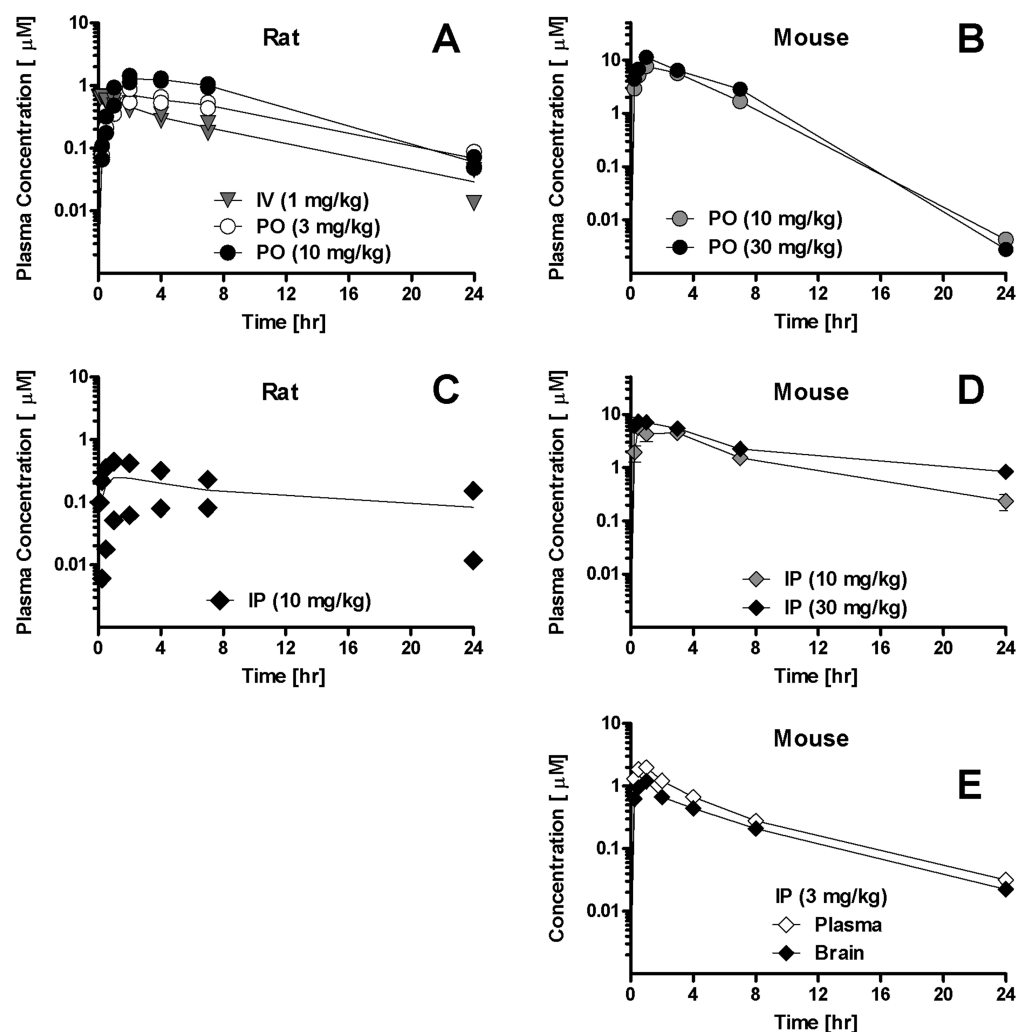


Figure 4. Time course of plasma and brain levels of VU0467154 following systemic administration to rats and mice. Panels A–D show plasma concentrations after IV (1 mg/kg) and PO (3 and 10 mg/kg) (A) or IP (10 mg/kg) (C) dosing of VU0467154 in rats or following PO (10 and 30 mg/kg) (B) and IP (10 and 30 mg/kg) (D) dosing of the compound in mice. Panel E shows the time course of plasma and brain levels of the compound after IP administration of VU0467154 (3 mg/kg) in mice. Data are means \pm SEM ($N \geq 3$) or means and individual data points ($N = 2$).

(C_{\max}) in plasma of $0.7 \mu\text{M}$ with a time to reach C_{\max} (T_{\max}) of approximately 3 h and an area-under-the-curve from 0 to ∞ ($AUC_{0-\infty}$) of $9.0 \mu\text{M}\cdot\text{h}$; a similar disposition was observed following a 10 mg/kg dose but with a less than linear increase in exposure (Table 2 and Figure 4A). Similar kinetics were also observed following IP administration but with lower dose-normalized exposure (Table 2 and Figure 4C).

In vitro rat plasma protein binding and brain homogenate binding equilibrium dialysis assays demonstrated that VU0467154 has a moderate fraction unbound in plasma and brain, suggesting a brain-to-plasma partition coefficient at unrestricted equilibrium ($[f_u]_p/[f_u]_b$) of 0.46. *In vivo* distribution of VU0467154 to rat brain was assessed at 0.5 h following single IP administration of 10 mg/kg, which revealed a K_p of 0.49 and a corresponding unbound brain-to-unbound plasma partition coefficient ($K_{p,uu}$) of 1.1 (see Table 2), suggesting that the compound permeates the blood–brain barrier, and the unbound concentration in brain freely and rapidly equilibrates with that in plasma. In addition, biotransformation experiments in hepatocytes (cryopreserved; male, Sprague–Dawley rat) revealed low turnover of VU0467154 through several NADPH-dependent metabolic pathways, which produced multiple distinct mono-oxidation metabolites (data not shown).

In mice, VU0467154 exhibited an extravascular pharmacokinetic profile similar to that in rat following IP or PO administration of 10 mg/kg, which provided a C_{\max} of $5.6 \mu\text{M}$ (IP) and of $7.6 \mu\text{M}$ (PO) at a T_{\max} of 0.5 h (IP) and of 1 h (PO) with high systemic exposure (IP area-under-the-curve from 0 to 24 h, $AUC_{0-24h} = 39 \mu\text{M}\cdot\text{h}$, PO $AUC_{0-24h} = 46 \mu\text{M}\cdot\text{h}$; see Table 3 and Figure 4B,D). A similar pharmacokinetic profile was observed following a single IP or PO administration of a higher dose (30 mg/kg) of VU0467154 to mice; however, less than linear increases in exposure were observed after this dose relative to the exposure observed at the 10 mg/kg dose (Table 3 and Figure 4B,D). VU0467154 exhibited an *in vitro* f_u of 0.022 and f_u of 0.014 in mouse, suggesting a brain-to-plasma K_p of 1.6 at unrestricted equilibrium. *In vivo* distribution of VU0467154 to mouse brain was determined over time following a single IP administration of 3 mg/kg, revealing a brain AUC_{0-24h} ($5.9 \mu\text{M}\cdot\text{h}$)-to-plasma AUC_{0-24h} ($9.2 \mu\text{M}\cdot\text{h}$) K_p of 0.64 and a corresponding $K_{p,uu}$ of 0.41 (Table 3 and Figure 4E), thus suggesting moderate peripheral restriction of unbound concentrations at equilibrium.

VU0467154 Reverses Amphetamine-Induced Hyperlocomotion in Rats after IP and PO Dosing. We have previously shown that the M_4 PAM VU0152100, like the M_1/M_4 -

Table 3. Mouse Pharmacokinetic Properties of VU0467154

route (dose, n)	mouse PK parameter ^a	value
PO (10 mg/kg, 4)	$C_{\max,p}$ (μM)	7.6
	$T_{\max,p}$ (h)	1
	$\text{AUC}_{0-24\text{h}}$ ($\mu\text{M}\cdot\text{h}$)	46
PO (30 mg/kg, 4)	$C_{\max,p}$ (μM)	11
	$T_{\max,p}$ (h)	1
	$\text{AUC}_{0-24\text{h}}$ ($\mu\text{M}\cdot\text{h}$)	66
IP (3 mg/kg, 3)	$C_{\max,p}$ (μM)	2.0
	$T_{\max,p}$ (h)	1
	$\text{AUC}_{0-24\text{h}}$ ($\mu\text{M}\cdot\text{h}$)	9.2
	brain/plasma K_p ^b	0.64
	brain/plasma $K_{p,uu}$ ^c	0.41
IP (10 mg/kg, 4)	$C_{\max,p}$ (μM)	5.6
	$T_{\max,p}$ (h)	0.5
	$\text{AUC}_{0-24\text{h}}$ ($\mu\text{M}\cdot\text{h}$)	39
IP (30 mg/kg, 4)	$C_{\max,p}$ (μM)	7.3
	$T_{\max,p}$ (h)	0.5
	$\text{AUC}_{0-24\text{h}}$ ($\mu\text{M}\cdot\text{h}$)	59

^aAUC, area-under-the curve; CL_p , plasma clearance; $C_{\max,p}$, maximum plasma concentration; %F, bioavailability (%); K_p , partition coefficient; MRT, mean residence time; $T_{1/2}$, half-life; $T_{\max,p}$, time to reach maximum plasma concentration; V_{ss} , volume of distribution at steady-state. ^bBrain $\text{AUC}_{0-24\text{h}}$ and plasma $\text{AUC}_{0-24\text{h}}$. ^c*In vitro* mouse $f_{u,\text{plasma}}$ (0.022), $f_{u,\text{brain}}$ (0.014).

preferring agonist xanomeline, has an APD-like profile, including reversal of amphetamine-induced hyperlocomotion.^{18,24} To confirm and extend these findings using a more potent M_4 PAM, we assessed the ability of VU0467154 to counteract the motor stimulant effects of amphetamine. We first evaluated the dose–response relationship for amphetamine in inducing hyperlocomotion in rats to select an optimal dose for subsequent reversal studies with VU0467154 (Figure 5A). Amphetamine produced a dose- and time-dependent increase in locomotor activity ($F_{\text{dose}(5,1008)} = 96.37, p < 0.001$; $F_{\text{time}(23,1008)} = 58.99, p < 0.001$; and $F_{\text{dose}\times\text{time}(115,1008)} = 6.64, p < 0.001$), significant at doses of 0.3–1 mg/kg ($F_{5,47} = 29.47, p < 0.001$). As shown in Figure 5B–C, VU0467154 produced a robust dose-dependent reversal of amphetamine-induced hyperlocomotion after IP ($F_{\text{dose}(6,1032)} = 40.97, p < 0.001$; $F_{\text{time}(23,1032)} = 42.78, p < 0.001$; and $F_{\text{dose}\times\text{time}(138,1032)} = 2.66, p < 0.001$) and PO ($F_{\text{dose}(6,1176)} = 45.54, p < 0.001$; $F_{\text{time}(23,1176)} = 65.09, p < 0.001$; and $F_{\text{dose}\times\text{time}(138,1176)} = 2.55, p < 0.001$) routes of administration. Total locomotor activity was significantly reversed with doses of 10–56.6 mg/kg IP ($F_{6,49} = 9.51, p < 0.001$) and 3–56.6 mg/kg PO ($F_{6,55} = 11.10, p < 0.001$).

To establish the *in vivo* concentration–effect relationship for VU0467154 in reversing amphetamine-induced hyperlocomotion in rats, terminal brain concentrations were correlated with the percent reversal of amphetamine-induced hyperlocomotion in each treatment group after PO dosing (see Table 4). Mean brain concentrations rose linearly between doses of 1 and 3 mg/kg and in a less linear fashion between 3 and 10 mg/kg doses; however, brain concentrations reached a plateau of 1.4 μM at the 30 and 56.6 mg/kg doses. Terminal unbound brain concentrations for all treatment groups were plotted versus the efficacy in reversing amphetamine-induced hyperlocomotion in each animal, revealing an *in vivo* EC_{50} of 710 nM (48 nM unbound) for reversal of amphetamine-induced hyperlocomotion (see Table 4). The more moderate effects of VU0467154 on amphetamine-induced hyperlocomotion following IP administration are

consistent with the relatively lower exposure achieved following IP administration in rats.

VU0467154 Reverses Amphetamine-Induced Hyperlocomotion in Wild-type but Not in M_4 KO Mice. Similar to our studies in rats, we first determined the dose–response relationship for the effects of amphetamine on locomotion in wild-type and M_4 KO mice to allow selection of an optimal dose for subsequent reversal studies with VU0467154 across both genotypes (Figure 6A).

Amphetamine produced a dose- and time-dependent increase in locomotor activity in both wild-type and M_4 KO mice ($F_{\text{dose}(7,3696)} = 337.8, p < 0.001$; $F_{\text{time}(47,3696)} = 31.77, p < 0.001$; and $F_{\text{dose}\times\text{time}(329,3696)} = 9.99, p < 0.001$). The effect of amphetamine treatment on locomotor activity was significant at a dose of 1.8 mg/kg in M_4 KO mice and at a dose of 3 mg/kg in both genotypes; however, there was no significant main effect of genotype on the amphetamine response ($F_{\text{dose}(3,77)} = 85.81, p < 0.001$; $F_{\text{genotype}(1,77)} = 1.08, \text{ns}$; and $F_{\text{dose}\times\text{genotype}(3,77)} = 1.02, \text{ns}$; Figure 6A). VU0467154 (0.3–30 mg/kg) produced a robust reversal of the amphetamine-induced hyperlocomotion in wild-type mice ($F_{\text{dose}(5,2550)} = 68.42, p < 0.001$; $F_{\text{time}(47,2550)} = 68.73, p < 0.001$; and $F_{\text{dose}\times\text{time}(235,2555)} = 2.91, p < 0.001$), significant at doses of 3–30 mg/kg ($F_{5,59} = 9.13, p < 0.001$; Figure 6B). In contrast, VU0467154 had no effect on the time course of amphetamine-induced hyperlocomotion in M_4 KO mice ($F_{\text{treatment}(2,1296)} = 0.86, \text{ns}$; $F_{\text{time}(47,1296)} = 15.33, p < 0.001$; and $F_{\text{dose}\times\text{time}(94,1296)} = 0.47, \text{ns}$), nor did it reduce the total number of beam breaks compared with the animals treated with vehicle plus amphetamine ($F_{(2,29)} = 0.87, \text{ns}$; Figure 6C).

The VU0467154 dose–concentration–efficacy relationship observed in amphetamine-induced hyperlocomotion studies in mice was less graded than that observed in rat, since relatively lower brain concentrations were required for robust reversal effects, and the top three dose levels (3, 10, and 30 mg/kg) produced similarly high efficacy (see Table 5).

VU0467154 Reverses MK-801-Induced Hyperlocomotion in Wild-type but Not in M_4 KO Mice. Previous clinical studies have demonstrated that noncompetitive NMDAR antagonists produce abnormalities in affective and cognitive functions and corresponding alterations in cortical and subcortical neuronal signaling thought to underlie many of the symptoms associated with neuropsychiatric disorders like schizophrenia.^{1–3,25} In rodents, acute administration of the noncompetitive NMDAR antagonist MK-801 produces behavioral hyperactivity and attentional and memory deficits that correlate with disinhibition of PFC pyramidal cell firing and increased extracellular glutamate and DA levels in the PFC and subcortical structures.^{25–29} Here we examined the effects of VU0467154 on hyperlocomotion induced by MK-801. MK-801 alone produced a dose- and time-dependent increase in locomotor activity in both the wild-type and M_4 KO mice ($F_{\text{dose}(7,4173)} = 137.3, p < 0.001$; $F_{\text{time}(47,4173)} = 103.9, p < 0.001$; $F_{\text{dose}\times\text{time}(329,4173)} = 4.22, p < 0.001$; see Figure 7A). The effect of MK-801 treatment on locomotor activity was significant at a dose of 0.18 mg/kg in M_4 KO mice and at a dose of 3 mg/kg in both genotypes. In addition, MK-801, at the top dose tested, elicited a greater locomotor response in M_4 KO mice compared with wild-type mice ($F_{\text{dose}(3,87)} = 33.61, p < 0.001$; $F_{\text{genotype}(1,87)} = 8.69, p < 0.01$; $F_{\text{dose}\times\text{genotype}(3,87)} = 3.58, p < 0.05$; Figure 7A). Pretreatment with VU0467154 dose-dependently reversed MK-801-induced locomotor activity in wild-type mice ($F_{\text{dose}(3,1872)} = 44.27, p < 0.001$; $F_{\text{time}(47,1872)} = 20.54, p < 0.001$; $F_{\text{dose}\times\text{time}(141,1872)} = 1.44, p < 0.001$), significant at doses of 10 and 30 mg/kg ($F_{(3,43)} = 4.66, p <$

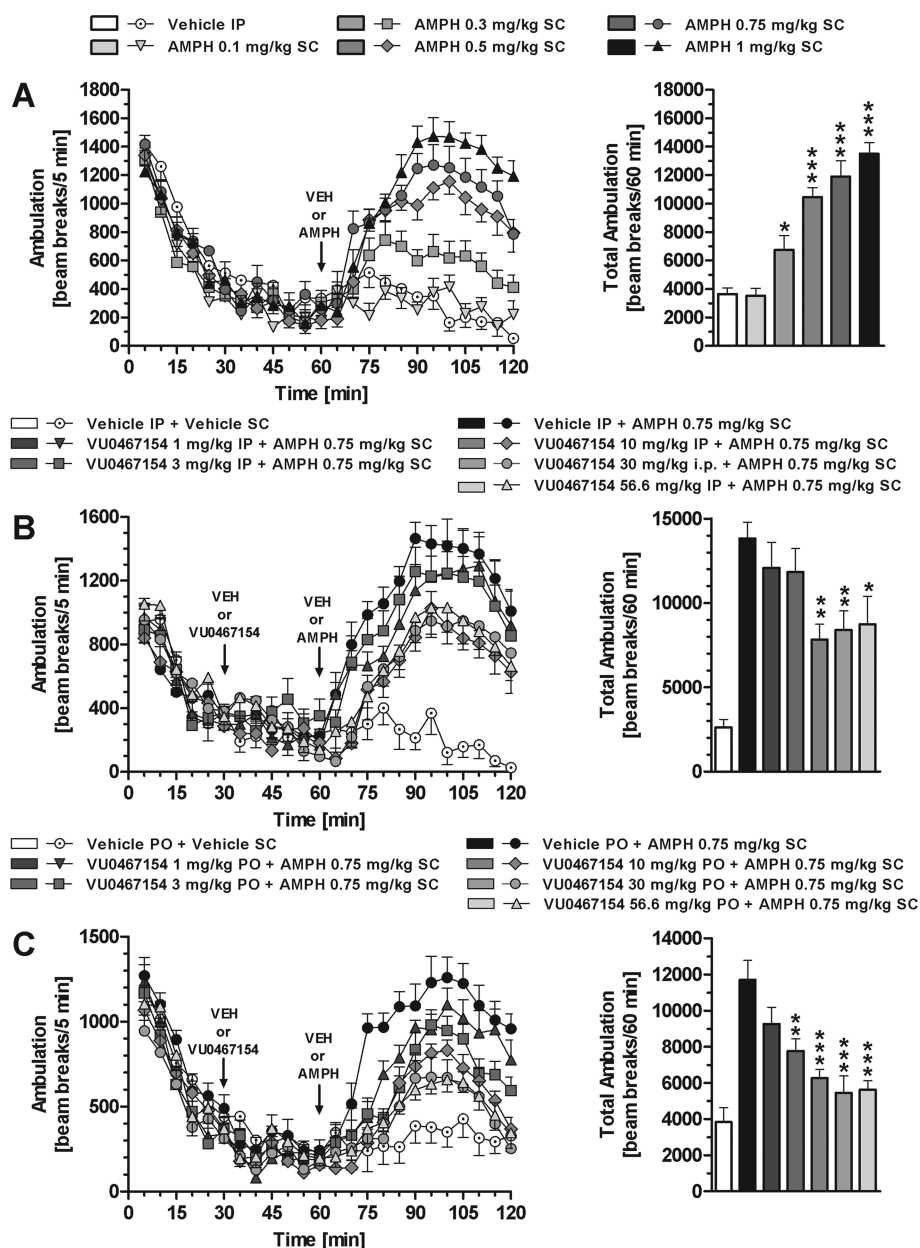


Figure 5. VU0467154 reverses amphetamine-induced hyperlocomotion in rats. (A) Amphetamine (AMPH) dose-dependently increases open field locomotor activity. Systemic (IP [B] or PO [C]) administration of VU0467154 dose-dependently reverses amphetamine-induced hyperlocomotion. The time course of locomotor activity is shown on the left and total locomotor activity during the 60 min period following amphetamine administration on the right. Data are means \pm SEM of 6–8 animals per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs vehicle (A) or vs vehicle + amphetamine (B and C) (ANOVA followed by Dunnett's test).

Table 4. Relationship between Total (Mean C_{brain}) and Unbound (Mean $C_{\text{brain,u}}$) Brain Concentration of VU0467154 and Pharmacodynamic Effects on Amphetamine (0.75 mg/kg, SC)-Induced Hyperlocomotion in Rats

PO dose, (mg/kg)	mean reversal of AHL [%]	mean C_{brain}^a [nM]	mean $C_{\text{brain,u}}^b$ [nM]
1	21	220	15
3	33	750	50
10	46	1100	74
30	53	1400	94
56.6	42	1400	94

^aAt 1.5 h postadministration. ^b*In vitro* rat $f_{\text{u,brain}}$ (0.067).

0.01; Figure 7B). In contrast to its effects in wild-type mice, VU0467154 had no effect on the time course of MK-801-induced hyperlocomotion in the M_4 KO mice ($F_{\text{dose}(2,1344)} = 0.84$, ns; $F_{\text{time}(47,1344)} = 30.31$, $p < 0.001$; $F_{\text{dose} \times \text{time}(94,1344)} = 0.79$, ns), nor did it alter the total activity after MK-801 administration ($F_{(2,30)} = 0.18$, ns; Figure 7C).

VU0467154 Reverses Various Amphetamine- and MK-801-Induced Motor Behaviors in Wild-type Mice. The induction of hyperlocomotion and stereotypy are hallmark actions of amphetamine and other psychostimulant drugs that are thought to be mediated by activation of the mesolimbic and nigrostriatal DA system, respectively.^{30–32} The ability to reverse amphetamine-induced stereotypy, hyperlocomotion, or both has also been used to assess motor side effect liability of antipsychotic

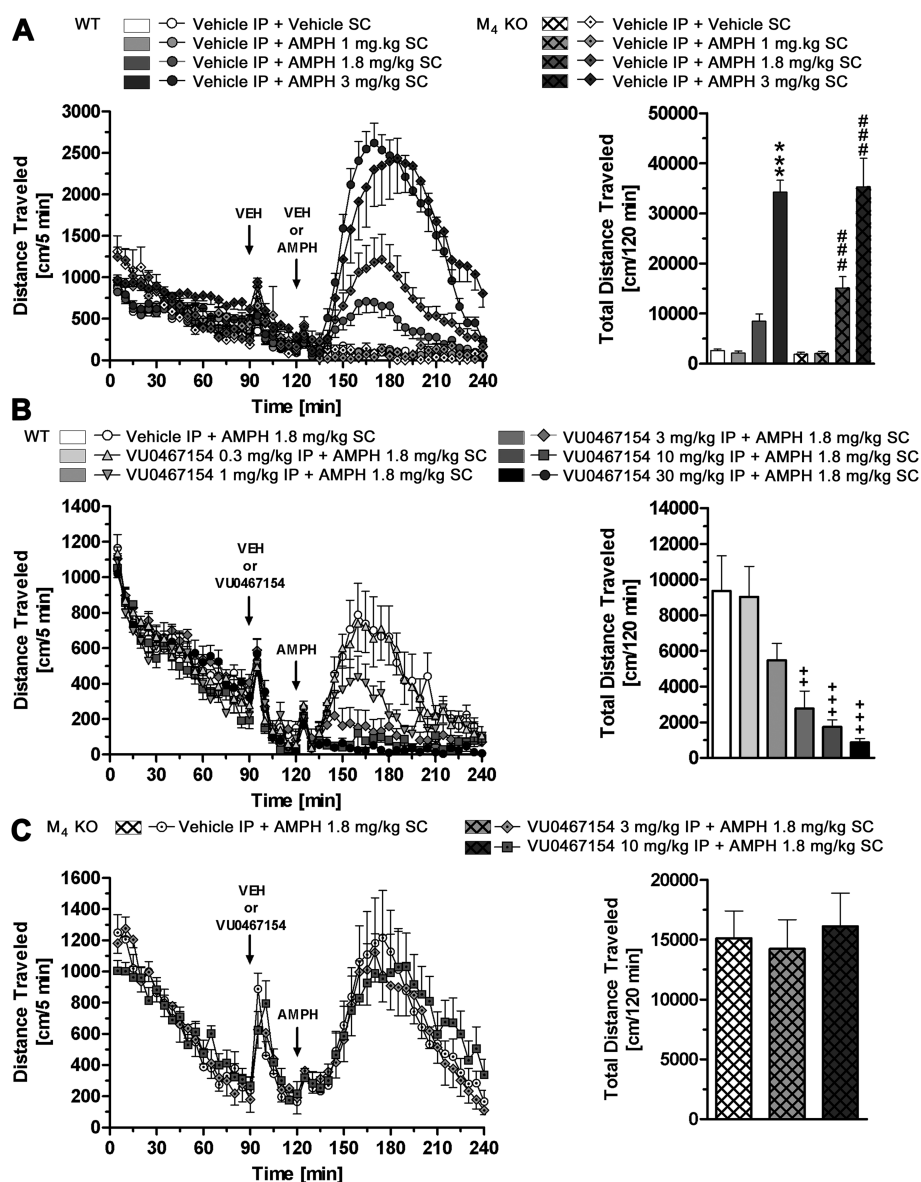


Figure 6. VU0467154 reverses amphetamine-induced hyperlocomotion in wild-type, but not M₄ KO mice. (A) Amphetamine (AMPH) dose-dependently increases open field locomotor activity in wild-type (WT) and M₄ KO mice. (B) In wild-type mice, administration of VU0467154 dose-dependently reverses amphetamine-induced hyperlocomotion. (C) VU0467154 does not reverse amphetamine-induced hyperlocomotion in M₄ KO mice. The time course of locomotor activity is shown on the left and total locomotor activity during the 120 min period following amphetamine administration on the right. Data are means \pm SEM of 9–13 wild-type mice and 10–11 M₄ KO mice per group. *** $p < 0.001$ vs wild-type vehicle + vehicle (A); ### $p < 0.001$ vs M₄ KO vehicle + vehicle (A); ++ $p < 0.01$, +++ $p < 0.001$ vs wild-type vehicle + amphetamine (B) (ANOVA followed by Bonferroni's test).

medications.³³ In an effort to further characterize the profile of action of M₄ PAMs, we therefore examined the ability of VU0467154 to counteract psychostimulant-induced changes in stereotypy, rearing, and resting time.

As shown in Figure 8, VU0467154 exhibited similar potency in reversing increases in the number of ambulatory episodes ($F_{(6,66)} = 7.50, p < 0.001$), stereotypic counts ($F_{(6,66)} = 14.65, p < 0.001$), and decreases in resting time ($F_{(6,66)} = 11.88, p < 0.001$), elicited by amphetamine. VU0467154 also potently reversed the MK-801-induced increases in ambulatory episodes ($F_{(4,49)} = 3.97, p < 0.01$), and rearing and jumping, as measured as the number of vertical counts ($F_{(4,49)} = 5.04, p < 0.01$). However, VU0467154 appeared to be less potent in reversing the MK-801-mediated increase in stereotypic counts ($F_{(4,49)} = 18.96, p < 0.001$) and decreases in resting time ($F_{(4,49)} = 16.95, p < 0.001$). Its ability to

counteract psychostimulant-induced hyperlocomotion and stereotypy suggests that VU0467154 acts at M₄ receptors both in the nucleus accumbens (NAS) and in the dorsal striatum (CP).

VU0467154 Does Not Reverse the Effects of MK-801 on Dopamine Utilization in the Forebrain of Mice. Using microdialysis, we have previously shown in rats that the M₄ PAM VU0152100 reverses amphetamine-induced hyperlocomotion and reduces amphetamine-induced increases in extracellular DA levels in the NAS and CP.¹⁸ Since acute NMDAR blockade by MK-801 also increases subcortical DA activity in rodents,^{34–36} we performed a neurochemical study to determine whether VU0467154 alters MK-801-induced changes in DA utilization—measured as the metabolite-to-DA ratios of the acidic metabolites dihydroxyphenylacetic acid [DOPAC] and homo-

Table 5. Relationship between Total (Mean C_{brain}) and Unbound (Mean $C_{\text{brain,u}}$) Brain Concentration of VU0467154 and Pharmacodynamic Effects on Amphetamine (1.8 mg/kg, SC)-induced Hyperlocomotion (AHL) in Wild-type and M_4 KO Mice

genotype	dose [mg/kg IP]	N	mean reversal of AHL [%]	mean C_{brain}^a [nM]	mean $C_{\text{brain,u}}^b$ [nM]
WT	0.3	10	3.7	24	0.3
WT	1	9	41.6	69	1.0
WT	3	9	70.4	157	2.2
WT	10	11	81.5	398	5.6
WT	30	10	90.7	579	8.1
M_4 KO	10	10	-6.6	217	3.0

^aAt 2.5 h postadministration. ^b*In vitro* mouse f_{ubrain} (0.014).

vanillic acid [HVA]—in the NAS and CP. As shown in Figure 9, the increases in accumbal and striatal DA utilization elicited by single administration of MK-801 were not altered by pretreatment with VU0467154 (NAS DOPAC/DA [$F_{3,37} = 6.21, p < 0.01$]; CP DOPAC/DA [$F_{3,37} = 5.63, p < 0.01$] and HVA/DA [$F_{3,37} = 9.70, p < 0.001$]). Neither single nor combined administration of VU0467154 with MK-801 altered serotonin utilization in the NAS and CP (data not shown).

VU0467154 Reverses MK-801-Induced Deficits in a Touchscreen Pairwise Visual Discrimination Task in Wild-type but Not in M_4 KO Mice. In recent years, touchscreen-based cognitive tasks have been developed for mice and rats to provide a better translational approach across species for further understanding the cognitive impairments observed in various neuropsychiatric disorders and for testing potential pharmacological interventions.^{37–40} Here, we used a touchscreen visual

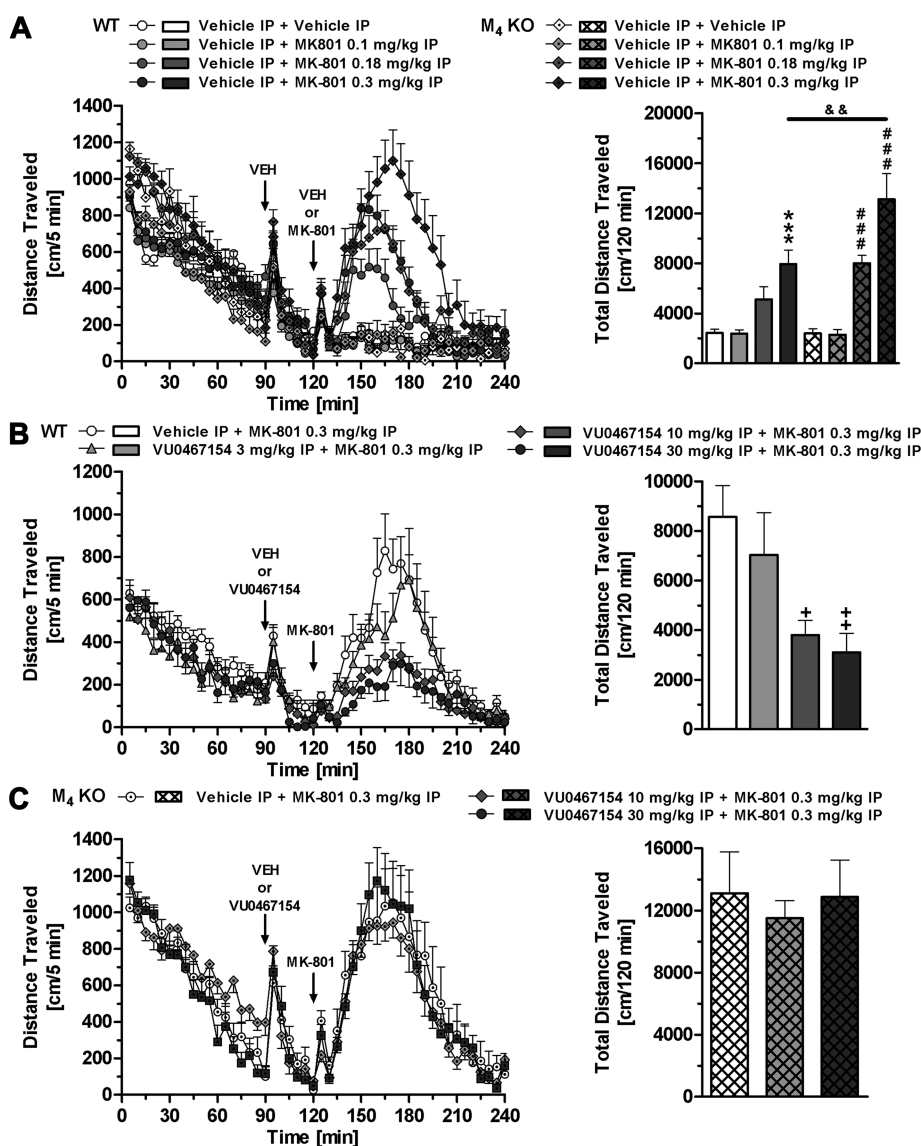


Figure 7. VU0467154 reverses MK-801-induced hyperlocomotion in wild-type but not M_4 KO mice. (A) MK-801 dose-dependently increases open field locomotor activity in wild-type (WT) and M_4 KO mice. (B) In wild-type mice, IP administration of VU0467154 dose-dependently reverses MK-801-induced hyperlocomotion. (C) VU0467154 does not reverse MK-801-induced hyperlocomotion in M_4 KO mice. The time course of locomotor activity is shown on the left and total locomotor activity during the 120 min period following MK-801 administration on the right. Data are means \pm SEM of 10–13 animals per group. *** $p < 0.001$ vs wild-type vehicle + vehicle (A); ### $p < 0.001$ vs M_4 KO vehicle + vehicle (A); && $p < 0.01$ vs wild-type vehicle + 0.3 mg/kg MK-801 (A); + $p < 0.05$, ++ $p < 0.01$ vs wild-type vehicle + MK-801 (B) (ANOVA followed by Bonferroni's test).

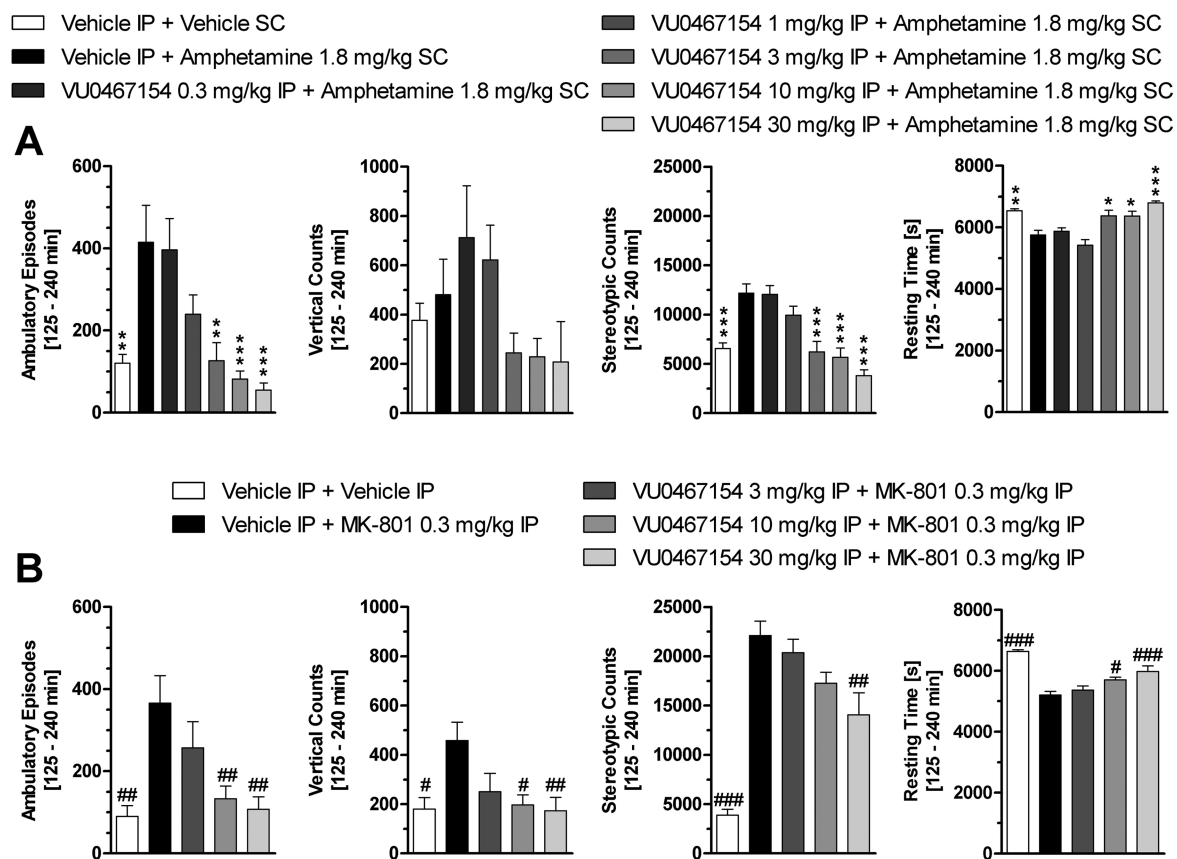


Figure 8. VU0467154 reverses amphetamine- (A) and MK-801-induced (B) locomotor and nonlocomotor open field activity in wild-type mice. The total number of locomotor episodes, rearings (vertical counts), and stereotypic counts and the duration of resting time during the 120 min period following amphetamine or MK-801 administration are shown. Data are means \pm SEM of 7–11 (A) and 7–13 (B) mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs vehicle + amphetamine; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs vehicle + MK-801 (ANOVA followed by Bonferroni's test).

pairwise discrimination task, one model of associative learning and memory functions that operates under a schedule of positive reinforcement, to assess whether selective potentiation of M_4 could reverse performance deficits induced by acute MK-801 challenge.

Training. Independent unpaired t tests showed that wild-type and M_4 KO mice did not differ in the number of total training days (mean \pm SEM; wild-type = 14.1 ± 1.7 ; M_4 KO = 13.0 ± 1.0 ; $t_{18} = 0.58$, $p > 0.05$) or in the latency to respond on the touchscreen after trial initiation on the last day of training (wild-type = 5.1 ± 1.6 s; M_4 KO = 5.9 ± 1.9 s; $t_{18} = 0.31$, $p > 0.05$). M_4 KO mice took significantly longer to retrieve a reward following correct responses on the last day of training (wild-type = 1.6 ± 0.2 s; M_4 KO = 2.2 ± 0.12 s; $t_{18} = 2.47$, $p < 0.05$), which is most likely not an effect of reward salience as previous studies have shown an increased motivation to respond for liquid reinforcers in M_4 KO mice.⁴¹

Response Accuracy. Wild-type and M_4 KO mice did not differ in the acquisition of the pairwise visual discrimination task ($F_{\text{genotype}(1,216)} = 0.15$, ns; $F_{\text{days}(11,216)} = 19.40$, $p < 0.001$; $F_{\text{genotype} \times \text{days}(11,216)} = 1.14$, ns; see Figure 10D). Mice of both genotypes reached a response accuracy of 85–95% to distinguish the S^+ (marbles) from the S^- (fan) (see Figure 10E). Following three days of stable task performance, acute administration of MK-801 impaired response accuracy ($F_{\text{treatment}(3,135)} = 11.94$, $p < 0.001$; $F_{\text{day}(4,135)} = 18.74.11$, $p < 0.001$; $F_{\text{treatment} \times \text{day}(12,135)} = 8.48$, $p < 0.001$; see Figure 10A). On the test day, 0.3 mg/kg MK-801, but not lower doses, reduced response accuracy from 92%

(vehicle group) to 63% ($F_{3,30} = 18.66$, $p < 0.001$). When animals were tested 24 h after drug treatment, their performance returned to baseline, irrespective of the treatment condition. Treatment with VU0467154 alone did not affect performance of pairwise discrimination with mean response accuracy of vehicle- and VU0467154-treated animals ranging from 90.6% to 93.3% correct ($F_{4,35} = 0.38$, ns; data not shown). Pretreatment with VU0467154 dose-dependently reversed the MK-801-induced pairwise discrimination deficit ($F_{\text{treatment}(4,170)} = 11.52$, $p < 0.001$; $F_{\text{day}(4,170)} = 81.92$, $p < 0.001$; $F_{\text{treatment} \times \text{day}(16,165)} = 7.90$, $p < 0.001$, see Figure 10B). On the test day, 5.6 and 10 mg/kg, but not 3 mg/kg of VU0467154, reversed impaired performance of MK-801-treated animals ($F_{4,38} = 25.60$, $p < 0.001$). However, it must be noted that while treatment with the highest dose of VU0467154 significantly improved the MK-801-induced deficit, these animals did not perform as well as vehicle-treated controls.

To corroborate that VU0467154 improves MK-801-induced cognition deficits through an M_4 -dependent mechanism, we examined the effects of VU0467154 and MK-801, alone and in combination, on cognition in M_4 KO mice. In contrast to wild-type mice, pretreatment with VU0467154 did not reverse the MK-801-induced pairwise discrimination deficit in M_4 KO mice ($F_{\text{treatment}(3,179)} = 2.62$, $p = 0.05$; $F_{\text{day}(4,179)} = 39.67$, $p < 0.001$; $F_{\text{treatment} \times \text{day}(12,165)} = 10.87$, $p < 0.001$, see Figure 10C). On the test day, the response accuracy of M_4 KO animals treated with MK-801 alone (73%) or in combination with VU0467154 (64%) did not differ but was significantly reduced compared with vehicle-treated animals (91% [$F_{3,39} = 13.92$, $p < 0.001$]).

- Vehicle IP + Vehicle IP
- VU0467154 30 mg/kg IP + Vehicle IP
- ▨ Vehicle IP + MK-801 0.3 mg/kg IP
- ▩ VU0467154 30 mg/kg IP + MK-801 0.3 mg/kg IP

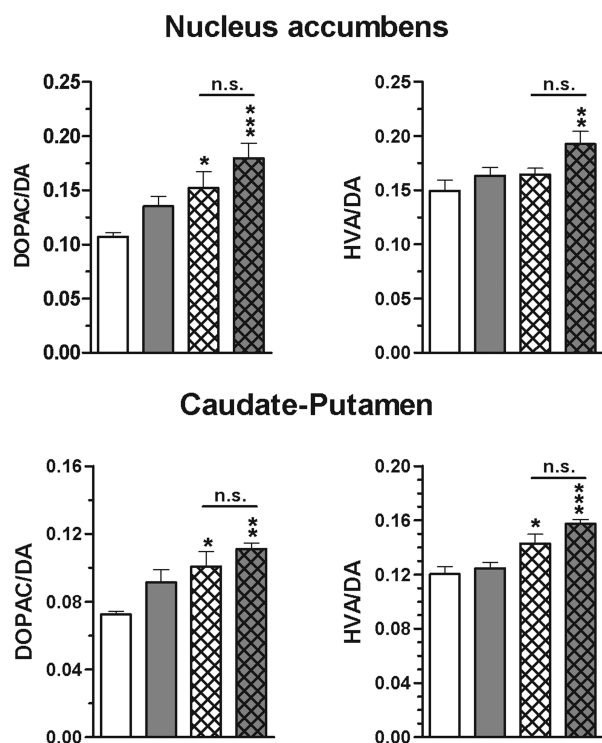


Figure 9. Effects of MK-801 on subcortical dopamine utilization in wild-type mice are not reversed by VU0467154. Effects of single and combined treatment with MK-801 and VU0467154 on DOPAC/DA and HVA/DA ratios in the nucleus accumbens and caudate-putamen are shown. Data are means \pm SEM of 9–12 animals per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs vehicle + vehicle (ANOVA followed by Bonferroni's test).

Response Latencies and Trial Completion. To determine whether changes in response accuracy are mediated, in part, by drug effects on motor function or motivation, we examined additional parameters, such as response latencies and number of completed trials per session (see Table 6). Interestingly, both the highest dose of MK-801 ($F_{3,30} = 8.75$, $p < 0.001$) and the 10 or 30 mg/kg doses of VU0467154 ($F_{4,35} = 3.85$, $p < 0.05$) increased the latency from initiating a trial to performing a correct touch screen response. However, since none of the treatments altered the latency between performing a correct response and collection of the food reward, it is unlikely that motor alterations contributed to drug-induced changes in response accuracy. Finally, in the groups treated with 10 mg/kg VU0467154 alone, 30 mg/kg VU0467154 alone, and 3 mg/kg VU0467154 in combination with 0.3 mg/kg MK-801, respectively, 25%, 33%, and 14% of the animals did not complete all 60 trials in the 60 min session. As observed in wild-type mice, treatment with VU0467154 alone increased both the time to complete 60 trials ($F_{3,39} = 3.43$, $p < 0.05$) and correct touch latency ($F_{3,36} = 3.19$, $p < 0.05$) in M_4 KO mice suggesting that these effects are not M_4 -mediated (see Table 6).

VU0467154 Reverses MK-801-Induced Impairments in the Acquisition of Contextual Fear Conditioning Behaviors. Contextual fear conditioning is another associative learning

and memory task in which a response to an aversive foot shock stimulus becomes associated with a specific neutral context or testing environment; acquisition of this task is dependent on intact NMDAR signaling in the hippocampus.^{42,43} Here we assessed whether VU0467154 could reverse the detrimental effects of MK-801 on the acquisition of contextual fear conditioning. Administration of MK-801 (0.1–0.3 mg/kg) prior to the four-shock conditioning trial dose-dependently impaired the acquisition of contextual fear conditioning measured 24 h later under drug free conditions as shown by a decrease in percent freezing behaviors ($F_{3,35} = 42.42$, $p < 0.001$; see Figure 11A). VU0467154 alone had no effect on contextual fear conditioning; however, pretreatment with VU0467154 (3–10 mg/kg) dose-dependently reversed the MK-801-induced deficits in the acquisition of contextual fear conditioning ($F_{4,46} = 9.36$, $p < 0.001$; see Figure 11B). Animals pretreated with the highest dose of VU0467154 followed by MK-801 did not differ from vehicle-treated controls. We also confirmed the direct involvement of M_4 in the acquisition of contextual fear conditioning using M_4 KO mice. In comparison with wild-type mice, the M_4 KO mice displayed severe deficits in percent freezing behavior ($t_{26} = 5.07$, $p < 0.001$; see Figure 11C) consistent with a role of M_4 in the acquisition of contextual fear conditioning. To determine whether VU0467154 (10 mg/kg) or the deletion of the M_4 gene altered sensitivity to the footshock stimulus, the nociceptive response to increasing shock current intensities was determined in a separate cohort of animals. As shown in Figure 11D, VU0467154 treatment did not alter the animals' twitching, flinching, and vocalizing responses to increasing foot shock intensities but increased the threshold to elicit jumping ($t_{13} = 2.197$, $p < 0.05$). These observations do not support the interpretation that VU0467154 reverses MK-801-induced conditioning deficits by simply lowering pain sensitivity to the footshock stimulus. As shown in Figure 11E, the shock threshold for flinching ($t_{27} = 2.53$, $p < 0.05$) and vocalizing ($t_{27} = 2.93$, $p < 0.01$) responses was lower in M_4 KO mice than in wild-type mice, indicating that failure to acquire the contextual fear conditioning response was not due to decreased salience of the footshock.

VU0467154 Enhances the Acquisition of Contextual and Cue-Dependent Fear Conditioning Behaviors. In the aforementioned contextual fear conditioning studies, the training parameters were selected to achieve greater than 70% freezing or pairwise visual discrimination accuracy to provide a sufficient signal window for disruptions with MK-801. However, this experimental design limited the potential assessment of any cognitive enhancing effects of VU0467154 due to a ceiling effect in the task performance. Using a one-shock paradigm, which resulted in ~50% freezing, wild-type mice treated with VU0467154 (3–30 mg/kg) before the conditioning session exhibited a dose-dependent enhancement in the acquisition of contextual fear conditioning behaviors that were measured 24 h later under drug-free conditions ($F_{5,68} = 38.39$, $p < 0.001$; see Figure 12A). Administration of VU0467154 (3–30 mg/kg) prior to the conditioning session also enhanced acquisition of cue-dependent fear conditioning in wild-type mice as shown by a dose-related increase in freezing behaviors ($F_{5,77} = 17.35$, $p < 0.001$; see Figure 12B). In contrast, M_4 KO mice exhibited pronounced deficits in the acquisition of both contextual and cue-dependent fear conditioning behaviors that were not ameliorated by administration of VU0467154 (see Figure 12A,B).

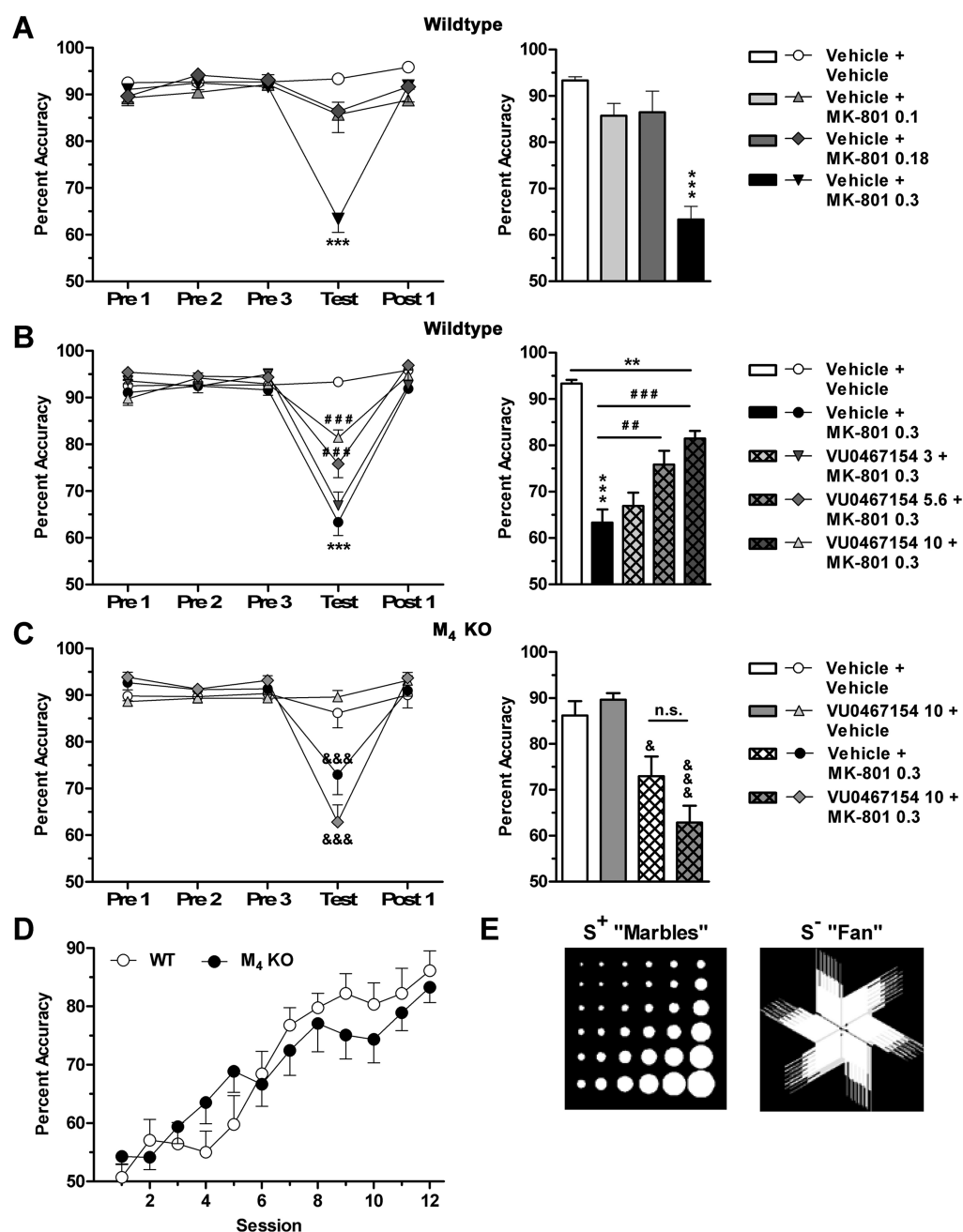


Figure 10. VU0467154 reverses MK-801-induced performance deficits in a touchscreen visual discrimination task. (A) Dose-dependent disruption of stable baseline performance (85–95% accuracy) of wild-type (WT) mice by MK-801. (B) Dose-dependent reversal of MK-801-induced disruption by pretreatment with VU0467154. (C). Failure of VU0467154 to reverse MK-801-induced deficits in M₄ KO mice. (D) Equal acquisition rate of pairwise discrimination in wild-type and M₄ KO mice. (E) Visual stimuli used in the pairwise discrimination task. In panels A–C, the time course of task performance is shown on the left and the performance on the test day on the right. Data are means \pm SEM of 6–8 (A), 7–9 (B), 10 (C), and 9–11 (D) animals per group. ** $p < 0.01$, *** $p < 0.001$ vs wild-type vehicle + vehicle (A, B); ### $p < 0.01$, ### $p < 0.001$ vs wild-type vehicle + MK-801 (B); & $p < 0.05$, &&& $p < 0.001$ vs M₄ KO vehicle + vehicle (C) (ANOVA followed by Bonferroni's test).

VU0467154 Does Not Induce Adverse Side Effects Associated with Nonselective Activation of Peripheral mAChRs Nor Impair Motor Coordination at Doses That Produce Antipsychotic-like Activity. In clinical studies, treatment with mAChR agonists or acetylcholinesterase inhibitors has been confounded by dose-limiting adverse effects that are mainly attributed to activation of peripheral M₂ and M₃ mAChRs.^{44,45} We therefore assessed the potential adverse side effect liability of VU0467154 in both the modified Irwin neurological test battery⁴⁶ and the rotarod test. Mice treated

with VU0467154 (10–30 mg/kg) did not exhibit autonomic and somatosensory side effects, whereas the nonselective mAChR agonist oxotremorine (1 mg/kg) induced time-dependent changes in autonomic function (e.g., increased lacrimation and salivation, diarrhea, and loss of the cornea and pinna reflexes) as well as somatomotor deficits (e.g., hypoactivity, tremor, and loss of the tail pinch response; see Table 7). In the rotarod test, which reveals adverse motor side effects associated with administration of a variety of antipsychotic drugs,^{47,48} VU0467154 (3–30 mg/kg [IP]) did not alter the latency to fall ($F_{3,49} = 1.283$, ns; see

Table 6. Effects of VU0467154 and MK-801, Alone or in Combination, on Performance of Wild-type and M₄ KO Mice in the Pairwise Visual Discrimination Task

pretreatment	dose [mg/kg IP]	treatment	dose [mg/kg IP]	N	time to complete 60 trials ^a [s]	correct touch latency ^a [s]	correct reward collection latency [s]
Wild-type							
vehicle		vehicle		8	842 ± 70.3	2.01 ± 0.16	1.35 ± 0.24
vehicle		MK-801	0.10	7	1004 ± 124.3	2.95 ± 0.57	1.32 ± 0.15
vehicle		MK-801	0.18	8	745 ± 38.6	2.39 ± 0.27	0.98 ± 0.04
vehicle		MK-801	0.30	8	1118 ± 139.2	8.65 ± 1.99 ^{***}	5.58 ± 2.58
VU0467154	3.0	vehicle		8	1061 ± 130.2	2.01 ± 0.16	1.13 ± 0.07
VU0467154	5.6	vehicle		6	1256 ± 211.5	2.24 ± 0.36	2.09 ± 0.50
VU0467154	10.0	vehicle		8	2171 ± 424.8 ^{**b}	18.58 ± 8.50 [*]	9.24 ± 6.71
VU0467154	30.0	vehicle		6	2557 ± 397.1 ^{***c}	20.89 ± 4.17 [*]	2.82 ± 0.71
VU0467154	3.0	MK-801	0.30	7	1239 ± 397.7 ^d	18.14 ± 14.17	3.34 ± 2.06
VU0467154	5.6	MK-801	0.30	8	856 ± 58.4	4.10 ± 0.63	1.23 ± 0.08
VU0467154	10.0	MK-801	0.30	8	1017 ± 89.1	6.39 ± 1.11	1.33 ± 0.08
M ₄ KO							
vehicle		vehicle		10	1142 ± 116.7	5.68 ± 1.10	2.00 ± 0.19
VU0467154	10.0	vehicle		10	2080 ± 384.9 ^{#e}	22.81 ± 8.86 [#]	3.19 ± 0.73
vehicle		MK-801	0.30	10	1264 ± 280.5 ^f	8.76 ± 2.19	1.54 ± 0.15
VU0467154	10.0	MK-801	0.30	10	1120 ± 95.5	8.45 ± 1.57	1.57 ± 0.07

^a* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs vehicle + vehicle (wild-type); [#] $p < 0.05$ vs vehicle + vehicle (M4 KO). ^b2 out of 8 WT mice did not complete all 60 trials per session. ^c2 out of 6 WT mice did not complete all 60 trials per session. ^d1 out of 7 WT mice did not complete all 60 trials per session. ^e3 out of 10 M4 KO mice did not complete all 60 trials per session. ^f1 out of 10 M4 KO mice did not complete all 60 trials per session.

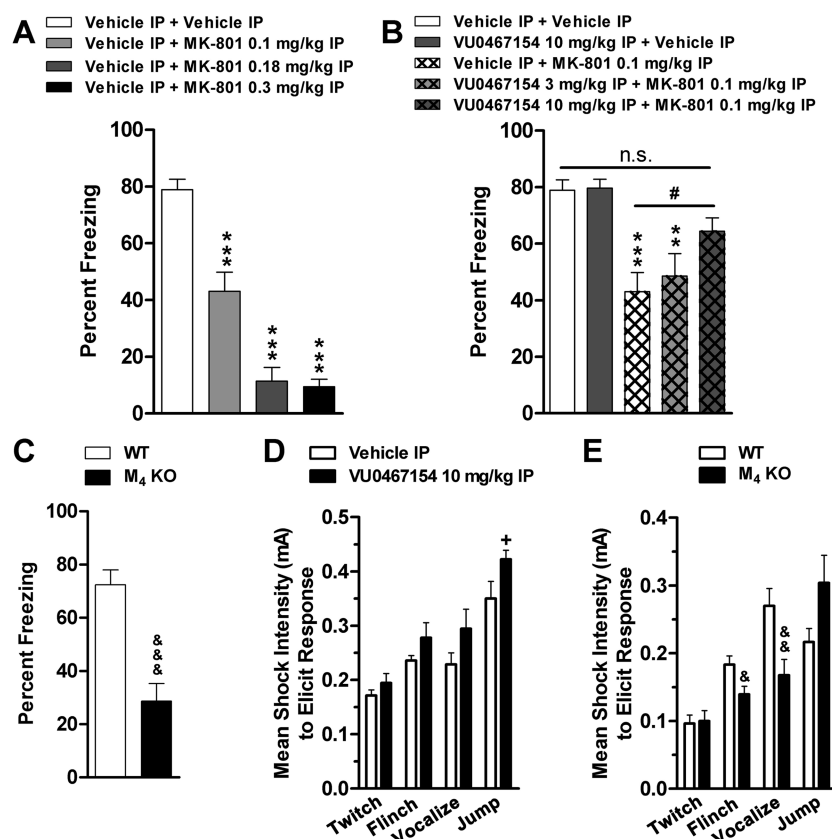


Figure 11. VU0467154 reverses MK-801-induced deficits in the acquisition of context-dependent fear conditioning in mice. (A) MK-801 dose-dependently disrupts the acquisition of contextual fear conditioning. (B) Pretreatment with VU0467154 reverses the MK-801-elicited deficit in contextual fear conditioning. (C) M₄ KO mice exhibit marked deficits in the acquisition of context-dependent fear conditioning. (D) VU0467154 increases the footshock threshold to elicit jumping behavior, and (E) M₄ KO mice have lower shock threshold for evoking flinching and vocalization. Data are means ± SEM of 7–10 (A), 9–10 (B), 14 (C), 6–10 (D), and 13–15 (E) animals per group. ** $p < 0.01$, *** $p < 0.001$ vs wild-type vehicle + vehicle (A, B); # $p < 0.05$ vs wild-type VU0467154 10 mg/kg + MK-801 0.1 mg/kg (B) (ANOVA followed by Bonferroni's test); & $p < 0.05$ && $p < 0.01$ &&& $p < 0.001$ vs. wild-type mice (C, E), + $p < 0.05$ vs. wild-type vehicle (D) (t -test).

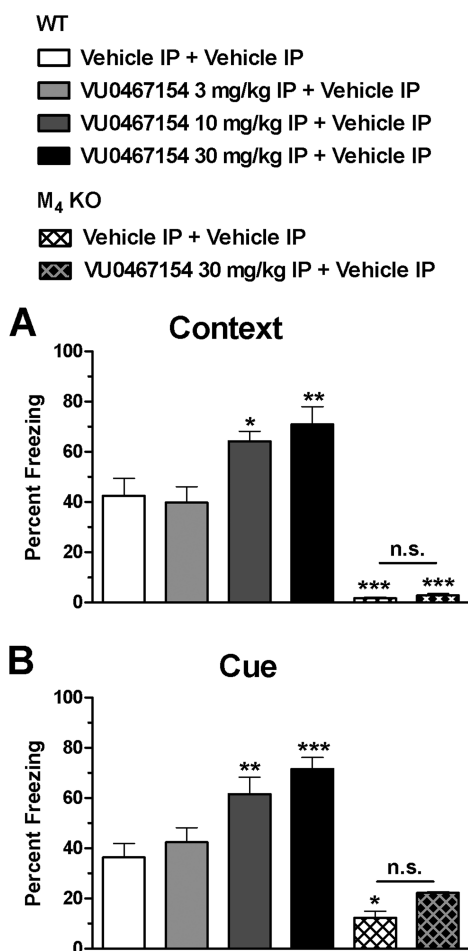


Figure 12. VU0467154 enhances the acquisition of contextual (A) and cue-dependent fear conditioning (B) in wild-type but not in M₄ KO mice. Data are means ± SEM of 10–15 wild-type and 8–13 M₄ KO mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs wild-type vehicle + vehicle (ANOVA followed by Bonferroni's test).

Figure 13). These data indicate that VU0467154 does not cause gross disturbances in motor coordination at doses that are efficacious in rodent models indicative of enhancement of cognition or antipsychotic-like activity.

Augmentation of central cholinergic neurotransmission has been shown to enhance cognition and to potentially restore cognitive impairments due to disruptions in NMDAR signaling. While accumulating evidence has historically supported the view that the M₁ receptor is the primary mAChR subtype involved in the modulation of cognitive functions,^{5,6} the present findings suggest a new potential role for M₄ in the modulation of the neural circuitry involved in learning and memory. Here we provide the first report that selective potentiation of M₄ produces robust reversals of MK-801-induced hyperactivity and disruptions in associative learning and memory tasks in wild-type mice using the novel M₄ PAM VU0467154. Moreover, VU0467154 improved the acquisition of hippocampal- and nonhippocampal associative learning tasks, specifically contextual and cue-dependent fear conditioning, when administered alone in wild-type mice. Importantly, the cognitive enhancing and antipsychotic-like activity of VU0467154 was observed within a dose range that did not produce any dose-limiting adverse side effects linked with nonselective activation of peripheral mAChR subtypes. Our data offer new evidence to

support a broader role for M₄ modulation in the neural circuits mediating the affective and cognitive functions that are disrupted in neuropsychiatric disorders such as schizophrenia.

In comparison with previously described M₄ ligands, the novel M₄ PAM VU0467154 represents a substantial improvement in both rodent *in vitro* potency (17.7 nM EC₅₀) and PK properties, including low clearance, favorable $t_{1/2}$, and excellent oral bioavailability. These optimized properties allowed the determination of the *in vivo* concentration–effect relationship for VU0467154 in reversing amphetamine-induced hyperlocomotion in rats after oral dosing and revealed an *in vivo* EC₅₀ of 710 nM (48 nM unbound) that is aligned with its *in vitro* EC₅₀. Moreover, the reversal of amphetamine-induced hyperactivity in rats and wild-type mice but not in M₄ KO mice by VU0467154 confirms and extends previous studies demonstrating that M₄ PAMs exhibit an APD-like activity profile comparable to the effects observed with the M₁/M₄-preferring mAChR agonist xanomeline in rodents.^{16,18–20,49–51} VU0467154 also displayed no interaction with numerous GPCRs, ion channels, transporters, and enzymes within the CNS that might confound the interpretation of the *in vivo* effects in the present study. However, VU0467154 did exhibit a K_i of 98 nM to the guinea pig adenosine transporter and an IC₅₀ of 240 nM for functional inhibition of the human adenosine transporter in recombinant systems. In order to control for potential off-target activity at the adenosine transporter *in vivo* and to definitively confirm a role for M₄ in the actions of VU0467154, we established that the activity of VU0467154 in reversing the amphetamine- and MK-801-induced hyperactivity and disruptions in pairwise visual discrimination was absent in the M₄ KO mice. Moreover, VU0467154 did not affect the impairments in the acquisition of contextual or cue-dependent fear conditioning in M₄ KO mice. Collectively, these studies indicate that the observed APD-like and cognition enhancing activity of VU0467154 is mediated through an M₄-dependent mechanism.

We also found that the M₄ PAM VU0467154 has efficacy in reversing the behavioral alterations associated with a preclinical model of acute NMDAR hypofunction, specifically MK-801-induced hyperlocomotion. Previous studies have shown that acute MK-801 challenge increases DA utilization in the NAS and CP through activation of midbrain DA neurons as measured by increased DA synthesis and turnover and DA cell firing.^{34–36,52} However, in contrast to our recent findings that potentiation of M₄ reverses amphetamine-induced increases in striatal DA release,¹⁸ VU0467154 did not appear to alter MK-801-induced hyperactivity through reductions in subcortical DA release. In light of these findings, it is worth noting that hyperlocomotion induced by MK-801 is not blocked by DA depletion in the NAS.^{53,54} One possible explanation for these results may be that VU0467154 counteracts the MK-801-induced behavior by enhancing M₄-mediated inhibition of medium-spiny striatal and accumbal neurons that coexpress M₄ and D₁ DA receptors. This interpretation is in line with studies showing that loss of M₄ by targeted genetic deletion of M₄ in D₁ receptor-expressing cells enhances amphetamine-, cocaine-, and D1-like DA receptors agonist-induced locomotor activity, without altering the effects of the D2-like DA receptors agonist quinpirole.^{55,56} Alternatively, since acute MK-801 administration can also result in disinhibition of cortical pyramidal cell firing and increased extracellular glutamate in the PFC and subcortical structures,^{25–29} one alternative site of action for the effects of VU0467154 in reversing MK-801-induced hyperactivity may involve the modulation of M₄ at corticostriatal synapses. Recent

Table 7. Adverse Side Effect Profiling of VU0467154 in Mice Using the Modified Irwin Neurological Test Battery^a

	vehicle					10 mg/kg VU0467154					30 mg/kg VU0467154					1 mg/kg oxotremorine				
	5	15	30	60	180	5	15	30	60	180	5	15	30	60	180	5	15	30	60	180
Autonomic Nervous System																				
ptosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
exophthalmus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++++**	++++**	+++*	+	-
miosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
mydriasis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
corneal reflex loss	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+++*	+++*	+	-
pinna reflex loss	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++++**	++++**	++++**	++++**	+++*
piloerection	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++*	+	+	+	-
respiratory rate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
writhing	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
tail erection	-	-	-	-	-	-	-	+	+	+	-	-	+	+	+	-	+	+	-	-
lacrimation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++++**	++++**	++++**	+++*	+
salivation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++++**	++++**	++++**	+++*	+++*
vasodilation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	**	-	-
skin color	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++*	-	-	-	-
irritability	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Somatomotor Systems																				
motor activity	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++++**	+++*	+++*	+++*	+++*
convulsions	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
arch/roll	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
tremors	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++++**	++++**	++++**	++++**	-
leg weakness	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
rigid stance	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
spraddle	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+++*	+++*	+	-
placing loss	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
grasping loss	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++*	+++*	+++*	+	-
righting loss	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
catalepsy	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
tail pinch	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++++**	++++**	++++**	++++**	+++*
escape loss	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-

^aThe effects of VU0467154 (10 and 30 mg/kg, IP) or vehicle (10% Tween 80) on autonomic nervous system and somatomotor function were compared with oxotremorine (1 mg/kg, SC). The mean scores of five animals per treatment are classified as follows: -, no effect; +, 0.01–0.50; ++, 0.51–1.0; +++, 1.01–1.50; +++++, 1.51–2.0. * $p < 0.05$, ** $p < 0.01$ vs vehicle (Dunnett's test).

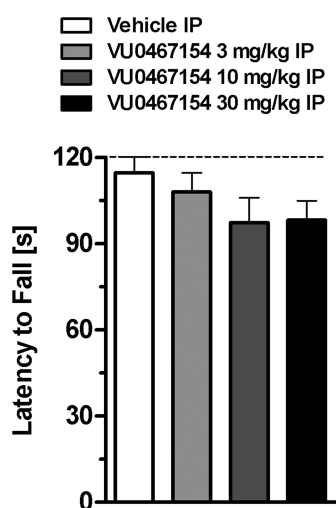


Figure 13. VU0467154 does not impair rotarod performance in wild-type mice. The latency of wild-type mice to fall from a rotarod turning at 20 rpm was not affected by VU0467154. Data are means \pm SEM of 10–16 wild-type mice per treatment group (ANOVA, not significant).

physiology studies have revealed that activation of presynaptic M_4 receptors decreases glutamate release from corticostriatal

terminals,⁵⁷ indicating that M_4 is the primary mAChR mediating cholinergic inhibition of corticostriatal transmission onto striatonigral and striatopallidal medium spiny neurons. Therefore, the ability of VU0467154 to reverse MK-801-induced hyperactivity may involve actions at corticostriatal terminals to normalize the function of overactive (disinhibited) excitatory projections from the cortex to the striatal complex. Additional electrophysiology studies from our group have also demonstrated that M_4 PAMs can potentiate M_4 -mediated depression of excitatory synaptic transmission at hippocampal CA1 synapses, which may serve as another important site of action for the effects of VU0467154.¹⁷ Ongoing studies are evaluating the effects of VU0467154 on MK-801-induced cortical and subcortical glutamate release and in other preclinical models predictive of antipsychotic-like activity, including prepulse inhibition of the acoustic startle reflex.

The present study provides evidence for a crucial involvement of M_4 in the modulation of NMDAR-dependent associative learning and memory processes.^{4,58} Acute challenge with the NMDAR antagonist MK-801 induced cognitive deficits in wild-type mice comparable to documented impairments reported in mice with genetic deletions or reductions in the NMDAR subunits NR1, NR2A, or NR2C.^{38,59–61} Our data revealed that selective potentiation of M_4 by VU0467154 is sufficient to

reverse cognitive impairments induced by acute MK-801 challenge. Interestingly, the M_4 KO mice showed no disruptions in the acquisition and performance of the visual pairwise discrimination task consistent with previous reports that M_4 KO mice exhibit normal working and spatial hippocampal memory in the Morris water maze test.⁶² In contrast, the M_4 KO mice exhibited robust deficits in the acquisition of both contextual and cue-dependent fear conditioning, effects that were not confounded by a shift in sensitivity to the shock stimulus. These outcomes suggest differential effects of M_4 on the aversive versus positive reinforcing nature of the two cognitive tests. Taken together, our data suggest that highly selective M_4 PAMs may prove beneficial in treating some of the cognitive impairments associated with alterations in glutamatergic signaling thought to underlie at least in part the cognitive deficits in schizophrenia patients.^{1–3,25} Future studies are needed to determine whether potentiation of M_4 can also ameliorate the cognitive deficits in chronic NMDAR hypofunction models, such as chronic NMDAR blockade or the NR1 transgenic knockdown mouse model.

Integrity of the hippocampus and PFC is required for the acquisition of contextual and cue-dependent fear conditioning, respectively.^{42,63} However, prior to the present studies, the potential contribution of M_4 to the acquisition of prefrontal versus hippocampal-dependent fear conditioning in intact animals remained unknown. Here we established that selective potentiation of M_4 with VU0467154 produced robust dose-dependent increases in the acquisition of both contextual and cue-dependent fear conditioning. Importantly, VU0467154 did not normalize the disruptions in the acquisition of either conditioning tasks observed in M_4 KO mice. As shown previously, M_4 activation can selectively potentiate mAChR-mediated reductions in glutamatergic, but not GABAergic, signaling in hippocampal neurons,¹⁷ indicating a possible key role for M_4 in regulating hippocampal function, and possibly in mediating the cognitive effects observed with VU0467154. Our findings are also in agreement with a recent study showing that activation of M_4 enhances performance in a PFC-mediated object recognition task.⁶⁴ The present findings are particularly important in light of the fact that clinically used APDs disrupt hippocampal and prefrontal cortical mediated cognitive tasks in rodents and have little or no effect on the cognitive deficits in schizophrenia patients.^{5,65,66}

In summary, selective positive allosteric modulation of M_4 muscarinic receptors by VU0467154 reverses the behavioral and cognitive deficits in an acute model of NMDAR hypofunction, while enhancing the acquisition of hippocampal and non-hippocampal-dependent associative learning and memory functions. The present findings provide strong evidence for the broader therapeutic utility for M_4 PAMs in the treatment of affective and, more importantly, cognitive impairments observed in neuropsychiatric disorders such as schizophrenia.

METHODS

Chemicals and Biological Materials. Chemicals for the synthesis of VU0467154, VU0152100, and LY2033298, molecular pharmacology, and pharmacokinetic studies were obtained from Sigma-Aldrich or made in-house. Large scale syntheses were conducted at NAEJA Pharmaceutical (Edmonton, Canada) using chemical methods developed at Vanderbilt University. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Fluo-4AM, and antibiotics were purchased from Invitrogen (Carlsbad, CA). Rodent plasma and brains were purchased from BioreclamationIVT (Westbury, NY).

Synthesis of VU0467154. 5-Amino-3,4-dimethyl-*N*-(4-((trifluoromethyl)sulfonyl)benzyl)thieno[2,3-*c*]pyridazine-6-carboxamide (VU0467154) was synthesized according to the scheme shown in Figure 1.

5-Amino-3,4-dimethylthieno[2,3-*c*]pyridazine-6-carboxylate (A). To a 20 mL microwave vial fitted with a stir bar were added 3-chloro-5,6-dimethylpyridazine-4-carbonitrile (580 mg, 3.50 mmol) and methanol (7 mL). Methyl thioglycolate (325 μ L, 3.60 mmol) was added followed by an aqueous solution of sodium hydroxide (1 M, 7.6 mmol). The microwave vial was sealed and heated to 150 °C for 30 min. The vial was cooled, and the solution was concentrated to provide sodium 5-amino-3,4-dimethylthieno[2,3-*c*]pyridazine-6-carboxylate, which was used without further purification (LCMS, R_T = 0.320 min, >99% @ 254 nm, >99% @ 220 nm; m/z ($M + 1$)⁺ = 224).

4-((Trifluoromethyl)sulfonyl)phenylmethanamine (B). To a flame-dried flask equipped with a magnetic stir bar was added 4-((trifluoromethyl)sulfonyl)benzotrile (1 g, 4.3 mmol) and tetrahydrofuran (THF; 13 mL). To this solution was added a 1 M solution of borane-THF in THF (8.4 mL, 8.4 mmol). The flask was fitted with a condenser, and the mixture was heated to reflux for ~18 h. After the mixture was cooled to ambient temperature, excess borane-THF was quenched by careful addition of methanol. Volatiles were removed under reduced pressure, and the resulting residue was dissolved in methanol and loaded onto an SCX cartridge. Elution with methanolic ammonia afforded 4-((trifluoromethyl)sulfonyl)phenylmethanamine as a yellow oil, which was used without further purification (LCMS, R_T = 0.46 min, >99% @ 254 nm, >99% @ 215 nm; m/z ($M + 1$)⁺ = 240).

VU0467154. VU0467154 was prepared from precursors **A** and **B** as follows: To a suspension of **A** (50 mg, 0.22 mmol) in dimethylformamide (1 mL) was added *N,N*-diisopropylethylamine (90 μ L, 0.66 mmol), followed by *O*-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU; 100 mg, 0.26 mmol). This mixture was allowed to stir at ambient temperature for 45 min; then **B** (58 mg, 0.24 mmol) was added. After stirring for an additional 20 min, the mixture was diluted with dimethyl sulfoxide (1 mL) and purified by reversed-phase HPLC, eluting with acetonitrile/water (with 0.1% ammonium hydroxide), to afford VU0467154 (LCMS, R_T = 0.70 min, >99% @ 254 nm, >99% @ 215 nm; m/z ($M + 1$)⁺ = 445). ¹H NMR (400 MHz, *d*₆-DMSO, δ (ppm)): 8.8 (t, *J* = 5.8 Hz, 1H), 8.1 (d, *J* = 8.4 Hz, 2H), 7.8 (d, *J* = 8.4 Hz, 2H), 7.0 (br. s, 2H), 4.6 (d, *J* = 5.8 Hz, 2H), 2.7–2.8 (m, 6H). HRMS calculated for C₁₇H₁₆F₃N₄O₃S₂ ($M + H$)⁺ m/z : 445.0616. Measured: 445.0616).

The comparator compounds VU0152100 and LY2033298 were synthesized in-house as described previously.^{16,67}

Calcium Mobilization Assays. Compound-evoked increases in intracellular calcium were measured using Chinese hamster ovary (CHO) cells stably expressing rat, human, or cynomolgus monkey muscarinic receptors (M_1 – M_5 ; M_2 and M_4 cells were cotransfected with G_{q15}).¹⁶ Cells were plated in 384-well, black-walled, clear-bottomed plates in 20 μ L of assay medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% dialyzed fetal bovine serum, 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), and 1 mM sodium pyruvate) at a density of 15000 cells/well and grown overnight at 37 °C/5% CO₂. The next day, medium was removed, and the cells were incubated with 20 μ L/well of 1 μ M Fluo-4AM (Invitrogen, Carlsbad, CA) prepared as a 2.3 mM stock in dimethyl sulfoxide (DMSO), mixed in a 1:1 ratio with 10% (w/v) pluronic acid F-127, and diluted in calcium assay buffer (Hank's balanced salt solution [HBSS], Invitrogen, Carlsbad, CA) supplemented with 20 mM HEPES and 2.5 mM probenecid, pH 7.4, for 50 min at 37 °C. Dye was removed and replaced with 20 μ L/well of assay buffer. For PAM potency curves, M_4 compounds were diluted in calcium assay buffer and added to the cells followed by the addition of an EC₂₀ concentration of ACh 140 s later and then an EC₈₀ concentration of ACh 120 s later. For fold shift experiments, multiple fixed concentrations (50 nM to 30 μ M) of M_4 compound or vehicle was added followed by the addition of a concentration–response curve of ACh 140 s later. Calcium flux was measured over time as an increase in fluorescence using a functional drug screening system 6000 (FDSS 6000, Hamamatsu, Japan). The change in relative fluorescence over basal was calculated before

normalization to the maximal response to ACh. As described previously, shifts of ACh concentration–response curves by the M_4 modulators were globally fitted to an operational model of allosterism.²¹ Data (means \pm SEM, $n = 3$) were analyzed using GraphPad Prism V.5.04 (GraphPad Software, San Diego, CA).

Radioligand Binding. Cell membranes were prepared from CHO cells expressing rat M_4 receptors cotransfected with $G_{i\alpha 5}$. Cells were harvested, collected by centrifugation, resuspended in ice-cold homogenization buffer (50 mM Tris-HCl, 0.9% NaCl, pH 7.4), and then homogenized by 3×10 s bursts with a homogenizer. Cell fractions were separated by centrifugation, and the resulting pellet was resuspended in ice-cold assay buffer (100 mM NaCl, 10 mM $MgCl_2$, 20 mM HEPES, and 10 mM EDTA, pH 7.4). For inhibition binding experiments, membranes (10 μ g/well) were incubated with 300 pM [³H]NMS, a fixed concentration of M_4 PAM (300 nM to 10 μ M) or vehicle, and a range of concentrations of ACh (1 nM to 1 mM) for 3 h at room temperature with shaking in assay buffer. Nonspecific binding was determined using 10 μ M atropine. Assays were terminated by rapid filtration using a Brandel 96-well plate harvester and washed three times with ice-cold assay buffer. The next day MicroScint20 was added, and radioactivity was counted. Data (mean \pm SEM, $n \geq 2$) were analyzed using GraphPad Prism 5.04.

Ancillary Pharmacology Screening. VU0467154 was tested at a concentration of 10 μ M in the Ricerca Lead Profiling Screen (Ricerca Biosciences, Taipei, Taiwan), a radioligand binding assay panel consisting of 57 GPCRs, ion channels, and transporters. Displacement of $\geq 50\%$ radioligand binding at a panel target was considered significant. Detailed assay procedures are available at <https://www.eurofinspanlabs.com/Catalog/Products/ProductDetails.aspx?prodId=0aCrd3Mu4RA%3d&path=128&leaf=128&clear=1>. Additionally, VU0467154 was tested at multiple concentrations in a larger radioligand binding panel comprised of 84 common and diverse molecular targets (Cerep, Inc., Poitiers, France). Following this screen, a secondary functional assay was performed by Eurofins Panlabs (Taipei, Taiwan) to determine the VU0467154 IC_{50} for inhibition of the human adenosine transporter (S-(4-nitrobenzyl)-6-thioinosine (NBTI)-sensitive equilibrative nucleoside transporter). For assay details, see http://www.cerep.fr/cerep/users/pages/Catalog/Affiche_CondExp_Test.asp?test=7 and <https://www.eurofinspanlabs.com/catalog/Products/ProductDetails.aspx?prodId=MFz7iKhlilo%3D>.

Animal Care and Housing. All *in vivo* studies were carried out using adult male Sprague–Dawley rats (Harlan, Indianapolis, IN), age-matched adult male wild-type C57BL/6 mice (Taconic Farms, Hudson, NY), and adult male M_4 KO mice with a C57BL/6 background (Dr. Jürgen Wess, National Institute of Diabetes and Digestive and Kidney Disorders, Bethesda, MD). Animals were group-housed under a 12/12 h light–dark cycle (lights on at 6 AM) with food and water available *ad libitum* unless stated elsewhere. All animal experiments were approved by the Vanderbilt University Animal Care and Use Committee, and experimental procedures conformed to guidelines established by the National Research Council *Guide for the Care and Use of Laboratory Animals*. All efforts were made to minimize animal suffering and the number of animals used.

Pharmacokinetic Studies in Rats. All pharmacokinetic studies in rats were performed in male Sprague–Dawley rats weighing 250–350 g. Rats used in serial sampling studies were surgically implanted with carotid artery and jugular vein catheters. All rats were acclimated to their environment for approximately 1 week. For the intravenous (IV) pharmacokinetic studies, VU0467154 was formulated as a 1 mg/mL solution in vehicle (ethanol, PEG400, 0.9% saline [10%/50%/40%]) and administered IV via jugular vein catheter to two rats at a dose of 1 mg/kg bodyweight. At multiple time points (2, 7, 15, and 30 min and 1, 2, 4, 7, and 24 h), blood was serially collected via carotid artery catheter into chilled, EDTA-fortified tubes. Blood samples were centrifuged for 10 min (3000 RCF, 4 °C), and plasma samples were stored at -80 °C until analysis. Pharmacokinetic parameters were determined using noncompartmental analysis of individual animal concentration–time profiles using WinNonlin v.5.3 (Pharsight Corp., Mountain View, CA).

For the oral (PO) pharmacokinetic studies, VU0467154 was formulated as a microsuspension at concentrations of 0.3 and 1 mg/

mL in vehicle (10% Tween 80 in water) and administered to rats ($N = 2$ /group) at doses of 3 and 10 mg/kg, respectively. Serial blood samples were obtained at multiple time points (15 and 30 min; 1, 2, 4, 7, and 24 h) and processed as described above. For the intraperitoneal (IP) plasma and brain distribution studies, VU0467154 was formulated as a microsuspension at 4 mg/mL in 10% Tween 80 in water and administered IP to two rats at a dose of 10 mg/kg. Plasma samples were collected as described above at a single time point (0.5 h). Whole brain samples were also collected simultaneously (0.5 h) and rinsed with cold phosphate-buffered saline before being snap frozen in dry ice and stored at -80 °C until analysis. K_p was determined by dividing the mean brain concentration by the mean plasma concentration, which itself was divided by the K_p predicted for unrestricted equilibrium ($[fu_p]/[fu_{br}]$) to obtain $K_{p,aur}$. Data are presented as means and individual data points ($n = 2$), and pharmacokinetic parameters were determined by noncompartmental analysis of individual animal concentration–time profiles using WinNonlin v.5.3 (Pharsight Corp., Mountain View, CA).

Pharmacokinetic Studies in Mice. Mouse PK studies were conducted with Frontage Laboratories (Exton, PA) and performed in adult male C57BL/6 mice ($n \geq 3$). For the PO and IP pharmacokinetic studies, VU0467154 was formulated as a microsuspension in 10% Tween 80 in water and administered at 10 or 30 mg/kg bodyweight; serial plasma samples were obtained at multiple time points (15 and 30 min and 1, 3, 7, and 24 h). For the IP plasma and brain distribution studies, VU0467154 was formulated as a microsuspension in 10% Tween 80 in water and administered at 3 mg/kg bodyweight, and plasma and brain samples were obtained at multiple time points (0.25, 0.5, 1, 2, 4, 8, and 24 h; nonserially). K_p was determined by dividing the mean brain AUC_{0-24h} by the mean plasma AUC_{0-24h} , which itself was divided by the K_p predicted for unrestricted equilibrium ($[fu_p]/[fu_{br}]$) to obtain $K_{p,aur}$. Data are presented as means \pm SEM ($n = 4$), and pharmacokinetic parameters were determined by noncompartmental analysis as described above.

Determination of *in Vivo* Concentration–Effect Relationship.

To determine the relationship between *in vivo* efficacy of VU0467154 and brain concentrations in rats, the efficacy of VU0467154 (1, 3, 10, 30, and 56.6 mg/kg; PO; $n \geq 8$ per dose level) in reversing amphetamine-induced hyperlocomotion (see below) was correlated to the brain concentrations of VU0467154 in the same animals upon study completion (1.5 h postadministration). In mice, the *in vivo* concentration–effect relationship for VU0467154 was determined by correlating the efficacy of VU0467154 in reversing amphetamine-induced hyperlocomotion (0.3, 1, 3, 10, and 30 mg/kg; IP; see below) to the brain concentrations of VU0467154 in the same animals upon study completion (2.5 h postadministration). Terminal unbound brain concentrations for all treatment groups were plotted versus each animal's efficacy in reversing amphetamine-induced hyperlocomotion. Nonlinear regression analysis (sigmoidal dose–response; variable slope and no constraints) of the plotted data were calculated to determine the *in vivo* EC_{50} value (nM) for VU0467154 in reversing amphetamine-induced hyperlocomotion in rats using GraphPad Prism 5.0 (GraphPad Inc., La Jolla, CA).

In Vitro Pharmacokinetic Assays and Analytical Procedures.

All *in vitro* rodent plasma protein binding and brain homogenate binding assays, sample preparation for bioanalysis, and associated LC-MS/MS analyses were performed as previously described.⁶⁸ Samples from rodent *in vivo* PK and brain distribution studies were prepared for bioanalysis and LC-MS/MS analyses were performed as previously described⁶⁸ but with the following modifications. A 10% B gradient was held for 0.2 min and was linearly increased to 90% B over 1.0 min with an isocratic hold for 0.3 min prior to transitioning to 10% B over 0.1 min. The column was re-equilibrated for 0.4 min prior to the next sample injection, and the total run time was 2.0 min.

Behavioral and Neurochemistry Studies. *Drugs.* D-Amphetamine hemisulfate, MK-801 ((5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5,10-imine maleate), and oxotremorine sesquifumarate were obtained from Sigma-Aldrich. VU0467154 was prepared as a microsuspension in vehicle (10% Tween 80 in sterile water) and administered IP in a volume of 2 mL/kg (rats) or 10 mL/kg (mice). For oral (PO) dosing in rats, VU0467154 was administered in a

volume of 10 mL/kg. Drugs were dissolved in sterile water (amphetamine and oxotremorine) or in 0.9% saline (MK-801). In rats, amphetamine was administered subcutaneously (SC) at 1 mL/kg. In mice, drugs were administered in a volume of 10 mL/kg; amphetamine and oxotremorine were administered SC, while MK-801 was administered IP.

Locomotor Activity Studies in Rats. Open field activity was tested using a SmartFrame Open Field System (Kinder Scientific, San Diego, CA) with a 16 × 16 array of infrared photobeams located 2.5 cm above the floor of the chamber as previously described.^{9,18} To establish the dose–response relationship for amphetamine on locomotor activity, rats were habituated for 60 min in the open field and then injected with vehicle (sterile water SC) or dose of amphetamine (0.1–1 mg/kg, SC), and locomotor activity was recorded for an additional 60 min. To determine the effects of VU0467154 on reversing amphetamine-induced hyperlocomotion, rats were habituated in the open field for 30 min, followed by administration of vehicle (10% Tween 80 in sterile water) or a dose of VU0467154 (1–56.6 mg/kg, IP or PO). After an additional 30 min, vehicle (sterile water) or amphetamine (0.75 mg/kg SC) was injected, and locomotor activity was recorded for another 60 min. The time course of drug-induced changes in ambulation is expressed as mean number of beam breaks per 5 min bin over the 120 min session. Total locomotor activity was calculated as the total number of beam breaks from the time of amphetamine administration [$t = 60$ min] to the end of the experiment [$t = 120$ min]. Total activity and time course data (means ± SEM) were analyzed by one-way and two-way ANOVA, respectively, and *post hoc* comparisons were made by Dunnett's test using GraphPad Prism.

Locomotor Activity Studies in Mice. Open field activity was tested in wild-type and M_4 KO mice, 8–10 weeks old, using an open field system (OFA-510, MedAssociates, St. Albans, VT) with three 16 × 16 arrays of infrared photobeams as previously described (see ref 18). The following paradigm was used to assess drug effects on amphetamine- or MK-801-induced locomotor activity: Wild-type and M_4 KO mice animals were habituated for 90 min in the open field before being injected with vehicle or VU0467154; 30 min later, vehicle, amphetamine, or MK-801 were administered, and locomotor activity was recorded for an additional 120 min. To assess the dose–response relationship for amphetamine-induced hyperlocomotion, mice were administered vehicle (10% Tween 80 in sterile water, IP) followed 30 min later by an injection of vehicle (sterile water SC) or a dose of amphetamine (1–3 mg/kg SC). The effects of VU0467154 on amphetamine-induced hyperlocomotion were examined by pretreating mice with vehicle (10% Tween 80 in sterile water IP) or a dose of VU0467154 (0.3–30 mg/kg IP) followed 30 min later by an injection of vehicle (sterile water SC) or amphetamine (1.8 mg/kg SC).

To assess the dose–response relationship of MK-801, wild-type and M_4 KO mice were injected with vehicle (10% Tween 80 IP) followed 30 min later by saline (IP) or a dose of MK-801 (0.1–0.3 mg/kg IP). The effects of VU0467154 on MK-801-induced hyperlocomotion were tested by pretreating mice with vehicle (10% Tween 80 IP) or VU0467154 (3–30 mg/kg IP) followed 30 min later by administration of vehicle (saline) or a dose of MK-801 (0.3 mg/kg IP).

The time course of drug-induced changes in ambulation is expressed as distance traveled (cm) per 5 min bin over the 4-h session. Total activity data were calculated as the total distance traveled from the time of amphetamine or MK-801 administration [$t = 120$ min] to the end of the experiment [$t = 240$ min]. To assess whether VU0467154 alters other amphetamine- or MK-801-elicited behaviors, we examined its effects on the following parameters: number of ambulatory episodes, vertical counts (rearing and jumping), stereotypic counts (activity while the animal is stationary), and the total resting time (time spent without photobeam breaks). Data are presented as means ± SEM and were analyzed by one-way and two-way ANOVA, respectively; *post hoc* comparisons were made by Bonferroni's test.

Monoamine Neurochemistry. Wild-type mice were injected with vehicle (10% Tween 80 IP) or VU0467154 (30 mg/kg, IP), followed 30 min later by administration of vehicle (saline IP) or MK-801 (0.3 mg/kg, IP). Thirty minutes after treatment with MK-801, mice were anaesthetized with isoflurane and decapitated, and brains were rapidly

extracted. Brain regions (NAS and CP) were dissected on ice from 1 mm thick coronal slices. Samples were frozen on dry ice and homogenized, and monoamines and their acidic metabolites were analyzed by HPLC with electrochemical detection as described previously.⁶⁹ Data were analyzed by one-way ANOVA followed by Bonferroni's test using GraphPad Prism and are shown as means ± SEM.

Touchscreen Pairwise Visual Discrimination Task. Wild-type and M_4 KO mice, 8–11 weeks old, were maintained at ~85% free-feeding weight with *ad libitum* access to water. Mice were trained to perform a touchscreen pairwise visual discrimination task as previously described^{37,39,40} in which breaking an infrared beam with a nosepoke on a computer touchscreen that was mounted on one wall of an operant chamber (Bussey-Saksida mouse touchscreen system chambers; Campden Instruments, England) on one of two stimuli (S^+ , marbles) resulted in reinforcement (delivery of 30 μ L of vanilla-flavored Ensure [Abbot Laboratories, Columbus, OH]) and a nosepoke on the other (S^- , fan) resulted in trial termination and extinction of the house light for 5 s (see Figure 9F for images). Images were pseudorandomly distributed on the left or right side of the screen for 60 trials or 60 min, whichever occurred first. Mice were trained daily until they reached an accuracy at 85% or above for three consecutive days. On the day after achieving criterion, mice were injected 60 min before testing with either vehicle (10% Tween 80 in sterile water IP) or VU0467154 (3–30 mg/kg, IP) followed 30 min later by vehicle (saline) or MK-801 (0.1–0.3 mg/kg IP). On the following day, mice were tested under drug-free conditions. Mice that received MK-801 treatment during an experiment were not reused for subsequent drug studies. Statistical analysis on the percentage of correct responses, total session length, and response and reinforcement retrieval latencies in the pairwise visual discrimination task were analyzed by one- or two-way ANOVA followed by Bonferroni's test.

Contextual Fear Conditioning. Studies were conducted using conditioning chambers in sound attenuating cubicles equipped with a stainless steel grid floor for shock delivery and a video camera for recording freezing behavior as previously described (MedAssociates, Allentown, NJ, see⁷⁰). One milliliter of 10% vanilla extract was used as an odor cue. Wild-type and M_4 KO mice, 8–11 weeks old, were handled for 3 days prior to conditioning and injected with saline for 1 day prior to conditioning. On the conditioning day, mice were pretreated for 30 min with vehicle (10% Tween 80 in sterile water IP) or a dose of VU0467154 (3–10 mg/kg IP) followed by an injection of vehicle (saline IP) or MK-801 (0.1–0.3 mg/kg IP) for an additional 30 min, then placed into the conditioning chamber. After a 2 min habituation period, four presentations of an unconditioned stimulus (0.7 mA 1-s footshock; 89-s intertrial interval) were delivered followed by a 90-s interval without stimuli (8 min total). Approximately 24 h after conditioning, mice were exposed to the same conditioning context (identical conditioning chamber and odor cue) under drug-free conditions. Freezing behavior, defined as motionless posture, excluding respiratory movements, was measured in the absence of any shock stimuli for 8 min. To assess effects of VU0467154 alone on the acquisition of contextual fear conditioning, parameters were optimized to obtain a baseline measure of <50% freezing. The training session was decreased to 3 min total; a 2 min habituation period was followed by 1 shock (0.4 mA) followed by 59 s post shock, prior to removal from the chamber. Mice were returned to the chamber 24 h later, and the percent time freezing over the 3 min session was examined. Dosing (vehicle [10% Tween 80 in sterile water] and 3, 10, and 30 mg/kg VU0467154 IP) and testing environment were identical to the initial contextual fear conditioning study. To determine whether VU0467154 or the deletion of the M_4 gene altered the sensitivity to footshock stimulus, thus potentially modifying the strength of the unconditioned stimulus, the nociceptive response to increasing shock current intensities was determined in a separate cohort of animals. Wild-type and M_4 KO mice were injected with vehicle (10% Tween 80 in sterile water IP) or VU0467154 (10 mg/kg IP) and 30 min later placed in the conditioning chambers. The behavioral changes to increasing current intensities (0–0.5 mA in 0.05 mA increments) were determined as previously reported.⁷¹ Data are presented as means ± SEM and analyzed by one-way ANOVA followed by Bonferroni's test.

Cue-Dependent Fear Conditioning. Wild-type and M_4 KO mice, 8 weeks old, were handled for 3 days prior to conditioning and injected with saline for 1 day prior to conditioning. On the conditioning day, mice were habituated for 1 h in the anteroom. Mice were pretreated with vehicle (10% Tween 80 in sterile water IP) or a dose of VU0467154 (3–10 mg/kg IP) 1 h prior to conditioning. Mice were then placed into the chamber, scented with 1.0 mL of 10% vanilla extract and illuminated by a white house light, and exposed to the following events during an 8 min session: 90 s habituation followed by four 30 s tone presentations (85 dB, 2500 Hz) coterminating with a shock (0.7 mA, 1 s) with an intertrial interval of 60 s, followed by a 90-s interval without stimuli. Approximately 24 h after conditioning, mice were returned to the anteroom where they were habituated under infrared light for 60 min. The test room and chamber were also illuminated by an infrared light only. The context of the chamber was altered with the addition of a white plexiglass floor on top of the shock grid, a black teepee to alter the shape/size of the chamber, and a 0.5 mL 10% *Eucalyptus* oil odor cue. Mice were exposed to the identical testing paradigm as on conditioning day but without the shock stimuli. Again, freezing behavior was measured in the absence of any shock stimuli for 8 min. Data are presented as means \pm SEM and analyzed by one-way ANOVA followed by Bonferroni's test.

Modified Irwin Neurological Test Battery. Effects of vehicle and VU0467154 on autonomic and somatomotor function of wild-type mice were compared with those of the nonselective muscarinic agonist oxotremorine sesquifumarate using the modified Irwin neurological test battery.⁴⁶ Assessments were performed 5, 15, 30, 60, and 180 min after IP administration of vehicle (10% Tween 80 in sterile water IP), VU0467154 (10–30 mg/kg IP), or oxotremorine (1 mg/kg SC) as described previously.^{9,18} The mean scores of the treatment groups were compared by one-way ANOVA followed by Dunnett's test.

Rotarod. The effects of VU0467154 on motor coordination were tested using a rotarod (model 4600, Ugo Basile, Comerio, Italy) with a rod diameter of 3 cm. During two training sessions that were separated by at least 20 min, mice were trained to walk on a rotarod that was rotating at a constant speed of 20 rpm. Only mice that walked on the rotarod for 120 s during the second training session advanced to drug treatment, which commenced in the afternoon of the same day. Vehicle (10% Tween 80 in sterile water IP) or a dose of VU0467154 (3–30 mg/kg IP) were administered, and 60 min later mice were gently placed on the rotarod, and the latency to fall off the rod was measured. Animals that did not fall of the rotarod were given a score of 120 s. Data were analyzed using one-way ANOVA followed by Dunnett's test.

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Author Contributions

M.B., T.M.B., D.D., R.W.G., M.J.N., C.M.N., J.S.D., M.R.W., and C.K.J. designed the experiments. M.B., T.M.B., D.D., R.W.G., M.G., M.J.N., A.L., and F.W.B. performed the experiments. M.S.P., B.J.M., J.C.T., J.W., M.E.D., J.D., N.J.B., M.W.W., M.R.W., and C.W.L. contributed reagents and other resources. M.B., T.M.B., D.D., R.W.G., M.J.N., and C.K.J. performed data analyses. M.B., T.M.B., R.W.G., M.J.N., M.R.W., P.J.C., and C.K.J. wrote the manuscript.

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Notes

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ABBREVIATIONS

ACh, acetylcholine; APD, antipsychotic drug; CHO, Chinese hamster ovary; CNS, central nervous system; CP, caudate-putamen; DA, dopamine; DOPAC, dihydroxyphenylacetic acid; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; GPCR, G-protein-coupled receptor; HATU, 1-[Bis-(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate; HVA, homovanillic acid; HPLC, high-performance liquid chromatography; KO, knock-out; LCMS, liquid chromatography-coupled mass spectroscopy; mAChR, muscarinic acetylcholine receptor; NAS, nucleus accumbens; NMDAR, N-methyl-D-aspartate subtype of the glutamate receptor; NMS, N-methylscopolamine; PAM, positive allosteric modulator; PFC, prefrontal cortex; Pr₂NET, N,N-diisopropylethylamine

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